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Demonstration by a nested PCR for *Mycoplasma pneumoniae* that *M. pneumoniae* load in the throat is higher in patients hospitalised for *M. pneumoniae* infection than in non-hospitalised subjects

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A nested PCR protocol to detect *Mycoplasma pneumoniae* DNA in throat specimens was developed. An amplification control (AC) template, which is amplified by the same primers as the *M. pneumoniae* target sequence, was constructed. The assay allowed highly specific and sensitive detection of *M. pneumoniae* DNA. In all, 305 throat samples, 62 from hospitalised patients and 243 from non-hospitalised subjects, were analysed by the nested PCR. Inhibition of the PCR was observed in 20% of the samples, but was abolished after a 1 in 10 dilution. Throat samples from 5 (8%) of the hospitalised patients and from 7 (3%) of the non-hospitalised subjects were positive for *M. pneumoniae* DNA. To investigate the relationship between *M. pneumoniae* load and the severity of disease, the *M. pneumoniae* load in 10 throat samples from *M. pneumoniae*-positive subjects was assessed semi-quantitatively by application of the nested PCR to a series of limiting dilutions of nucleic acid extracted from these throat samples. The calculated *M. pneumoniae* load varied from 20 to 3830 cfu/ml of throat sample. The mean *M. pneumoniae* load in samples from the hospitalised patients was significantly higher than that in samples from the non-hospitalised subjects. The nested PCR is a useful tool to detect *M. pneumoniae* DNA in the throat and to study the relationship between the load of *M. pneumoniae* in throat samples and severity of disease due to *M. pneumoniae* infection.

Introduction

Mycoplasma pneumoniae is a common cause of upper and lower respiratory tract infections in man. The majority of these infections are relatively mild, but serious disease like pneumonia can require hospitalisation. Complications of the primary infection, e.g., neurological and cardiovascular complications, can also occur [1–3]. *M. pneumoniae* infection is diagnosed

routinely by serological methods or by culture, or by both. Because these diagnostic approaches have disadvantages, such as repeated blood sampling to perform reliable serology [4] and the slow growth of *M. pneumoniae* in culture, molecular diagnostic techniques have been applied to the laboratory diagnosis of *M. pneumoniae* infection. Both DNA hybridisation techniques [5–8] and the PCR [5, 9–14] have been used.

The clinical significance of a positive PCR result for *M. pneumoniae* in throat specimens obtained from patients can be obscured by the fact that *M. pneumoniae* can persist in the throat after symptomatic infection [15] and even after antibiotic treatment [8]. As a relationship between high levels of *M. pneumoniae* in the throat and clinically significant respiratory tract infection has been observed [8, 13], molecular diagnostic methods that allow detection of *M. pneu-*

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moniae and estimation of its load are required. Therefore, a nested PCR assay for rapid and sensitive detection of *M. pneumoniae* in throat specimens was developed. The PCR assay was designed with an amplification control (AC) template to verify amplification from clinical specimens. The method was applied to the laboratory diagnosis of *M. pneumoniae* infection in a group of hospitalised patients, in patients who attended their general practitioner (GP) and in household contacts of patients with a positive PCR for *M. pneumoniae*. Subsequently, the *M. pneumoniae* load in the severely ill hospitalised patients and in the patients with relatively mild or no symptoms was estimated by applying the nested PCR to a series of limiting dilutions of nucleic acid isolated from throat swabs.

Materials and methods

Patients and throat sample preparation for nested PCR

Samples were obtained from patients admitted to hospital with community-acquired pneumonia, from patients visiting their GP because of a respiratory tract infection and from household contacts of *M. pneumoniae* DNA-positive patients in the hospitalised and non-hospitalised groups. Between Feb. 1997 and Aug. 1997 a total of 302 throat samples was collected from 62 hospitalised patients, 208 non-hospitalised patients and 32 household contacts. A further three follow-up throat samples were collected from asymptomatic, but *M. pneumoniae*-positive, household contacts 3–4 weeks after the first sample. Patients who had to be hospitalised because of community-acquired respiratory tract infection were defined as more severely ill than non-hospitalised patients. Hospital nursing staff collected the throat swabs from the hospitalised patients and the GPs or their staff collected the throat swabs from the non-hospitalised patients. In the hospital as well as at the GP practices patients were sampled according to a standard protocol. Throat samples were collected on cotton-tipped swabs, which subsequently were agitated in 2 ml of transport medium (PPLO-broth, yeast extract 10%, unheated horse serum 20%, glucose 0.5%, phenol red 0.002% and penicillin 1000 U/ml). A 200- μ l sample of the transport medium in which the throat swab had been agitated was centrifuged at 12 000 *g* for 30 min. The remaining 1.8 ml was stored at -70°C . Pellets were resuspended in 100 μ l of lysis buffer (50 mM KCl, 10 mM Tris-HCl,

pH 8.3, NP40 0.45%, Tween 20 0.45%, proteinase K 10 mg/ml) (Boehringer Mannheim GmbH, Mannheim, Germany) and incubated at 50°C for 2 h. Subsequently, samples were heated at 95°C for 15 min to inactivate proteinase K. Ten μ l of the lysed sample were used as template in the first reaction of the nested PCR (see below). The proteinase K protocol was used because the detection limits of *M. pneumoniae* DNA in throat swab specimens prepared by proteinase K lysis or by the more elaborate DNA extraction protocol of Boom *et al.* [16] were identical [17].

Nested PCR protocol

A nested PCR for amplification of *M. pneumoniae* DNA was designed, based on the P1 cytoadhesin gene PCR of Ursi *et al.* [18]. Primers P1-1 and P1-6 (Table 1), which amplify a 272-bp fragment of the P1 gene, were used in the first PCR [19]. The nested primers P1-2 and P1-3 (Table 1) used in the second PCR amplify a 133-bp fragment. Primers were obtained from Perkin Elmer Applied Biosystems (Nieuwerkerk a/d IJssel, The Netherlands). Amplifications were performed in a final volume of 50 μ l containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 200 mM of each deoxynucleoside triphosphate (Perkin Elmer), 20 pmol of each primer, 1 U AmpliTaq DNA polymerase (Roche Molecular Systems, Branchburg, NJ, USA), 2 μ l AC template and 10 μ l of sample. Amplification was performed in a 9600 Thermocycler (Perkin Elmer) with a programme of 5 min at 95°C , followed by 30 cycles of 15 s at 95°C , 1 min at 65°C , 1.5 min at 72°C and a final step of 10 min at 72°C . One μ l of the first PCR product was added to the reaction mixture of the second PCR. The second PCR was performed under the same conditions as the first PCR in a 20-cycle procedure. Ten μ l of the reaction products were analysed on Metaphor agarose (FMC Bioproducts, Rockland, USA) 2% gels containing ethidium bromide 0.5 $\mu\text{g}/\text{ml}$ for visualisation of amplicons by UV transillumination.

Prevention of PCR contamination

Preparation of the patient samples by lysis or DNA extraction and preparation of the PCR mixtures were performed in safety hoods equipped with UV germicidal lamps, in separate dedicated positive pressure laboratories (> 10 Pa) with lock-gates. Addition of the patient samples to the PCR reaction mixtures was

Table 1. Primers used for the detection of *M. pneumoniae* DNA

Primer name	Nucleotide sequence	Corresponding region of the P1 gene*	Reference no.
P1-1	5'-GCCACCCTCGGGGGCAGTCAG-3'	2757–2777	18
P1-6	5'-ATCGCATTGCCGTCCTGCGTGG-3'	3028–3007	This study
P1-2	5'-CTGAACGGGGGCGGGGTGAAGG-3'	2834–2855	10
P1-3	5'-GAGTCGGGATTCCCCGCGGAGG-3'	2965–2944	18

*Nucleotide position within the P1 gene sequence [19] (Genbank accession number M 18639).

performed in a third room. To check for cross-contamination of samples and for amplicon contamination during the procedure, negative controls were included after every fourth patient sample from the start of the preparation procedure. These negative controls consisted of transport medium without patient material and were processed in the same way as the patient samples. Pipetting of amplicons from the first into the second PCR reaction mixture was performed in a fourth room. During this part of the procedure gloves were changed after pipetting each sample. Finally, gel electrophoresis of the amplification products was performed in a fifth laboratory, located in a different building.

AC template for nested PCR

To enable detection of PCR inhibition in clinical specimens, an AC template was constructed for the nested PCR. The AC DNA template was a PCR product of the P1 gene into which had been inserted, between the sites recognised by the internal nested primers, a 210-bp sequence from the *lacZ* gene of *Escherichia coli*. As this target generates a longer PCR product than the naturally occurring template, it can be used to spike clinical specimens for PCR and produce a positive control band which can be distinguished easily from the natural product on the basis of size. The absence of the AC product in a spiked reaction thus signals the likely presence of PCR inhibitors in the clinical sample. Extended primers designated P1-1* (5'-GCCGGAATTCGCCACCCTCGGGGCGAGTCAG-3') and P1-6* (5'-GGTGCTGCAGATCGCATTGCCGTCCTGCGTGG-3') containing terminal *EcoRI* and *PstI* restriction sites, respectively, were used to amplify a 292-bp P1 gene fragment from DNA of *M. pneumoniae* strain PI 1428. The reaction was performed with 1 ng of purified genomic DNA [16] as template and the PCR procedure was performed as described for the P1-1/P1-6 PCR. The PCR fragment was digested with *EcoRI* and *PstI* (Boehringer Mannheim), purified from a Metaphor agarose 2% gel with QiaEx (Qiagen GmbH, Hilden, Germany) and ligated to *EcoRI*- and *PstI*-digested plasmid pUC18Sfi [20]. Enzymic reactions and transformation of *E. coli* were performed according to standard molecular biological procedures [21]. The ligation mixture was used to transform *E. coli* JM101 competent cells. From ampicillin-resistant transformants, plasmids were isolated with the Qiagen plasmid mini kit (Qiagen). One plasmid isolate, pMM1, containing the *EcoRI*-*PstI* gene fragment, was used in further experiments. A fragment of the *E. coli lacZ* gene (bp 815–1018) was amplified from plasmid pMP220 [22] with *KpnI*-tailed primers PMM2lac1 (5'-TCATGGTACCCGCCTTTCGGCGGTGAAATTATCG-3') and PMM2lac2 (5'-AGTAGGTACCCATTTTCAATCCGCACCTCGCGG-3'). Amplification was performed in a programme of 30 cycles of 15 s at 95°C, 1 min at 55°C, 1.5 min at 72°C and a final step of 10 min at 72°C. The fragment

was digested with *KpnI*, purified from Metaphor agarose 2% gel with QiaEx and ligated to *KpnI*-digested plasmid pMM1. The size of the inserted *lacZ* fragment, including nucleotides of the added *KpnI* sites, was 210 bp. After transformation of *E. coli* JM101, plasmids from ampicillin-resistant colonies were isolated and analysed by restriction digestion for the presence of the *lacZ* fragment. One plasmid containing the proper insertion, pMM2, was selected for further experimentation. Primers P1-1 and P1-6 amplified a 482-bp fragment from plasmid pMM2. This fragment was purified from an agarose gel with QiaEx. The minimum concentration of this AC construct required to generate a visible 482-bp band in gel after amplification was determined.

M. pneumoniae strains and growth conditions

M. pneumoniae strains MAC (ATCC 15492) and PI-1428 (ATCC 29085) were cultured in modified Chanock broth [23] and in SP4 broth [24] at 36°C in a humidified atmosphere of CO₂ 5% in air until colour change occurred (after 6 and 7 days for strains MAC and PI-1428, respectively). To determine bacterial density, 10-fold serial dilutions of the mycoplasma suspensions were made in Chanock broth and SP4 broth, respectively, and 100- μ l samples were plated on modified Chanock agar and SP4 agar in triplicate. Plates were incubated at 36°C in CO₂ 5% in air for 7–24 days. Colonies were counted by examining the plates with an inverted microscope at 40 $\times$ magnification. At the time of colour change, cultures of *M. pneumoniae* strain MAC and PI 1428 contained 7×10^4 cfu/ml and 1.5×10^6 cfu/ml, respectively.

Specificity and detection limit of the nested PCR

DNA from clinical isolates of the following bacteria was used to test the specificity of the nested PCR: *M. orale*, *M. hominis*, *M. salivarium*, *M. fermentans* and *M. genitalium*, *Chlamydia pneumoniae*, *Legionella pneumophila*, viridans streptococci, *Staphylococcus aureus*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Moraxella catarrhalis*, *Streptococcus pneumoniae*, *E. coli* and *Klebsiella pneumoniae*. In addition, the nested PCR was applied to throat swab specimens obtained from 10 volunteers, who had been without respiratory illness 1 month before and after throat swab sampling.

To assess the detection limit of the nested PCR, throat samples from healthy volunteers were spiked with *M. pneumoniae* strain MAC (7×10^4 cfu/ml) or strain PI 1428 (1.5×10^6 cfu/ml). These spiked samples were serially diluted in 10-fold steps in transport medium, subjected to proteinase K lysis and the lysates were analysed in the nested PCR.

Semi-quantitative assessment of M. pneumoniae load in throat samples

There was sufficient material available to assess the *M. pneumoniae* load in 10 *M. pneumoniae* PCR-positive throat samples. To enable standardisation of the template input for the semi-quantitative PCR, total nucleic acid (NA) from 500 µl of the throat samples was extracted [16] and the concentration of purified NA was determined spectrophotometrically. NA concentrations were adjusted to 6 µg/ml for all samples; 10 µl of these adjusted samples and of 10-fold serial dilutions of the samples were used as input in the PCR. The highest 10-fold dilution which was PCR-positive was assumed to contain a number of cfu equal to the detection limit of the PCR. From this dilution, the number of *M. pneumoniae* cfu/µg of throat sample NA was calculated. Based on this value and the recorded concentration of NA in the original throat sample extract, the number of cfu/ml of throat sample was calculated.

Statistical analysis

Values of *M. pneumoniae* cfu(log₁₀)/ml in throat samples from hospitalised patients and non-hospitalised subjects were compared with the Student's *t* test.

Results

Primer specificity and detection limit of the nested PCR protocol in simulated clinical specimens

Specificity of the primer sets for *M. pneumoniae* DNA was confirmed by performing the nested PCR on various *Mycoplasma* spp., *C. pneumoniae*, *L. pneumophila* and on eight other bacteria commonly residing in the nasopharynx. The nested PCR was also performed on lysates of throat swab specimens from 10 healthy volunteers. None of these controls showed the *M. pneumoniae*-specific signal (data not shown). The detection limit in the lysates of simulated clinical specimens corresponded to 0.6 and 1.3 cfu of strains MAC and PI-1428, respectively. These detection levels were highly reproducible: the detection was identical in five independent experiments performed at different times. Based on these data, the average detection limit was defined as 1 cfu of *M. pneumoniae*.

Amplification control

A final AC concentration of 0.07 fg/µl still allowed detectable amplification in the absence of *M. pneumoniae* DNA. In the presence of *M. pneumoniae*, this concentration of AC did not interfere with PCR detection of *M. pneumoniae* DNA in simulated clinical specimens (Fig. 1), i.e., the *M. pneumoniae*-specific signal was observed in the same dilutions in the presence or absence of the AC.

Patients with M. pneumoniae-positive throat samples

For a 6-month period, from Feb. until Aug. 1997, 305 throat samples were analysed by the nested *M. pneumoniae* PCR. No PCR contamination was observed in negative controls included after each fourth patient sample. Inhibition of the PCR, as detected by the absence of the 482-bp band of the AC, occurred in 20% of the samples. After 1 in 10 dilution of these samples, no inhibition of the AC amplification was observed. Throat samples gave positive results for *M. pneumoniae* in 5 (8%) of the 62 hospitalised patients and in 7 (3%) of the 240 non-hospitalised subjects. In the non-hospitalised group, 3 (1.4%) *M. pneumoniae*-positive patients were identified among the 208 GP patients and 4 (13%) among the 32 household contacts of the eight *M. pneumoniae*-positive patients. Of the four *M. pneumoniae*-positive contacts, one subject had symptoms of a respiratory tract infection and three were asymptomatic before and at the time of sampling. Two of them remained asymptomatic and follow-up throat samples, taken 4 weeks after the first sample, were negative. The third apparently was in the incubation period of a *M. pneumoniae* infection, as he developed a respiratory tract infection 2 weeks after the first sample was taken. His follow-up throat sample, taken 3 weeks after the first sample, was still positive. The mean age of the hospitalised *M. pneumoniae*-positive patients was 8 (range 3–14) years and of the non-hospitalised *M. pneumoniae*-positive subjects 11 (range 2–26) years. The mean interval between occurrence of disease and sampling for the five hospitalised patients was 6 (range 5–11) days and for the three non-hospitalised patients and the two symptomatic household contacts this was 5 (range 2–8) days.

Semi-quantitative assessment of M. pneumoniae load in throat samples

The *M. pneumoniae* load was assessed semi-quantitatively in 10 *M. pneumoniae*-positive throat samples, five from five hospitalised patients and five from four non-hospitalised subjects, the latter comprising two GP patients and two household contacts. Two samples were analysed from one household contact. The first sample was obtained when this subject was asymptomatic and a follow-up sample was obtained 3 weeks later when he had mild symptoms of respiratory infection. The second household contact was asymptomatic. The NA contents of throat sample varied between 1.0 and 2.3 µg/ml (mean 1.6). The nested PCR produced easily interpretable all-or-none signals in the dilution series. The highest dilutions that showed the *M. pneumoniae*-specific PCR signal (Fig. 2) were used to calculate the *M. pneumoniae* load, based on the established average detection limit of 1 cfu. The calculated *M. pneumoniae* load varied from 20 to 3830 cfu/ml of throat sample. The mean *M. pneumoniae* load in the hospitalised

Fig. 1. Amplification of *M. pneumoniae* DNA in the presence and absence of AC. (a) First PCR: *M. pneumoniae* target, 272 bp; AC, 482 bp. (b) Second PCR: *M. pneumoniae* target, 133 bp; AC, 343 bp. Left side: PCR products of limiting 10-fold dilution series of a lysate of a throat swab specimen seeded with *M. pneumoniae* strain MAC (7×10^4 cfu/ml). A final AC concentration of $0.07 \text{ fg}/\mu\text{l}$ was added to all samples. Right side: PCR products of samples without AC, but otherwise identical to the left side. M, DNA mol. wt marker IX (Boehringer Mannheim).

patients was 2500 cfu/ml of throat sample, whereas the mean *M. pneumoniae* load in the non-hospitalised group was 100 cfu/ml of throat sample (Fig. 3). The *M. pneumoniae* load in the first and second sample of the person who was asymptomatic at first sampling but became symptomatic after 2 weeks was only slightly different (260 and 170 cfu/ml of throat sample, respectively). The difference in $\text{cfu}(\log_{10})/\text{ml}$ between the hospitalised and non-hospitalised group was 1.5 (95% CI 0.77–2.24) ($p = 0.0016$).

Discussion

In the present study a nested PCR for *M. pneumoniae*, with the target sequence in the P1 cytoadhesin gene, was developed. This PCR protocol was applied to proteinase K lysates of throat swab specimens, to assess rapid laboratory diagnosis of *M. pneumoniae* infection. Sensitivity of detection of *M. pneumoniae* by PCR depends on the sample preparation method [13], the primer pairs used [10, 14, 25], the type of clinical specimen on which the PCR is performed [26], the use

of genuine *versus* simulated clinical specimens [25] and signal enhancement by hybridisation after PCR [27]. Detection limits of 1.5–50 cfu, 10–100 colour changing units (ccu) and 5–50 fg of template DNA have been reported [3, 13, 14, 28]. This nested PCR allowed detection of 1 cfu of *M. pneumoniae* in proteinase K lysates of simulated *M. pneumoniae*-positive throat samples. In addition to a high sensitivity, the nested PCR protocol has the advantage of direct confirmation of the identity of the first PCR product, without the need for a time-consuming and labour-intensive hybridisation step. The risk of carry-over contamination associated with nested PCR was minimised by performing all steps of the procedure in separate rooms. Negative controls included after each fourth test sample were PCR negative in all cases, indicating that contamination was effectively prevented.

Clinical samples may contain compounds that inhibit Taq polymerase [18, 26]; many PCR protocols for detection of *M. pneumoniae* DNA lack an amplification control (AC) to check for such inhibition [9, 11, 27]. In the present study an AC template was constructed for

Fig. 2. Estimation of *M. pneumoniae* DNA in throat samples of four *M. pneumoniae*-positive patients. (a) First PCR; (b) second PCR. Serial 10-fold dilutions of a standard amount (6.0 µg/ml) of purified throat sample NA were subjected to the nested PCR. NH1 and NH4, non-hospitalised subjects with *M. pneumoniae* load of 33 and 170 cfu/ml throat sample respectively (NH1 was a GP patient and NH4 a symptomatic household contact). H1 and H2, hospitalised patients with *M. pneumoniae* load of 2500 and 3160 cfu/ml of throat sample, respectively. M, DNA mol. wt marker: 100-bp ladder.

the nested PCR protocol consisting of a P1-1/P1-6 *M. pneumoniae* P1 gene PCR fragment, enlarged by insertion of a 210-bp *E. coli lacZ* fragment. The concentration of this AC is critical: it has to be sufficiently high to generate a visible amplification product and sufficiently low not to interfere with the detection of *M. pneumoniae* DNA due to competition. A carefully selected AC concentration of 0.07 fg/µl met both criteria (Fig. 1). As inhibition was found in 20% of the samples investigated, the inclusion of an AC for diagnostic application of this PCR is essential.

The frequency of *M. pneumoniae* infections in the different patient populations varied. The incidence of *M. pneumoniae* pneumonia among the patients hospitalised because of community-acquired pneumonia was 8%, matching incidences reported by others in a non-epidemic situation [15]. In the population of patients visiting their GP because of a respiratory tract infection, the incidence of *M. pneumoniae* infection was lower (3%). Among the household contacts of *M. pneumoniae*-positive patients the study found a rela-

tively high percentage of *M. pneumoniae* PCR-positive subjects (13%), indicating a high level of transmission in the household setting.

It has been recognised that quantification of various infectious agents can have diagnostic and prognostic implications. This may also be the case for *M. pneumoniae*. Estimation of the *M. pneumoniae* load by amplification techniques might be a valuable approach to distinguish between *M. pneumoniae* carriers and patients with a clinically significant infection. In contrast to blood, plasma or serum specimens, which are used for the quantification of mostly viral nucleic acids [29–31], throat swabs are subject to variation in their composition. Therefore, a semi-quantitative approach, rather than a quantitative PCR, was developed to estimate the *M. pneumoniae* load. The nested PCR was applied to a dilution series of purified NA from throat swab samples and it was found that the *M. pneumoniae* load in five hospitalised patients was significantly higher than in four non-hospitalised subjects. As the interval between the

Fig. 3. The calculated number of cfu of *M. pneumoniae* based on semi-quantitative assessment by PCR in five *M. pneumoniae*-positive throat samples from five hospitalised patients (H) and in five samples from four non-hospitalised subjects (NH1–4). NH1 and NH2 were GP patients, NH3 was a household contact without symptoms and NH4 was a household contact first sampled when he was asymptomatic (4a), and 3 weeks later, when he had symptoms of a respiratory infection (4b). Bars represent mean numbers of cfu.

occurrence of respiratory tract infection and the time of throat sampling and the mean age was similar in the hospitalised and non-hospitalised groups, this finding suggests that more severe respiratory tract infection due to *M. pneumoniae* is associated with higher loads of *M. pneumoniae* in the throat. These results are in accordance with those of two other studies, in which the *M. pneumoniae* load was assessed in a semi-quantitative way in throat specimens from symptomatic patients and asymptomatic controls. Kleemola *et al.* used a commercial probe test during an epidemic of *M. pneumoniae* infections among army conscripts, and observed that 32 patients with clinically significant infection had higher probe test values than five asymptomatic controls [8]. Skakni *et al.* used a semi-quantitative PCR and reported high loads of *M. pneumoniae* in 8 of 10 patients with acute pneumonia, and low loads in four asymptomatic patients with chronic pulmonary disease, in three patients with cystic fibrosis and in two patients with acute asthma [13]. Although the total number of individuals studied is still small, the findings of the present study as well as those

discussed above strongly suggest a relationship between *M. pneumoniae* load and severity of disease. The *M. pneumoniae*-positive subjects in the present study were identified in a population of 302 subjects. A very large number of subjects would have to be studied to obtain a larger group of *M. pneumoniae*-positive hospitalised, non-hospitalised and asymptomatic persons.

In summary, a nested PCR protocol which allows the rapid, sensitive and specific detection of *M. pneumoniae* and the estimation of *M. pneumoniae* DNA load in throat swabs has been developed. With this method, a relationship between *M. pneumoniae* load in the throat and severity of disease was observed in a group of hospitalised patients, non-hospitalised patients and asymptomatic carriers.

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