

## Low prevalence of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* among patients with symptoms of respiratory tract infections in Dutch general practices

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**Abstract.** Acute respiratory disease is one of the most common reasons to consult a general practitioner. A substantial part of these diseases cannot be explained by an infection with a virus or a common pathogenic bacterium. To study this diagnostic deficit, the prevalence of *Chlamydia pneumoniae* and *Mycoplasma pneumoniae* infections was determined in two groups of patients consulting a general practitioner. DNA of *C. pneumoniae* and *M. pneumoniae* was detected by a polymerase chain reaction (PCR) in nose/throat swabs from six (1.1%), and from seven (1.3%) patients, respectively, of 557 patients consulting a general practitioner for complaints suggestive for a virus infection during the 1994/1995 respiratory infections season. Two patients remained *C. pneumoniae* PCR-positive for at least 4 weeks. All others were negative

within 3 weeks. Double infections of *C. pneumoniae* and influenza virus (3/6), and of *M. pneumoniae* and respiratory syncytial virus (1/7) or rhinovirus (1/7) were diagnosed. During the 1992/1993 season, attempts to isolate *C. pneumoniae* in cell culture or to detect *C. pneumoniae* DNA by PCR using throat swabs were all negative for 80 patients with a sore throat, although serological data suggested a *C. pneumoniae* infection in 13 (16%) patients. A specimen from another patient of this group was *M. pneumoniae* PCR-positive and the corresponding serum specimens showed a persistent high antibody titre. In summary, the prevalence of acute *C. pneumoniae* and *M. pneumoniae* infections was less than 2% in patients consulting a general practitioner.

**Key words:** Antibodies, *Chlamydia pneumoniae*, Family practice, *Mycoplasma pneumoniae*, Polymerase chain reaction, Respiratory tract diseases

**Abbreviations:** CI = confidence interval; EIA = enzyme immunoassay; IFU = inclusion forming unit; LPS = lipopolysaccharide; NIVEL = Netherlands Institute of Primary Health Care; PCR = polymerase chain reaction; RIVM = National Institute of Public Health and the Environment; rELISA = recombinant enzyme linked immunosorbent assay

### Introduction

*Chlamydia pneumoniae* and *Mycoplasma pneumoniae* have been recognised as important respiratory tract pathogens [1–3]. Most *C. pneumoniae* infections are thought to be asymptomatic [1]. Nevertheless, approximately 10% of all pneumonia cases, 5% of all bronchitis and sinusitis cases, and 2–8% of all pharyngitis cases have been attributed to *C. pneumoniae* [1]. Furthermore, *C. pneumoniae* infections have been associated with new-onset asthma and exacerbation's of chronic asthma, and, recently, also with coronary heart disease and atherosclerosis [1]. Some studies have indicated about one-fifth of all *M. pneumoniae* infections is asymptomatic [2]. Most symptomatic

infections (77%) are characterised by respiratory illnesses such as pharyngitis and tracheobronchitis and only a minor fraction (3%) by pneumonia [2, 3]. Complications associated with *M. pneumoniae* infection include arthritis, pleuritis, anemia, peri- and myocarditis, and encephalitis [3]. The clinical characteristics of patients presenting with serious respiratory infections caused by *C. pneumoniae*, *M. pneumoniae*, viruses, or unidentified agents, are very similar [4, 5]. Among respiratory illnesses, sore throat is one of the most common reasons to consult a general practitioner. Generally, diagnostic tests are not performed in Dutch general practices and the decision to treat patients is based mainly on clinical signs and symptoms. Recently, the clinical entity 'sore

throat' was investigated in Dutch general practice [6]. In that study, laboratory diagnosis of infection with *C. pneumoniae*, *M. pneumoniae* or viruses was not carried out. Seventy percent of the patients (n = 598) had positive cultures for pathogenic bacteria, of which 68% were  $\beta$ -haemolytic streptococci. For that reason, sore throat is often treated blindly with penicillin. However, these antibiotics are not effective against *C. pneumoniae* and *M. pneumoniae*. Thus, since *C. pneumoniae* or *M. pneumoniae* may be involved [4], some patients may receive inappropriate treatment and may be at risk for developing complications associated with *C. pneumoniae* or *M. pneumoniae*. In this report, we extend the study of the aetiology of sore throat to *C. pneumoniae* and *M. pneumoniae* in patients visiting a general practice.

## Materials and methods

### Study populations

Two groups of patients were studied. The first group consisted of patients consulting a general practitioner who participated in a surveillance programme of the Netherlands Institute of Primary Health Care (NIVEL). This programme consisted of 44 sentinel general practices distributed over the whole country proportionate to the population density, providing health care for about 1% of the Dutch population [7, 8]. The criteria for enrolment were symptoms of acute respiratory illness suggesting a virus infection. Patients were included in the study from October 1994 to May 1995. Five hundred fifty-seven patients were enrolled. From each patient a nose/throat swab in gelatin-lactalbumin-yeast extract medium was collected for isolation of respiratory viruses in cell culture, and for detection by polymerase chain reaction (PCR) assays of *C. pneumoniae*, *M. pneumoniae*, respiratory syncytial virus, rhinovirus, corona virus OC43 and 229E, and enterovirus [9]. Follow-up specimens were requested from PCR-positive patients, until the PCR assay became negative.

The second group consisted of patients with a sore throat, consulting one of 10 selected general practices located within 15 km distance from the laboratory. The criteria for enrolment were a sore throat with a duration of less than 15 days, and age between 10 and 60 years. Eighty patients were recruited from September 1992 to August 1993. From each patient, a throat swab in chlamydia transport-medium (16 mM phosphate buffer pH 7.2, 0.4 M sucrose, supplemented with 10% fetal calf serum and antibiotics), and paired serum specimens were collected. The throat swabs were delivered within 3 hours at the laboratory and stored at  $-70^{\circ}\text{C}$  until inoculated in cell culture. The throat swabs were used for detection of *C. pneumoniae* by PCR and isolation in cell culture, and for detection of *M. pneumoniae* by PCR.

The serum specimens were used for detection of antibodies to *C. pneumoniae* and *M. pneumoniae*.

### PCR assays

Throat swabs were vortex mixed for 30 sec in the transport-medium. A 600  $\mu\text{L}$  was centrifuged at  $12,500 \times g$  for 30 min in a microcentrifuge. The pellet was lysed in 25  $\mu\text{L}$  of lysisbuffer [10].

Primers and probe for the *C. pneumoniae* PCR assay were designed to detect the 16S rRNA gene (GenBank accession number L06108). The lower primer (5' AAT AAT GAC TTC GGT TGT TAT TTA G 3') was located at nucleotides 70–94, and the upper primer (5' AGA TAG TTT TAA ATG CTG ACT TGG 3') was located at nucleotides 656–633, yielding an amplicon of 587 basepairs. The specificity of the PCR product was confirmed by hybridisation with a biotin-labelled probe (5' biotin GTA GTG TAA TTA GGC ATC TAA TA 3'), located at nucleotides 174–196. Prevention of molecular contamination was as described previously [11]. A 5  $\mu\text{L}$  of the specimen was added to 20  $\mu\text{L}$  PCR mixture (final concentrations: 50 mM Tris/HCl pH 9.0, 50 mM KCl, 4.5 mM  $\text{MgCl}_2$ , 0.1% Triton X-100, 0.01% gelatine, 10  $\mu\text{M}$  tetramethylammonium chloride, 0.01% bovine serum albumin, 0.3 mM dATP, 0.3 mM dGTP, 0.3 mM dCTP, 0.9 mM dUTP, 5 units/ml uracil N-glycosylase [AmpErase UNG, Perkin Elmer, Gouda, The Netherlands], 10 units/ml Super Tth polymerase [HT Biotechnology LTD, Sphaero Q, Leiden, The Netherlands], and 0.8  $\mu\text{M}$  of each primer). The PCR was carried out in four steps: (i) 2 min  $50^{\circ}\text{C}$  followed by 10 min  $95^{\circ}\text{C}$ ; (ii) a touchdown protocol of 1 min  $95^{\circ}\text{C}$ , 1 min 65, 63, 61, 59, 58, 57, and  $56^{\circ}\text{C}$ , 1 min  $72^{\circ}\text{C}$ , each annealing temperature 2 cycles; (iii) 40 cycles of 1 min  $95^{\circ}\text{C}$ , 1 min  $55^{\circ}\text{C}$ , 1 min at  $72^{\circ}\text{C}$ ; (iv) 10 min extension at  $72^{\circ}\text{C}$ . The analytical sensitivity of the PCR assay was determined by testing a dilution series of DNA from *C. pneumoniae* isolate TW-183 [10]. To determine the specificity of the *C. pneumoniae* PCR assay, DNA from 12 *C. pneumoniae* isolates [10], 16 *Chlamydia trachomatis* isolates [11], six *Chlamydia psittaci* isolates [10], and one *Chlamydia pecorum* isolate [10] was used.

The *M. pneumoniae* PCR was carried out as described previously using primers P1-1 and P1-3, and a biotin-labelled probe [12, 13].

A  $\beta$ -globin PCR assay using the primerpair PC03/PC04 [14] was used to assess the presence of amplifiable eukaryotic DNA in the throat swab specimens of the second group only. The  $\beta$ -globin PCR assay was carried out as described above for *C. pneumoniae*.

A 10  $\mu\text{L}$  of the amplified specimen was analysed by electrophoresis on 2% multi-purpose agar (Boehringer Mannheim) using marker VIII (Boehringer Mannheim) as a reference. Amplicons were visualised by staining with ethidiumbromide and ultraviolet-

illumination. The gels were blotted onto nylon membrane (Boehringer Mannheim) and hybridised with the respective probes. The probes were detected using the chemoluminescence ECL kit (Amersham Nederland BV, 's-Hertogenbosch', The Netherlands) according to the instructions of the manufacturer.

#### *Isolation of C. pneumoniae in cell culture*

Isolation of *C. pneumoniae* from clinical specimens was carried out as described previously for *C. trachomatis* with minor modifications [11]. Briefly, 0.3 ml of the transport-medium was inoculated onto one-day-old monolayers of HEp2 (ATCC CCL 23), Vero (ATCC CCL 81), and HL [15] cells in shell vials and in 24-well microtiter plates, and incubated for 3 days in Iscove's modified Dulbecco medium (GIBCO BRL, Life Technologies B.V., Breda, The Netherlands) supplemented with 0.75 µg/ml cycloheximide, 10% foetal calf serum and antibiotics at 35 °C and 5% CO<sub>2</sub>. The monolayers in the microtiter plates were fixed with methanol and stained with fluorescein-labelled anti-lipopolysaccharide (LPS) monoclonal antibodies (PathoDX; Diagnostic Products Corporation, Los Angeles, California) to assess the presence of inclusions. All clinical specimens were passaged blindly four times. After the medium was replaced with fresh cold medium, the monolayers in the shell vials were disrupted by ultrasonic treatment for 1 min in a cup horn (Vibra Cell; Sonics & Materials, Inc., Danbury, Connecticut). The suspensions were inoculated onto fresh one-day-old monolayers. *Chlamydia pneumoniae* isolate CWL029 was propagated as described above in monolayers of HL cells in 6-well plates, for preparation of enzyme immunoassay (EIA) antigen as described previously [16].

#### *Detection of antibodies to C. pneumoniae*

IgM, IgG, and IgA antibodies to chlamydial LPS were determined using commercially available enzyme immunoassays (*Chlamydia* IgM, IgG, and IgA recombinant enzyme linked immunosorbent assay [rELISA], Medac GE-Diagnostika, Hamburg, Germany). The results of the Medac-rELISAs were interpreted according to the criteria of the manufacturer. A recent infection was defined as a seroconversion in IgM, IgG or IgA antibodies; a fourfold or greater rise in IgG titre, or a twofold or greater rise in IgA or IgM titre. A past infection was defined as the presence of any antibody not meeting these criteria. IgM, IgG, and IgA antibodies to *C. pneumoniae* elementary bodies were determined using an in house developed EIA, National Institute of Public Health and the Environment (RIVM-EIA) [16–18]. The RIVM-EIAs were carried out as previously described for *C. trachomatis*, with minor modifications [16–18]. Untreated and sodium periodate-treated antigens of

*C. pneumoniae* isolate CWL029 and control antigen of mock infected HL cells were used. Patient sera were tested diluted 1:100 for IgM, 1:500 for IgA, and 1:1000 for IgG determination. A reference serum was included in each assay. Titres of the patient specimens were calculated relative to this reference serum. A recent infection was defined as a seroconversion or a fourfold or greater rise in titre of IgM, IgG or IgA antibodies. A high titre was defined as 1:32,000 or higher. A past infection was defined as the presence of any antibody not meeting these criteria.

#### *Detection of antibodies to M. pneumoniae*

Antibodies to *M. pneumoniae* were determined using a complement fixation test as described previously [19]. A recent infection was defined as a seroconversion, or a fourfold or greater increase in titre.

#### *Statistical analysis*

The significance of the differences of two percentages was calculated using an approximation with the normal distribution. A *p*-value < 0.05 was considered statistically significant. The data were analysed using the computer programme Statistica for Windows, version 5.0 (StatSoft Inc., Tulsa, USA). The exact 95% confidence intervals (CIs) for percentages were calculated using the cumulative binominal distribution.

## **Results**

#### *Analytical sensitivity and specificity of the C. pneumoniae PCR assay*

Between 0.1 and 0.01 inclusion forming units (IFU) could be detected by the PCR assay. The specificity of the PCR assay was tested using DNA of isolates of all four *Chlamydia* species. All *C. pneumoniae* isolates were positive. All isolates of *C. trachomatis*, *C. psittaci*, and *C. pecorum* were negative.

#### *Patients consulting a sentinel general practitioner for acute respiratory illness*

The age of 550 patients ranged from zero to 94 years (mean age 33 years, median age 33 years). The group consisted of 276 females (age range 0–94 years; mean age 35 years, median age 33 years), and 264 males (age range 0–81 years; mean age 33 years, median age 33 years). The date of birth or the gender was not known of seven patients.

Six of the 557 patients specimens reacted positively in the PCR assay for *C. pneumoniae* (1.1%) and seven in the PCR assay for *M. pneumoniae* (1.3%). Four of the six patients positive for *C. pneumoniae* were females, age between seven and 68 years. The other two were male patients, one of 9 month old and one of

12 years old. Six of the seven patients positive for *M. pneumoniae* were females, age between three and 54 years. The seventh patient was a male, 43 years old. No clustering in time or region was observed.

Three of the six patients positive for *C. pneumoniae* (50%) were also positive for a virus, one for influenza A(H1N1) virus, one for influenza A(H3N2) virus, and one for influenza B virus. Two of the seven patients positive for *M. pneumoniae* (29%) were also positive for a virus, one for respiratory syncytial virus, and the other for rhinovirus.

Follow-up specimens were obtained from five patients positive for *C. pneumoniae* and from five patients positive for *M. pneumoniae*. Three of the five *C. pneumoniae* follow up specimens (from female patients of seven, eight, and 21 years old respectively) obtained after 10–20 days were negative in the PCR assay. The second and third specimen, obtained 15 and 31 days after the first, from a male patient of 9 months old were still positive for *C. pneumoniae* in the PCR assay, but the fourth specimen obtained after 65 days was negative. The second specimen obtained 31 days after the first, from a female patient of 68 years old was still positive for *C. pneumoniae* in the PCR assay, but the third specimen obtained after 88 days was negative. All *M. pneumoniae* second specimens, obtained 13–39 days after the first specimen, were negative in the PCR assay.

Sixty-two percent (343/557) of the patients presented with influenza-like illness, including four of the six patients positive for *C. pneumoniae* (three were also positive for influenza virus), and five of the seven patients positive for *M. pneumoniae* (one was also positive for rhinovirus). Thirty-three percent (181/557) of the patients presented with a non-influenza-like illness, including the other two *C. pneumoniae* positive patients and the other two *M. pneumoniae* positive patients (one was also positive for respiratory syncytial virus). For the remaining 5% of the patients, no clinical symptoms were reported.

Patients positive for *M. pneumoniae* in the PCR assay tend to report to cough more frequently than patients with a respiratory illness of unknown cause ( $p < 0.05$ ). Patients positive for *C. pneumoniae* in the PCR assay tend to have less frequently malaise and myalgia than patients with a respiratory illness of unknown or viral cause (both  $p < 0.05$ ).

#### *Patients consulting a general practitioner for a sore throat*

The age of the 80 patients ranged from 10 to 54 years (mean age 27 years, median age 26 years). The group of patients consisted of 55 females (age range 11–54 years; mean age 27 years, median age 25 years) and 25 males (age range 10–42 years; mean age 28 years, median age 32 years). The interval between the first and the second serum specimen varied between nine and 28 days (mean value 15 days,

median value 14 days). Of one of the patients, the date of the second specimen was not known.

To determine whether sodium periodate treatment of the antigen affected the outcome of the RIVM-EIA, the titres calculated using the untreated antigen were compared with the titres calculated using the treated antigen. For IgG 90.6% (95% CI: 85.0–94.7) and for IgA 89.4% (95% CI: 83.5–93.7) of the specimens the titres were within one dilution step. The IgG and IgA titres were in 8.8% (95% CI: 3.6–17.2) and 7.5% (95% CI: 2.8–15.6) of the acute phase specimens respectively, and in 8.8% (95% CI: 3.6–17.2) and 11.3% (95% CI: 5.3–20.3) of the convalescent phase specimens respectively, two dilution steps higher after treatment. The IgA titre of one acute phase specimen and of one convalescent phase specimen of two different patients became three dilution steps higher. The IgG titre of one acute phase specimen became two dilution steps lower after treatment. Fourfold or greater rises in titre were not affected by sodium periodate treatment. Only the data obtained with the sodium periodate treated antigen are presented.

Six patients showed a high titre to *C. pneumoniae* in the RIVM-EIA, and three patients showed a seroconversion (Table 1). Six patients showed a seroconversion in the Medac-rELISA, of which four showed optical density values close to the cut-off optical density value (Table 1). Two of the patients with a high titre in the RIVM-EIA showed a seroconversion in the Medac-rELISA. No IgM antibodies were detected by the RIVM-EIA, whereas the Medac-rELISA detected IgM antibodies in acute phase specimens of nine patients and in convalescent phase specimens of five patients. Of these patients one patient showed an IgM seroconversion and one had a persistent high IgM titre (Table 1).

The seroprevalence as determined by the RIVM-EIA differed significantly from that determined by the Medac-rELISA ( $p < 0.05$ ). There was no significant difference in seroprevalence of past infection, recent infection, or total seroprevalence between male patients and female patients, as determined by the RIVM-EIA or the Medac-rELISA (Table 2).

All throat swab specimens were negative for *C. pneumoniae* by isolation in cell culture and by PCR assay.

No seroconversions or rises in complement fixation titres to *M. pneumoniae* were observed. Nine patients showed steady complement fixation titres (1:4–1:32) to *M. pneumoniae* in both serum specimens. Of two patients, the complement fixation titre to *M. pneumoniae* was 1:64 in both serum specimens. The throat swab of one of these patients (female, age 13 years) was positive for *M. pneumoniae* in the PCR assay, whereas the throat swab of the other patient (female, age 30 years) was negative. Both patients showed no indication of a recent infection with *C. pneumoniae* by serology, culture or PCR assay. The throat swabs of

**Table 1.** Serological data of 13 patients presenting with a sore throat in Dutch general practices during the 1992/1993 season suggestive for recent infection with *C. pneumoniae* (underlined data)

Case no.	Age (years) /gender	Specimen	RIVM-EIA (reciprocal titer)		Medac-rELISA (reciprocal titer)		
			IgG	IgA	IgG	IgA	IgM
37	16/female	Acute	<u>64.00</u>	8.00	100	<50	<50
		Convalescent	<u>64.00</u>	16.00	100	<50	<50
43	30/female	Acute	<u>16.00</u>	8.00	100	<50	<50
		Convalescent	<u>32.00</u>	8.00	100	<50	<50
45	45/male	Acute	2.00	<500 <sup>a</sup>	<100	<50	<50
		Convalescent	1.00	<u>500<sup>a</sup></u>	<100	<50	<50
47	47/female	Acute	4.00	<500 <sup>a</sup>	100	<50	50
		Convalescent	2.00	<u>500<sup>a</sup></u>	100	<50	<50
133	31/female	Acute	<u>16.00</u>	2.00	<100	<50	<50
		Convalescent	<u>32.00</u>	2.00	<u>100</u>	<50	<50
141	24/female	Acute	8.00	<500	<100 <sup>a</sup>	<50	<50
		Convalescent	8.00	<500	<u>100<sup>a</sup></u>	<50	<50
145	37/male	Acute	2.00	<500	<100	<50	<50
		Convalescent	2.00	<500	<100	<50	<u>100</u>
153	30/female	Acute	1.00	1.00	100	<50 <sup>a</sup>	<50
		Convalescent	1.00	1.00	100	<u>50<sup>a</sup></u>	<50
155	21/male	Acute	<u>32.00</u>	8.00	<100	50	<u>200</u>
		Convalescent	<u>32.00</u>	8.00	<100	50	<u>200</u>
162	38/male	Acute	4.00	500	<100 <sup>a</sup>	<50	<50
		Convalescent	4.00	1.00	<u>100<sup>a</sup></u>	<50	<50
167	17/male	Acute	<u>16.00</u>	8.00	<100 <sup>a</sup>	<50	<50
		Convalescent	<u>32.00</u>	4.00	<u>100<sup>a</sup></u>	<50	<50
169	54/female	Acute	<u>32.00</u>	<500	<100	<50	<50
		Convalescent	<u>64.00</u>	<500	<100	<50	<50
192	54/female	Acute	<1.00	<500	<100	<50	<50
		Convalescent	<u>1.00</u>	<500	<100	<50	<50

<sup>a</sup> Optical density value of the acute specimens was just below and that of the convalescent specimen was just above the cutoff optical density value.

all other patients were negative for *M. pneumoniae* in the PCR assay. All throat swab specimens were positive in the  $\beta$ -globin PCR assay.

## Discussion

In our study, the overall prevalence of *C. pneumoniae* and *M. pneumoniae* infections as determined by PCR among patients presenting with acute respiratory

complaints in Dutch general practices was 0.9 and 1.3%, respectively. Since our largest group of patients was recruited from general practices providing health care for about 1% of the Dutch population [7, 8], distributed over the whole country and proportionate to the population density, we can conclude that approximately 1% of the patients presenting with a virus infection-like acute respiratory illness in Dutch general practices have a *C. pneumoniae* or a *M. pneumoniae* infection. Although serological data

**Table 2.** Summary of serological data for *C. pneumoniae* of 80 patients presenting with a sore throat in Dutch general practices during the 1992/1993 season

Gender	RIVM-EIA (periodate treated antigen)						Medac-rELISA							
	Past infection		High titer		Seroconversion		Total seropositive		Past infection		Recent infection		Total seropositive	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Male (n = 25)	20	(80) <sup>a</sup>	2	(8)	1	(4)	23	(92) <sup>b</sup>	7	(28) <sup>a</sup>	4	(16)	11	(44) <sup>b</sup>
Female (n = 55)	45	(82) <sup>c</sup>	4	(7)	2	(4)	51	(93) <sup>d</sup>	20	(36) <sup>c</sup>	3	(5)	23	(42) <sup>d</sup>
Total (n = 80)	65	(81) <sup>e</sup>	6	(8)	3	(4)	74	(93) <sup>f</sup>	27	(34) <sup>e</sup>	7	(8)	34	(43) <sup>f</sup>

a,b,c,d,e,f Corresponding values significantly different,  $p < 0.05$  (calculated using an approximation with the normal distribution).

suggested a prevalence of *C. pneumoniae* infections of 16% in a subgroup, this finding could not be confirmed by detection of the agent.

In 33% of our patients from the sentinel practices respiratory viruses were detected, in three cases together with *C. pneumoniae* and in another two cases together with *M. pneumoniae*. Previously, Dagnelie et al. [6] showed that 70% of similar patients carried pathogenic bacteria in their throats. Coexistence of *C. pneumoniae* or *M. pneumoniae* and other agents have been described before [4]. In these patients, it is difficult to assess the relative contributions of the two agents to the respiratory complaints. Animal models even suggest reactivation of a latent *C. pneumoniae* infection [20]. In agreement with previous reports [2, 4, 5], clinical characteristics were similar for viral, chlamydial and mycoplasmal infections.

Some studies reported higher prevalences (6–10%) of *C. pneumoniae* and *M. pneumoniae* infections [4, 21]. Our results are in agreement with prevalences (2–3%) reported by Thom et al. [5]. These differences might be caused by the presence or absence of epidemics. Both *C. pneumoniae* and *M. pneumoniae* infections occur in cycles of several years [2, 21]. Another explanation might be the use of different diagnostic techniques. If cross reactions in molecular or serological assays caused by *Chlamydia*-like organisms capable of infecting humans [22], fish [23], or free living amoebae [24, 25], of which recently several new types have been described [26], do occur, than the lower estimates of these prevalences are more likely to be true. Our PCR assays do not cross react with *Chlamydia*-like organisms (data not shown).

Specimens from two patients remained positive in the PCR assay for *C. pneumoniae* for at least 31 days after collection of the first specimen. Hammerschlag et al. [27] even detected *C. pneumoniae* up to 11 months after an acute infection, despite appropriate treatment. Since all patients became negative, positive results correlate with the period of clinical disease. Although positive cultures for *M. pneumoniae* might be obtained 6 weeks or more after infection [3], all second specimens in our study were negative in the PCR assay. However, since a control group was not included in this study, these findings don't prove a causal relationship.

Results based on detection of DNA alone should be interpreted cautiously, since detection of DNA does not discriminate between viable and nonviable or carrier state and clinical disease [3, 28]. In our second group serological assays were included. Although in 13 patients (16%) serological indications for a *C. pneumoniae* infection were observed, these data consisted of a high titer or seroconversions close to the cut-off values and cannot be considered hard evidence. A third serum specimen taken 6–8 weeks after the onset of the illness might improve serological diagnosis [1], but was not available. Serological evidence for *C. pneumoniae* infection is not always

confirmed by detection of the organism [5, 28, 29]. We used state of the art cell culture techniques [15, 30–32]. In addition, our PCR assay showed an analytical sensitivity of less than 0.1 IFU *C. pneumoniae* and no inhibitors were demonstrated by the  $\beta$ -globin PCR assay results. However, failure to sample the site of infection cannot be excluded.

A marked difference in seroprevalence between the RIVM-EIA and the Medac-rELISA was observed, 93 and 41%, respectively. The RIVM-EIA mainly detects antibodies to protein antigens [16–18], while the Medac-rELISA only detects antibodies to the LPS. Since most people are infected with *C. pneumoniae* at least once before the age of 20 years, these results suggest that the detection of anti-protein antibodies is a better indicator for a past infection than anti-LPS antibodies. Although it is clear that the kinetics of both types of antibodies are different [33], further studies are needed to find out if antibodies to LPS are a better indicator for a recent infection. The seroprevalence determined by the RIVM-EIA was higher than observed in the general population [34], but similar to that observed in elderly males [35]. The study population might be biased.

In our group of 80 patients, a specimen from only one patient (1.2%) was positive in the PCR assay for *M. pneumoniae*, but without serological evidence for a recent infection. Although the complement fixation test might underestimate the number of reinfections [36], the results of the serological assays and of the PCR assays are in agreement.

In conclusions, the endemic prevalence of *C. pneumoniae* and *M. pneumoniae* infections in patients visiting a general practitioner with complaints of a sore throat or virus-like disease is approximately 1%. These data can be used to infer guidelines for empirical antibiotic treatment.

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