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Real-time detection of *Mycoplasma pneumoniae* in respiratory samples with an internal processing control

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Real-time PCR was employed to detect a region of the P1 cytadhesin gene of *Mycoplasma pneumoniae* in clinical samples. An internal processing control was included that could be co-amplified simultaneously in the same reaction tube. The assay could reproducibly detect 1×10^3 *M. pneumoniae* organisms ml^{-1} in clinical samples. There was no amplification of DNA or signal production from 15 other species of human mycoplasmas and 19 other bacterial species. Using a panel of 175 respiratory samples taken from patients with pneumonia of proven aetiology, the sensitivity was found to be 60 % and the specificity of the assay 96·7 % when compared with serology. This assay is suitable for same-day diagnosis of *M. pneumoniae* infection and batch processing of respiratory samples for clinical screening.

INTRODUCTION

Mycoplasma pneumoniae is a common cause of community-acquired pneumonia transmitted by aerosol or close contact (Friedlaender *et al.*, 1976). Major outbreaks occur periodically and the severity of clinical symptoms ranges from asymptomatic to pronounced pneumonia (Clyde, 1993). A wide variety of extrapulmonary manifestations have been reported such as haematological, gastrointestinal, musculoskeletal, dermatological and neurological complications (McMillan, 1998). Traditionally, serological methods for detecting specific IgM, IgG and IgA have been used to establish diagnosis of infection. However, detectable levels of antibodies may not be attained until 7–10 days after the onset of symptoms (Sillis, 1993; McMillan, 1998). Culture requires specialized media, is time-consuming (up to 21 days) and is rarely undertaken in a routine context.

Due to the vast reduction in time in comparison with culture, PCR has been used increasingly for *M. pneumoniae* detection. Several gene targets have been used for amplification, including the 16S rRNA gene, the elongation factor *tuf*, the P1 cytadhesin gene and repetitive elements located within the latter (Razin, 1994; Ursi *et al.*, 2003; Michelow *et al.*, 2004; Miyashita *et al.*, 2004; Nour *et al.*, 2005). The P1 cytadhesin gene encodes the 169 kDa P1 protein of *M. pneumoniae*. This protein is the major virulence and adhesin factor of *M. pneumoniae* and is located at the terminal attachment organelle, wherein it attaches to host cells (Svenstrup *et al.*, 2002). Although similar genes are found in some other mycoplasma species, highly conserved regions of the sequence are unique to *M. pneumoniae* and this gene is therefore an attractive target for the design of species-specific PCR primers (Su *et al.*, 1987). The use of the Roche LightCycler with fluorescence resonance energy transfer hybridization probes has been applied to the detection of several bacterial pathogens including *Chlamydia pneumoniae* (Mygind *et al.*, 2001), *Neisseria gonorrhoeae* (Whiley *et al.*, 2002), *Bordetella* spp. (Cloud *et al.*, 2003) and *M. pneumoniae* (Ursi *et al.*, 2003). Multiplex assays have also been described that enable the simultaneous detection of *M. pneumoniae* with other pathogens (Khanna *et al.*, 2005; Raggam *et al.*, 2005; Stralin *et al.*, 2005). The purpose of this study was to establish a reliable, specific, sensitive and quantitative real-time PCR to detect *M. pneumoniae* in clinical specimens and to improve the current clinical diagnostic service. An internal processing control (IPC) was included to highlight amplification failure of the reaction due to inhibition.

Abbreviations: Cp, crossing point; IPC, internal processing control.

The sequence of the internal processing control bacteriophage lambda fragment is available as supplementary material in JMM Online.

METHODS

Clinical samples. A panel of 167 clinical respiratory samples (sputa and bronchoscopy specimens, group A), urine and acute and convalescent serum samples were taken on admission to hospital in 1991–1992 as part of a prospective clinical trial. Patients were excluded from the study if they were undergoing antibiotic therapy immediately prior to presentation at hospital. Samples were taken from adult patients (mean age 46 years, SD ± 19·6, 95 % CI 41·4–51·4, range 18–80) with radiologically confirmed acute community-acquired pneumonia and those of proven aetiology were made

available for retrospective examination in this study. Clinical samples from the patients had previously been examined immediately after collection for evidence of a range of respiratory pathogens by standard culture or serological methods as shown in Table 1. An additional eight sputum specimens (group B) from patients with pneumonia that had been serologically confirmed as *M. pneumoniae* positive were included in the study; four of these samples were also confirmed as positive for *M. pneumoniae* by culture. Samples were then frozen at −80 °C and later tested for *Legionella* and other atypical pathogens, including serological testing for *M. pneumoniae* IgG and IgM antibodies by indirect immunofluorescence (Wreghitt & Sillis, 1985). After several years, respiratory samples were thawed, treated with Sputasol (Oxoid) for 30 min at room temperature, heated for 10 min at 65 °C and heated for 15 min at 100 °C to ensure inactivation of any tubercle bacilli or other pathogens. DNA was then extracted from 200 µl of each respiratory sample using the QIAamp DNA Mini kit (Qiagen). The eluted samples (200 µl) were tested immediately for *M. pneumoniae* DNA.

Reference strains. The following reference strains were included in this study. Mollicute species: *Acholeplasma laidlawii* NCTC 10116^T, *Mycoplasma amphoriforme* NCTC 11740^T, *Mycoplasma buccale* NCTC 10136^T, *Mycoplasma faucium* NCTC 10174^T, *Mycoplasma fermentans* NCTC 10117^T, *Mycoplasma genitalium* NCTC 10195^T, *Mycoplasma*

Table 1. *M. pneumoniae* LightCycler PCR results on respiratory samples from adults with pneumonia of proven aetiology

Evidence for aetiology: *Chlamydophila* spp., *Coxiella burnetii*, RSV, influenza A and influenza B by complement-fixation test; *Streptococcus pneumoniae* cultured from blood, bronchoscopy samples, sputum or antigen in urine; *Enterobacteriaceae*, β-haemolytic streptococci, *H. influenzae*, *Moraxella catarrhalis* and *Staphylococcus aureus* cultured from bronchoscopy samples or sputum; *L. pneumophila* antigen in urine or serology. Sputum isolates were considered causative if $\geqslant 10^6$ c.f.u. ml^{-1} (*Streptococcus pneumoniae*) or $\geqslant 10^7$ c.f.u. ml^{-1} (other organisms) were detected with $\geqslant 25$ polymorphonuclear cells ml^{-1}. *M. pneumoniae*-positive samples were determined by immunofluorescence.

Causative organism	PCR positive/total (%)
Group A	16/167 (9·6)
Single infections	5/132
Chlamydophila spp.	1/9
Coxiella burnetii	0/1
Enterobacteriaceae	0/2
β-Haemolytic streptococci	0/3
Haemophilus influenzae	1/33
Influenza A virus	0/1
Influenza B virus	0/2
Legionella pneumophila	0/4
Moraxella catarrhalis	0/2
Respiratory syncytial virus (RSV)	0/2
Staphylococcus aureus	0/3
Streptococcus pneumoniae	3/70
Mixed infections	0/18
Coxiella burnetii and β-haemolytic streptococci	0/1
H. influenzae and *Moraxella catarrhalis*	0/1
Staphylococcus aureus and influenza B virus	0/1
Streptococcus pneumoniae and *Chlamydophila* spp.	0/1
Streptococcus pneumoniae and *H. influenzae*	0/8
Streptococcus pneumoniae and influenza B virus	0/2
Streptococcus pneumoniae and *Moraxella catarrhalis*	0/3
Streptococcus pneumoniae and RSV	0/1
M. pneumoniae confirmed	11/17
M. pneumoniae	10/15
M. pneumoniae and β-haemolytic streptococci	1/1
M. pneumoniae and RSV	0/1
Group B	4/8 (50·0)
M. pneumoniae serology-positive, culture-negative	0/4
M. pneumoniae serology-positive, culture-positive	4/4
Total PCR positive (Group A + B)	**20/175 (11·4)**
Total PCR positive, serology-positive	**15/25 (60·0)**
Total PCR positive, serology-negative	**5/150 (3·3)**

hominis NCTC 10111^T, *Mycoplasma lipophilum* NCTC 10173^T, *Mycoplasma orale* NCTC 10112^T, *Mycoplasma penetrans* ATCC 55252^T, *Mycoplasma pirum* NCTC 11702^T, *M. pneumoniae* NCTC 10119^T, *Mycoplasma salivarium* NCTC 10113^T, *Mycoplasma spermatophilum* NCTC 11720^T, *Ureaplasma parvum* ATCC 33697, *Ureaplasma urealyticum* NCTC 10177^T. Respiratory species: *Actinomyces odontolyticus* NCTC 9935^T, *Bordetella parapertussis* NCTC 5952^T, *Bordetella pertussis* NCTC 10739^T, *Burkholderia cepacia* NCTC 10743, *Chlamydophila pneumoniae* IOL-207, *Chlamydophila psittaci* 6BC^T, *Corynebacterium diphtheriae* NCTC 10356, *Enterococcus faecalis* NCTC 775^T, *Escherichia coli* NCTC 9001^T, *Haemophilus influenzae* NCTC 8143^T, *Klebsiella pneumoniae* subsp. *pneumoniae* NCTC 9633^T, *Legionella pneumophila* NCTC 11192^T, *Moraxella catarrhalis* NCTC 11020^T, *Pseudomonas aeruginosa* NCTC 10332^T, *Rothia dentocariosa* NCTC 10917^T, *Staphylococcus aureus* NCTC 8532^T, group G streptococcus NCTC 9603, *Streptococcus pneumoniae* NCTC 7465^T, *Streptococcus pyogenes* NCTC 12067.

Extraction of DNA from bacterial cultures. Cultures of mollicute species, *A. odontolyticus* and *R. dentocariosa* (5 ml) were concentrated by centrifugation at 8000 ***g*** for 15 min, resuspended in 180 µl nuclease-free water and extracted with 20 µl Instagene matrix (Bio-Rad) according to the manufacturer's instructions. All other strains used in the specificity panel were extracted by using the Roche MagNAPure robot using the DNA isolation kit III (Roche Diagnostics) according to the manufacturer's instructions. DNA concentration was adjusted to approximately 20 pg µl^{-1} for use in the LightCycler assay.

Primers and probes. The sequences of all primers and probes are listed in Table 2. Primers specific for the RepMP2 repetitive element (GenBank accession no. X13087) of the P1 cytadhesin gene of *M. pneumoniae* were designed using the primer selection program OLIGO (Medprobe) to amplify a PCR product of 141 bp. Within this sequence, two probes were designed to a 43 bp region with a 2 bp gap located proximal to the 3′ end of the sense sequence.

Construction of a PCR IPC. A 278 bp sequence from λ phage DNA into which *M. pneumoniae* primer sites were incorporated was amplified using primer sequences for *M. pneumoniae* with λ phage amplifying sequences at their 3′ ends (Table 2; full sequence available as supplementary material in JMM Online). λ DNA (1 µg; Sigma) was digested with *Eco*RI for 3 h. The restricted DNA (10 ng) was used as a template in a 50 µl PCR containing 150 mM NaCl, 1·5 mM $MgCl_2$, 10 mM Tris/HCl (pH 8·0), 1 U *Taq* DNA polymerase (Gibco-BRL), 10 pM each of primers M-IPC-1 and M-IPC-2, and 200 µM deoxyribonucleotides. PCR was performed on a Peltier Thermal Cycler (225) DNA Engine Tetrad and reaction conditions were 30 cycles of 95 °C for 30 s, 40 °C for 1 min and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The product size was confirmed by electrophoresis and the concentration of the product was estimated using a GeneQuant spectrophotometer (Pharmacia). The product was then cloned into the pCR2.1TOPO plasmid using the TOPO TA cloning kit (Invitrogen), transformed into *Escherichia coli* INVαF′ and positive transformants were selected according to the manufacturer's instructions. Positive transformants were cultured overnight in Luria–Bertani broth (5 ml) containing 50 µg ampicillin ml^{-1} and plasmid DNA was extracted using a Plasmid Mini kit (Qiagen), adjusted to a concentration of 1 ng µl^{-1} and then heated at 95 °C for 5 min. Plasmid preparations with inserts of the expected size (278 bp) were confirmed by PCR selection as follows. PCR reagents were as above with the inclusion of primers Mpn-3 and Mpn-4 (Table 2) and reaction conditions were 30 cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. The resulting IPC plasmid was linearized by digestion with *Xba*I, adjusted to 10^{10} copies µl^{-1} in TE buffer [10 mM Tris/HCl (pH 8·0), 1 mM EDTA] containing herring sperm DNA (10 ng µl^{-1}; Gibco-BRL) and stored as 35 µl aliquots at −80 °C. For use in the assay, stocks of IPC were diluted to 10^2 copies µl^{-1} in nuclease-free water.

Construction of a *M. pneumoniae* PCR positive control. To alleviate problems associated with growing large volumes of *M. pneumoniae* to generate stocks of standard DNA, a cloned template DNA sequence was prepared for use as a positive control. A 410 bp PCR product of the P1 cytadhesin gene, external to the amplicon in the LightCycler assay, was amplified using primers Mpn-1 and Mpn-2 (Table 2) from *M. pneumoniae* strain FH (NCTC 10119^T). This product was cloned and prepared as above and a stock solution containing 10^{10} copies µl^{-1} was prepared in TE buffer containing herring sperm DNA (10 ng µl^{-1}). The stock solution was serially diluted in nuclease-free water to give a concentration range that covered the expected dynamic range of the clinical specimens. Each LightCycler run included positive-control samples with 10^4, 10^3, 10^2 and 10^1 copies per PCR and a negative control (nuclease-free water). To assess reproducibility and stability, the crossing-point (Cp) values of these standards were monitored over several months.

LightCycler assay. Assays were carried out using a LightCycler (Roche) and the $MgCl_2$ concentration was optimized according to the manufacturer's instructions. Reaction volumes (20 µl in total) in glass capillary tubes (Roche) were used containing 2 µl of LightCycler FastStart DNA Master Hybridization Probes (which includes

Table 2. Primers and probes

Primers and purpose	Name	Sequence (5′→3′)
Cloning standard, forward	Mpn-1	AGGGGGTTCCTTCAGGCTCAG
Cloning standard, reverse	Mpn-2	GGATCAAACAGATCGGTGACTGGGT
Cloning internal processing control (IPC), forward*	M-IPC-1	TCTTCAGGCTCAGGTCAAatgcgtaagcgaaaca
Cloning IPC, reverse*	M-IPC-2	TTCCCCGTATTAGTATTAGGCgcataaacgaagcagtcgagt
LightCycler assay, forward	Mpn-3	TCTTCAGGCTCAGGTCAA
LightCycler assay, reverse	Mpn-4	TTCCCCGTATTAGTATTAGGC
Donor probe for *M. pneumoniae*	Mpn-DN	CAGTTACCAAGCACGAGTGAC-(FITC)
Acceptor probe for *M. pneumoniae*	Mpn-AC	(Red 640)-AAACACCTCCTCCACCAACA-(PO_4)
Donor probe for IPC	IPC-DN	GGTGCCGTTCACTTCACTTCCCGAATAAC-(FITC)
Acceptor probe for IPC	IPC-AC	(Red 705)-GATATTTTTGATCTGACCGAAGCGC-(PO_4)

*IPC: *M. pneumoniae* primer sequence in upper case, λ primer sequence in lower case.

reaction buffer, nucleotides and *Taq* polymerase; Roche), 1·0 μM Mpn-3 and Mpn-4 primers (Table 2), 5 mM $MgCl_2$, 0·25 μM each of the probes Mpn-DN, Mpn-AC, IPC-DN and IPC-AC (Table 2), 10^2 copies of the IPC, 1 U uracil-DNA glycosylase (Roche) and 5 μl sample or positive/negative control. LightCycler FastStart DNA Master Hybridization Probes (Roche) reagent contains dUTP (not dTTP), and uracil-DNA glycosylase (Roche) was added to each reaction to eliminate carry-over contamination (Longo *et al.*, 1990). Capillaries were sealed and placed in the LightCycler and the following cycling conditions applied: initial denaturation at 96 °C for 10 min (transition rate 20 °C s^{-1}; acquisition mode none); 45 quantification cycles of 95 °C for 10 s (transition rate 20 °C s^{-1}; acquisition mode none), 62 °C for 10 s (transition rate 20 °C s^{-1}; acquisition mode single) and 74 °C for 15 s (transition rate 3 °C s^{-1}; acquisition mode none); melting curve cycle of 95 °C for 0 s (transition rate 20 °C s^{-1}; acquisition mode none), 45 °C for 2 s (transition rate 20 °C s^{-1}; acquisition mode none) and 85 °C for 0 s (transition rate 0·1 °C s^{-1}; acquisition mode cont); cooling at 40 °C for 30 s (transition rate 0 °C s^{-1}; acquisition mode none).

Data were analysed using Roche LightCycler software version 3.5 using arithmetic baseline adjustment and second-derivative maximum analysis (Fig. 1). Copy number was estimated from the Cp threshold relative to positive standards; samples that did not give a positive result and in which the IPC did not amplify were recorded as inhibitory and were repeated undiluted and diluted 1/10 in nuclease-free water (Promega).

RESULTS AND DISCUSSION

The use of real-time PCR was employed to detect *M. pneumoniae* in clinical samples using a Roche LightCycler. In total, 30/175 (17·1 %) samples were positive by PCR, culture or serology. This figure could not be linked to prevalence or carriage in the population, as all patients had clinical signs of pneumonia and control samples from healthy patients were not available for study.

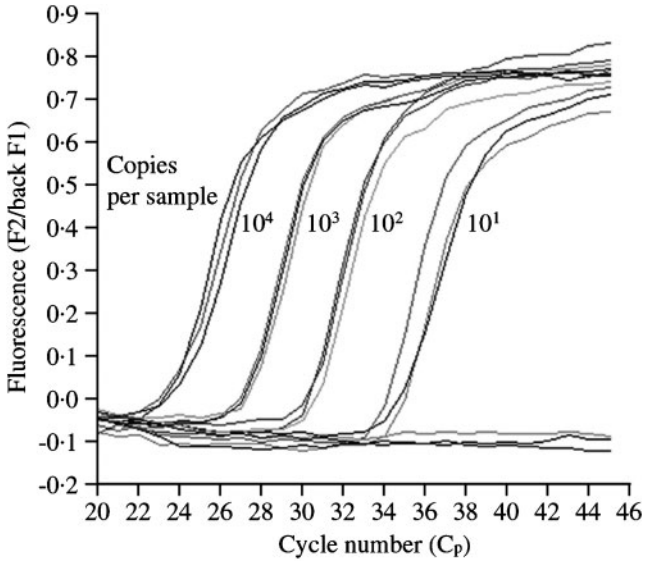

Fig. 1. Replicate standards of *M. pneumoniae* cloned DNA from 10^1 to 10^4 copies per sample. Channel F2, Red 640 fluorescence.

Sensitivity

The assay detected 10 copies or more of the target sequence in the 5 μl samples used in the assay [corresponding to $2{\cdot}0 \times 10^3$ organisms (ml extracted concentrated DNA)$^{-1}$ and 1×10^3 organisms (ml actual clinical sample)$^{-1}$]. The estimated copy number in actual clinical samples from proven *M. pneumoniae* patients ranged from $5{\cdot}0 \times 10^3$ to $6{\cdot}5 \times 10^7$ organisms ml^{-1}. Samples with a Cp equal to or less than the lowest standard in the assay were deemed negative. Due to the absence of control specimens from healthy individuals, this study could not ascertain the level of *M. pneumoniae* in subclinical colonization and the use of the method in population screening is therefore limited. The LightCycler is capable of measuring the genome load in clinical specimens accurately, yet in practice, it is of little use in applying quantitative analyses to bacterial numbers in the respiratory tract, as specimens vary considerably in volume, consistency and composition and contain substances inhibitory to PCR. The measurement of an estimated bacterial load in this case can only be considered as semi-quantitative, and without a case–control study examining *M. pneumoniae* load in clinical specimens from both healthy and pneumonic specimens over the infective period little clinical relevance can be drawn other than the presence or absence of detectable *M. pneumoniae* DNA. None the less, of the 175 pneumonic patient samples tested, 20 (11·4 %) were positive in the real-time assay (Table 1). In 25 of 175 patients, a diagnosis of *M. pneumoniae* infection had been previously established by serology, of which 15/25 (60·0 %) were positive by this real-time method (groups A and B): four had also previously been confirmed by culture (group B). Four samples were serologically positive, yet negative by culture and PCR (group B). This may be due to cross-reactive antibody tests, increased sensitivity of antibody detection methods in comparison with PCR and culture, clearance of the micro-organism from the body or early treatment with antibiotics. The latter is unlikely as patients receiving antibiotic therapy were excluded from the study. It is possible that a period occurs during the infection process that is optimal for the detection of *M. pneumoniae* by PCR. Specimens were taken on admission to hospital, which corresponded to between 0 and 17 days after the onset of clinical signs. Those found to be positive by PCR in group A were taken on average 7 days after onset (2–17 days, SD ± 4·48) of symptoms, in comparison with PCR-negative samples taken 3 days post-onset (0–12 days, SD ± 2·36).

The sensitivity was estimated at 60·0 % in comparison with serological positives obtained from paired acute and convalescent serum samples. Paired serum samples are not often received by clinical laboratories and comparison of the PCR assay with serology on single serum samples may have reflected use in practice more accurately. This level of sensitivity may be considered low; however, reports from other publications and the basis for calculating sensitivity have varied considerably. Michelow *et al.* (2004) reported a respiratory-sample PCR with a sensitivity of 57 % and specificity of 98 % when compared with *M. pneumoniae*

ELISA. Hardegger *et al.* (2000) reported a P1 gene TaqMan real-time assay to detect *M. pneumoniae* that could detect a 1 : 100 dilution of a DNA sample. However, no indication of DNA concentration or target copy number in positive-control samples was given and it is not possible to make a direct comparison of sensitivity between this and other methods. Miyashita *et al.* (2004) noted that their multiplex assay could detect 100 copies ml^{-1}, and Dorigo-Zetsma *et al.* (1999) reported an estimated sensitivity of 78 % in a block-based PCR. Using a similar assay to that described in this study, Ursi *et al.* (2003) noted a sensitivity of 5×10^3–5×10^4 organisms ml^{-1}, indicating that the assay described here is 5–50 times more sensitive. Our assay could be improved further by the addition of a second *M. pneumoniae* target, allowing confirmation of positive results simultaneously with detection. Sensitivity can be dependent on several factors, such as the method used for comparison and selection criteria for evidence of infection (PCR, culture or sero-conversion as the 'gold standard'), the presence of competitive DNA and inhibitors, sample type (swab, aspirate, sputum), method, age, transport and storage, operator variance, sample dilution and the method of extraction. Ievens *et al.* (1996) compared the PCR sensitivities of a simple boiling/freezing extraction and a guanidium thiocyanate/phenol extraction in two separate laboratories and found that results varied considerably between techniques and laboratories. This retrospective study utilized archived samples that had been stored at −80 °C for several years and had been heat-treated; therefore deterioration in the quality of the DNA in the samples could have occurred. Furthermore, immunofluorescence for *M. pneumoniae* antibody detection is highly subjective and, due to the lack of standardization across the techniques, caution should be employed when comparing techniques for *M. pneumoniae* detection (Loens *et al.*, 2003). These factors make inter-assay comparisons complex. Furthermore, no formal external quality assessment schemes for *M. pneumoniae* serology or molecular detection exist. Such a scheme could provide highly beneficial data regarding the detection of infection with this pathogen.

Specificity

The assay was found to be highly specific in that DNA extracted from reference cultures (15 human mollicute species and 19 common respiratory bacteria) did not give a positive signal in the assay, even when tested at concentrations significantly higher than the limit of detection. A search of GenBank using the BLAST algorithm (Altschul *et al.*, 1990) revealed no significant homology of the target sequence with any other known genes (E value = 5·8). Of the 150 patients where the primary diagnosis was not infection with *M. pneumoniae* infection, five (3·3 %) were PCR positive for *M. pneumoniae*. One had been serologically confirmed as positive for *Chlamydophila* spp. by complement fixation tests, one as *H. influenzae* positive by isolation from sputum and three as *S. pneumoniae* positive by isolation from sputum or antigen detection. This gives an overall percentage specificity of 96·7 % (5/150). These results could represent either false-positive reactions with other bacterial DNA, co-infection with *M. pneumoniae* prior to the formation of a detectable serum-antibody response or detection of residual *M. pneumoniae* DNA from a past infection. The former is unlikely, as none of the DNA from these three pathogens resulted in amplification in the assay. In addition, all five samples had low concentrations of detectable *M. pneumoniae* DNA (< 260 copies per reaction). Low levels of colonization may be detected by PCR prior to a detectable serological response in patients with or without symptoms. Indeed, several specimens from patients that were negative serologically exhibited Cp values close to the limits of detection (< 10 copies per sample). This may represent a technical artefact or could be detection of low levels of infection. Asymptomatic carriage has been reported to occur within the community and up to 13 % of healthy adults may act as carriers of *M. pneumoniae*, particularly during epidemic periods (Gnarpe *et al.*, 1992; Foy, 1993). Interestingly, Principi *et al.* (2001) detected *M. pneumoniae* by PCR in 3·8 % (16/419) of children without serological evidence of acute infection. Patients positive for *M. pneumoniae* had a mean age of 37·8 (SD ± 18·3, 95 % CI 29·2–46·3, range 18–78), significantly younger than patients in which *M. pneumoniae* was not detected (mean age of 50·6, SD ± 19·1, 95 % CI 44·6–56·7, range 18–80) (Fisher's exact test, $P = 0{\cdot}014$).

Control of inhibition

It is essential that respiratory samples inhibitory to real-time PCR are identified to ensure that they are not falsely reported as negative. Single-tube methods that simultaneously detect IPC and target DNA amplicons of differing lengths with the same primers have been used in isothermal amplification and real-time methods for *M. pneumoniae* (Loens *et al.*, 2002; Ursi *et al.*, 2003). We developed a similar IPC consisting of a cloned PCR fragment of the λ phage genome flanked by primer sites for the *M. pneumoniae* PCR. When used with target and λ-specific probes that fluoresce at different wavelengths, the same set of primers amplifies both *M. pneumoniae* and IPC, detecting both products simultaneously. Furthermore, the size of the IPC amplicon (278 bp) is larger than that of the target (141 bp) amplicon and the latter reaction is driven preferentially at the expense of the former (Fig. 2). Only when target DNA is low in concentration or absent will any IPC sequence be amplified, and inhibition is detected in reactions with an absence of both target DNA and IPC amplicons. The IPC was stable when Cp values were monitored over several months with a SD of approximately 1·0 for 10 sets of standards (10^1 copies per reaction: Cp 32·1–35·4, mean 33·5, SD ± 1·03; 10^2 copies per reaction: Cp 28·8–31·3, mean 30·0, SD ± 0·87; 10^3 copies per reaction: Cp 24·5–27·2, mean 25·9, SD ± 0·99; 10^4 copies per reaction: Cp 21·5–24·4, mean 22·4, SD ± 0·98; 10^5 copies per reaction: Cp 16·1–19·8, mean 17·9, SD ± 0·97).

In the current study, 40/167 (24·0 %) of undiluted samples were inhibitory to the assay (note that eight samples were

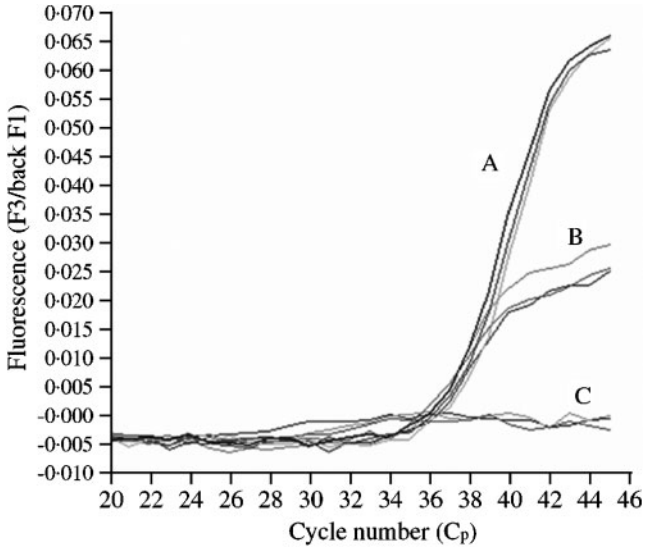

Fig. 2. Internal processing control (IPC) at a concentration of 10^2 copies per PCR in the presence of no target DNA (A), 10^1 copies target DNA (B), or 10^2 copies target DNA (C). Channel F3, Red 705 fluorescence.

only tested at a 1/10 dilution due to limited sample volume). By retesting a 10-fold dilution, this was reduced to 7/175 (4·0 %). These results support those of Dorigo-Zetsma *et al.* (1999), in which 20 % of throat-swab extracts inhibited amplification for *M. pneumoniae* and this inhibition was removed by sample dilution. Abele-Horn *et al.* (1998) reported inhibition in 10 % of samples, which was reduced to 2 % by the use of a 1 day culture enhancement. Raggam *et al.* (2005) and Stralin *et al.* (2005) found no inhibition with respiratory samples. Ursi *et al.* (2003) reported that 2/115 (1·7 %) respiratory samples were inhibitory. Both sample viscosity (Loens *et al.*, 2002) and the pretreatment of samples with mucolytic agents (dithiothreitol) used to reduce viscosity (Deneer & Knight, 1994) have been reported to cause false-negative results. In this study, differing extraction methods and the resulting effect on assay performance were not examined. A single method of extraction was employed that is well established in the laboratory for extracting DNA from respiratory specimens (QiAamp DNA Mini kit; Qiagen). Total DNA content may also affect PCR whereby increasing amounts of non-target DNA inhibit amplification of the target DNA. In respiratory specimens, human DNA, as well as DNA from other micro-organisms, may cause inhibition (Ludwig & Schleifer, 2000).

This study is similar to that by Ursi *et al.* (2003) in that a real-time assay for the P1 gene of *M. pneumoniae* with the same primer IPC was employed to compare detection of *M. pneumoniae* in respiratory specimens. Both studies used the same platform, similar methods of DNA extraction and a similar methodology and detected comparable concentrations of *M. pneumoniae* in respiratory samples (Ursi *et al.*, 2003: 5×10^3–5×10^{10} organisms ml^{-1}; this study: 5×10^3–6×10^7organisms ml^{-1}). However, our study examined a larger number of patients (175 compared with 82), improved the limit of detection by at least fivefold (1×10^3 compared with 5×10^3–5×10^4 organisms ml^{-1}) and was tested against a comprehensive panel of human *Mycoplasma* species and respiratory pathogens. Another important difference to note is that this study compared real-time PCR with serologically positive patients (and some that were culture-positive), whereas Ursi *et al.* (2003) compared the real-time assay with isothermal PCR using exactly the same target. Ursi *et al.* (2003) recommended testing lower respiratory tract specimens rather than upper respiratory tract specimens for *M. pneumoniae* due to the higher concentrations of detectable *M. pneumoniae* DNA. Here, lower respiratory tract samples from patients with clinically defined pneumonia that had been screened previously for a large panel of respiratory pathogens were tested. This may account for the difference noted in the number of inhibitory samples: Ursi *et al.* (2003) tested throat swabs, throat washings and sputa, with 2/115 (1·7 %) showing inhibition that was eliminated by sample dilution. This study comprised lower respiratory tract samples only, of which 24 % were inhibitory; this was reduced to 4 % by dilution. We therefore recommend testing of all lower respiratory tract DNA samples undiluted and at a 1/10 dilution for *M. pneumoniae* detection.

Conclusions

This assay for detecting *M. pneumoniae* in the respiratory tract is reproducible, with a sensitivity of 60·0 % in comparison with serology and a specificity of 96·7 %. The method is rapid and up to 27 extracted samples can be assayed in less than an hour, ideal for same-day screening of patients with respiratory symptoms. The use of a co-amplified IPC in the same tube and employing the same primers as the target sample increases the throughput of specimens for diagnosis and reduces cost. The use of undiluted and 1/10 dilutions of specimens decreased the observed inhibition from 24 to 4 %. Validation of extraction methods to resolve sample inhibition would be beneficial, but would require large volumes of clinical specimens. Dual infections were found in five (3·3 %) of the samples tested, and overall 20/175 (11·4 %) samples were PCR positive for *M. pneumoniae*. This study included validation of the assay on respiratory specimens that are highly defined for other respiratory infections, but was limited by the absence of control specimens from healthy individuals and the age of the specimens under test. It is difficult to gain access to respiratory specimens that have been tested for a full range of respiratory specimens with relevant clinical and matching serological data. Such a collection could be invaluable for assay comparison to ensure the best patient service as new techniques for *M. pneumoniae* and other fastidious respiratory pathogens are described.

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REFERENCES

Abele-Horn, M., Busch, U., Nitschko, H., Jacobs, E., Bax, R., Pfaff, F., Schaffer, B. & Heesemann, J. (1998). Molecular approaches to diagnosis of pulmonary diseases due to *Mycoplasma pneumoniae*. *J Clin Microbiol* **36**, 548–551.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.

Cloud, J. L., Hymas, W. C., Turlak, A., Croft, A., Reischl, U., Daly, J. A. & Carroll, K. C. (2003). Description of a multiplex *Bordetella pertussis* and *Bordetella parapertussis* LightCycler® PCR assay with inhibition control. *Diagn Microbiol Infect Dis* **46**, 189–195.

Clyde, W. A., Jr (1993). Clinical overview of typical *Mycoplasma pneumoniae* infections. *Clin Infect Dis* **17** (Suppl. 1), S32–S36.

Deneer, H. G. & Knight, I. (1994). Inhibition of the polymerase chain reaction by mucolytic agents. *Clin Chem* **40**, 171–172.

Dorigo-Zetsma, J. W., Zaat, S. A. J., Wertheim-van Dillen, P. M. E., Spanjaard, L., Rijntjes, J., van Waveren, G., Jensen, J. S., Angulo, A. F. & Dankert, J. (1999). Comparison of PCR, culture and serological tests for diagnosis of *Mycoplasma pneumoniae* respiratory tract infection in children. *J Clin Microbiol* **37**, 14–17.

Foy, H. M. (1993). Infections caused by *Mycoplasma pneumoniae* and possible carrier state in different populations of patients. *Clin Infect Dis* **17** (Suppl. 1), S37–S46.

Friedlaender, R. P., Grizzard, M. B., Helms, C. M., Barile, M. F., Senterfit, L. & Chanock, R. M. (1976). Experimental production of respiratory tract infection with *Mycoplasma pneumoniae* in rhesus monkeys. *J Infect Dis* **133**, 343–346.

Gnarpe, J., Lundback, A., Sundelof, B. & Gnarpe, H. (1992). Prevalence of *Mycoplasma pneumoniae* in subjectively healthy individuals. *Scand J Infect Dis* **24**, 161–164.

Hardegger, D., Nadal, D., Bossart, W., Altwegg, M. & Dutly, F. (2000). Rapid detection of *Mycoplasma pneumoniae* in clinical samples by real-time PCR. *J Microbiol Methods* **41**, 45–51.

Ievens, M., Ursi, D., van Bever, H., Quint, W., Niesters, H. G. M. & Goossens, H. (1996). Detection of *Mycoplasma pneumoniae* by two polymerase chain reactions and role of *M. pneumoniae* in acute respiratory tract infections in pediatric patients. *J Infect Dis* **173**, 1445–1452.

Khanna, M., Fan, J., Pehler-Harrington, K., Waters, C., Douglass, P., Stallock, J., Kehl, S. & Henrickson, K. J. (2005). The pneumoplex assays, a multiplex PCR-enzyme hybridization assay that allows simultaneous detection of five organisms, *Mycoplasma pneumoniae*, *Chlamydia* (*Chlamydophila*) *pneumoniae*, *Legionella pneumophila*, *Legionella micdadei*, and *Bordetella pertussis*, and its real-time counterpart. *J Clin Microbiol* **43**, 565–571.

Loens, K., Ursi, D., Ieven, M., van Aarle, P., Sillekens, P., Oudshoorn, P. & Goosens, H. (2002). Detection of *Mycoplasma pneumoniae* in spiked clinical samples by nucleic acid sequence-based amplification. *J Clin Microbiol* **40**, 1339–1345.

Loens, K., Ursi, D., Goossens, H. & Leven, M. (2003). Molecular diagnosis of *Mycoplasma pneumoniae* respiratory tract infections. *J Clin Microbiol* **41**, 4915–4923.

Longo, M. C., Berninger, M. S. & Hartley, J. L. (1990). Use of uracil DNA glycosylate to control carry-over in polymerase chain reaction. *Gene* **93**, 125–128.

Ludwig, W. & Schleifer, K.-H. (2000). How quantitative is quantitative PCR with respect to cell counts? *Syst Appl Microbiol* **23**, 556–562.

McMillan, J. A. (1998). *Mycoplasma pneumoniae* infection in pediatrics. *Semin Pediatr Infect Dis* **9**, 112–119.

Michelow, I. C., Olsen, K., Lozano, J., Duffy, L. B., McCracken, G. H. & Hardy, R. D. (2004). Diagnostic utility and clinical significance of naso- and oropharyngeal samples used in a PCR assay to diagnose *Mycoplasma pneumoniae* infection in children with community-acquired pneumonia. *J Clin Microbiol* **42**, 3339–3341.

Miyashita, N., Saito, A., Kohno, S., Yamaguchi, K., Watanabe, A., Oda, H., Kazuyama, Y., Matsushima, T. & the CAP Study Group (2004). Multiplex PCR for the simultaneous detection of *Chlamydia pneumoniae*, *Mycoplasma pneumoniae* and *Legionella pneumophila* in community-acquired pneumonia. CAP Study Group. *Respir Med* **98**, 542–550.

Mygind, T., Birkelund, S., Falk, E. & Christiansen, G. (2001). Evaluation of real-time quantitative PCR for identification and quantification of *Chlamydia pneumoniae* by comparison with immunohistochemistry. *J Microbiol Methods* **46**, 241–251.

Nour, M., Trabelsi, A., Maatouk, N. & Hammami, M. (2005). Amplification of P1 and 16S rRNA genes by nested PCR for detection of *Mycoplasma pneumoniae* in paediatric patients. *Pathol Biol (Paris)* **53**, 9–14.

Principi, N., Esposito, S., Blasi, F., Allegra, L. & the Mowgli Study Group (2001). Role of *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in children with community-acquired lower respiratory tract infections. *Clin Infect Dis* **32**, 1281–1289.

Raggam, R. B., Leitner, E., Berg, J., Mühlbauer, G., Marth, E. & Kessler, H. H. (2005). Single-run, parallel detection of DNA from three pneumonia-producing bacteria by real-time polymerase chain reaction. *J Mol Diagn* **7**, 133–138.

Razin, S. (1994). DNA probes and PCR in diagnosis of mycoplasma infections. *Mol Cell Probes* **8**, 497–511.

Sillis, M. (1993). Modern methods for diagnosis of *Mycoplasma pneumoniae*. *Rev Med Microbiol* **4**, 24–31.

Stralin, K., Backman, A., Holmberg, H., Fredlund, H. & Olcen, P. (2005). Design of a multiplex PCR for *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma pneumoniae* and *Chlamydophila pneumoniae* to be used on sputum samples. *APMIS* **113**, 99–111.

Su, C. J., Tryon, V. V. & Baseman, J. B. (1987). Cloning and sequence analysis of cytadhesin P1 gene from *Mycoplasma pneumoniae*. *Infect Immun* **55**, 3023–3029.

Svenstrup, H. F., Nielsen, P. K., Drasbek, M., Birkelund, S. & Christiansen, G. (2002). Adhesion and inhibition assay of *Mycoplasma genitalium* and *M. pneumoniae* by immunofluorescence microscopy. *J Med Microbiol* **51**, 361–373.

Ursi, D., Dirven, K., Loens, K., Ieven, M. & Goossens, H. (2003). Detection of *Mycoplasma pneumoniae* in respiratory samples by real-time PCR using an inhibition control. *J Microbiol Methods* **55**, 149–153.

Whiley, D. M., LeCornec, G. M., Mackay, I. M., Siebert, D. J. & Sloots, T. P. (2002). A real-time PCR assay for the detection of *Neisseria gonorrhoeae* by LightCycler. *Diagn Microbiol Infect Dis* **42**, 85–89.

Wreghitt, T. G. & Sillis, M. (1985). A micro-capture ELISA for detecting *Mycoplasma pneumoniae* IgM: comparison with indirect immunofluorescence and indirect ELISA. *J Hyg* **94**, 217–227.