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1 ***Mycoplasma pneumoniae* infection in primary care investigated by real-time PCR**
2 **in England and Wales.**

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13

Abstract

Real-time PCR was employed to detect a conserved region of the P1 cytoadhesin gene of *Mycoplasma pneumoniae* in combined nose and throat swabs collected from patients attending GP surgeries during 2005-2009 with symptoms of respiratory tract infection (RTI). Samples were collected as part of an annual winter epidemiological and virological linked study in England and Wales. A total of 3987 samples were tested, 65 (1.7%, 95%CI 1.3-2.1) had detectable *M. pneumoniae* DNA. Positive patients were detected of both sex, aged from 9 months to 78 years, had clinical signs of upper RTI, fever and/or myalgia, influenza like illness to lower RTI. Mixed infections were identified in four cases, two with influenza A H1, one with H3 and one with influenza B. Children aged 5-14 years were more likely to have detectable *M. pneumoniae* in samples than all other age groups (Fishers $p = 0.03$), attributed to the 2005-2006 season in which 6.0% (12/200, 95%CI 3.4-10.3) of 5-14 year olds had detectable *M. pneumoniae* in comparison to 2.2% in 2006-2007 (3/141 95%CI 0.5-6.4), 2.2% in 2007-2008 (2/89 95%CI 0.1-8.3) and 0% in 2008-2009 (0/151 95%CI 0-2.9).

Key words *Mycoplasma pneumoniae*, surveillance, PCR

Introduction

Mycoplasma pneumoniae is a common cause of community-acquired pneumonia (CAP) transmitted by aerosol or close contact. Major increases and decreases in *M.pneumoniae* infection have occurred periodically in the UK and severity of clinical symptoms range from asymptomatic to pronounced pneumonia [2]. *Mycoplasma pneumoniae* is found in all age groups however it is one of the most common etiological agents of CAP found in children with pneumonia over 5 years old [1]. Admissions to a UK hospital in patients with CAP that were attributable to *M. pneumoniae* were estimated at 18% in 1982 and 4% in 1999 [11]. Historically within the UK *M. pneumoniae* epidemics have occurred at approximately four yearly intervals and have lasted 12 to 15 months, concurrent with sporadic infection at a lower level (Fig. 1). In England and Wales data from voluntary laboratory reports are collated by the Health Protection Agency Centre for Infections (HPA) to give a representative indication of the number of patients testing positive for *M. pneumoniae* per week, (Fig. 1). Data on patients testing negative is not available and because reporting is voluntary is it not known whether the data reported is a true representation of the number of positive cases in England and Wales. Furthermore, since 1999 the number of voluntary reports received has decreased and the reducing incidence of *M. pneumoniae* has mirrored the reduction in incidence of respiratory tract infections and consequent reduced antibiotic prescribing in England and Wales, leading to a need for a more robust surveillance system for respiratory infections (Fig. 1) [7, 8]. Nucleic acid based surveillance screening has been used at the HPA and Royal College of General Practitioners Birmingham Research Unit for a number of years examining annual epidemics in England and Wales of influenza, respiratory syncytial virus (RSV) and human metapneumovirus in symptomatic patients attending sentinel general practitioners surgeries with respiratory symptoms (http://www.rcgp.org.uk/bru/_bru_home.aspx). The England and Wales respiratory viral screening programme during 2005 to 2009 was extended to include the detection of *M. pneumoniae* by real-time polymerase chain reaction (PCR) amplifying the P1 cytoadhesin gene [17]. Data was collated regarding date of sampling, date of onset of respiratory

symptoms, respiratory symptoms, age, sex, geographical location, *M. pneumoniae* detection and mixed infections with respiratory viruses. To give an indication of carriage in the community this method was also used to detect *M. pneumoniae* in 428 individuals in households in Hertfordshire, UK. Ethical approval was obtained.

Materials and methods

Clinical samples – symptomatic patients

A total of 3987 anonymised combined nose and throat swabs were taken from patients attending GPs during the winter months (weeks ~35-20) of 2005-2009 as part of the annual viral epidemiological screen. All age groups were included except during the 2008-2009 period which included only children less than 15 years old. Samples were taken from patients (mean age 33.2, SD±21.3, range 0 to 99 years) with clinical symptoms in the following categories, upper respiratory tract infection (URTI, sore throat, runny nose ± otitis media); lower respiratory tract infection (LRTI, cough, dyspnoea, acute bronchitis, bronchitis or pneumonia); constitutional infection (fever, myalgia); influenza like illness (fever, myalgia and cough with duration of <5 days). A complete record of age, sex, onset and clinical signs was not available for every patient and antibiotic use prior to presentation was not known. Nucleic acid was extracted from the samples using the Roche MagNAPure robot using DNA isolation kit III (Roche Diagnostics) according to the manufacturers instructions and examined for evidence of respiratory viruses as described previously [5, 18]. Total nucleic acid extracts were stored at -80°C prior to testing for *M. pneumoniae* DNA by real-time PCR [17].

Clinical samples - community subjects

Subjects asymptomatic for respiratory infections during the same time period as above were not available for testing. Nasopharyngeal swabs were collected from 428 individuals from Hertfordshire, England in October 2001 as part of a study on pneumococcal carriage in healthy children and their families [12]. These samples were included in this study to ascertain the level of *M. pneumoniae* in the community population. Participants included family households with pre-school children, their

parents, grandparents and siblings and therefore sampling was biased towards households with younger average age groups. DNA was extracted from swabs as detailed above after microbiological culture plating for other pathogens and exclusion criteria have been previously described [12].

***Mycoplasma pneumoniae* PCR LightCycler assay**

Assays were carried out using a LightCycler™ (Roche, United Kingdom) to amplify both *M. pneumoniae* and an internal processing control that detects inhibitory samples, with detection of 10 copies or more of the *M. pneumoniae* target sequence in 5 µl sample DNA [17]. Uracil-DNA glycosylase (Roche) was added to each reaction to eliminate carry over contamination [15]. Only 0.1% (4/4448, 95%CI 0.01-0.5) of samples were inhibitory to the assay of which all were negative on repeat testing on 1/10 dilutions [17]. Data was analysed using Roche LightCycler software version 3.5 and an estimation of detectable *M. pneumoniae* DNA was made in direct comparison to a positive standard curve.

Statistical analysis

All statistical analysis was performed with GraphPad Quickcalcs (<http://www.graphpad.com/quickcalcs/index.cf>) (GraphPad Software Inc.) or in Microsoft Excel. The probability of a type I error (α) of 0.05 was taken for all analyses and 95% confidence intervals are included where relevant.

Results

Mycoplasma pneumoniae was detected in 1.7% (65/3987, 95% CI, 1.3-2.1) samples from patients presenting to general practitioners with influenza like illness and 0.2% (1/428, 95% CI 0.01-1.5) community subjects. Symptomatic patients were more likely to have detectable *M. pneumoniae* DNA than community subjects (Fishers Exact test $p=0.02$). Laboratory reports of *M. pneumoniae* to HPA Centre for Infections in England and Wales by date of report, 1990-2010, including serological, molecular and culture test results are shown (Fig. 1). Detailed results for all age groups and clinical signs are listed (Table 1). *M. pneumoniae* infection was detected in 41/2877 (1.4%, 95% CI 0.8-2.2)

symptomatic adult patients aged 15 years and older. Children aged 5 to 14 years presenting to general practitioners with evidence of respiratory infection were more likely to have detectable *M. pneumoniae* DNA than all other age groups (3.0%, 17/564, 95%CI 1.9-4.8, Fishers exact test $p=0.03$, Table 1, Fig. 2) irrespective of clinical signs. This was attributed to the 2005-2006 season in which 6.0% (12/200, 95% CI 3.4-10.3) of 5-14 year olds sampled had detectable *M. pneumoniae* in comparison to 2.2% in 2006-2007 (3/141 95% CI 0.5-6.4), 2.2% in 2007-2008 (2/89 95% CI 0.1-8.3) and 0% in 2008-2009 (0/151 95% CI 0-2.9). A significant number of positive cases in the 5-14 year olds (6.0%) in 2005-2006 was found when compared to all age groups in all years (1.3%, 53/3787, 95% CI 0.9-2.0, Fishers exact test $p=0.0001$). A decreasing incidence trend was seen in all age groups from 2005 to 2009 excepting those under 5 years (Fig. 2). The 5 to 14 year old age group was less likely to have *M. pneumoniae* in the 2008-2009 season than other seasons (0/151, 0%, 95% CI 0-2.9 vs 17/430, 4.0%, 95% CI 2.4-6.3 Fishers exact test $p=0.009$). Positive patients were on average 5 years younger than negative patients (positive $26.7 \pm \text{SD}19.5$, negative $32.1 \pm \text{SD}21.3$). The youngest infected patient was 9 months old. All *M. pneumoniae* positive cases presented and were detected within 17 days of onset of symptoms (0-17 days, mean $5 \pm \text{SD}3.8$), whereas negative patients presented up to 87 days post onset (0-87 days, mean $5 \pm \text{SD}6.3$).

In this study of patients presenting to GPs, those with LRT clinical signs only had higher *M. pneumoniae* loads ($1.1 \times 10^4 \pm \text{SD}2.8 \times 10^4$) than patients with URT clinical signs only ($4.4 \times 10^3 \pm \text{SD}7.3 \times 10^3$) or patients with both URT and LRT clinical signs ($4.2 \times 10^3 \pm \text{SD}5.9 \times 10^3$). The DNA load was also higher in patients with fever, without LRT or URT ($9.8 \times 10^3 \pm \text{SD}2.2 \times 10^4$). Load data was normally distributed if log transformed for all categories. A statistical difference was not found in either comparison (using Student's t-test). No difference was found in age groups and DNA load of *M. pneumoniae*.

The number of *M. pneumoniae* positives cases per week is shown in addition to influenza virus, human metapneumonvirus (HMPV) and RSV (Fig. 3). Mixed infections were detected in 4/65 (6.2%, 95%CI 1.9-15.2) symptomatic patients that were PCR

positive for *M. pneumoniae*. One patient had *M. pneumoniae* and influenza B, two *M. pneumoniae* and influenza A H1 and one *M. pneumoniae* and influenza A H3.

Discussion

The level of *M. pneumoniae* infection detected in symptomatic patients was low (65/3987; 1.7% 95% CI, 1.3-2.1) and all age groups had detectable *M. pneumoniae* infection. The level of *M. pneumoniae* infection detected in symptomatic adult patients aged 15 years and older (41/2877 1.4%, 95% CI 0.8-2.2) was similar to that found previously (1.3%) in throat swabs, nasal aspirates and sputum from adult patients with LRTI attending GP clinics in London [3].

The higher childhood incidence in 5-14 year olds (6.0%, 12/200, 95% CI 3.4-10.3) noted in 2005-2006 is consistent with a rise in national *M. pneumoniae* reports in the same period (Fig. 1) occurring four years following the previous epidemic period. This supports other studies in which children of school age present with higher infection rates with *M. pneumoniae* [19] and is consistent with the hypothesis that infection of children may drive epidemic cycles. The decreasing incidence trend seen in all age groups from 2005 to 2009 excepting those under 5 years (Fig. 2) was reflected by the observation that the 5 to 14 year old age group was less likely to have *M. pneumoniae* in the 2008-2009 season than other seasons. Conversely, in 2008-2009 an increase in the under 5 year age group was noted (Fig. 2), although not statistically significant in comparison to other age groups. The beginning of cycles of infection may start in the youngest age group or naive population. Natural immunity develops to *M. pneumoniae* with age and children may be more susceptible due to lack of previous exposure. However, immunity may be transitory and recurrent isolation and serological responses have been found [6] and persistent infections have been noted [9]. Younger children and infants have an increased severity of clinical signs when infected with *M. pneumoniae* in comparison to older children [19], again perhaps reflecting lack of previous exposure. Positive patients were on average 5 years younger than negative patients reinforcing *M. pneumoniae* as an infection more common in younger patients.

Kleemola et al. [13] found that 3 weeks after PCR confirmed infection during an outbreak of *M. pneumoniae* 93% of cases were negative by PCR, indicating clearance of detectable organisms occurs in under 21 days in most individuals. In this study all *M. pneumoniae* positive cases were detected within 17 days, however follow-up samples were not available to ascertain clearance of infection.

In this study the youngest infected patient was 9 months old. Defilippi et al. [4] recently reported infection in infants of only 1 month old and noted that children less than 5 years old had a differing clinical presentation to older children (tachypnoea, upper respiratory tract involvement, diarrhoea and vomiting were more common) and are more likely to have concurrent RSV infection [4]. By comparing observed peaks of infected individuals (Fig. 3) *M. pneumoniae* infection seems to run concurrently with RSV rather than with influenza annual peaks, however, specific association has not been made. Mixed infections were detected in symptomatic patients that were PCR positive for *M. pneumoniae* with influenza B, influenza A H1 or influenza A H3. Similar to this study mixed infections have been documented in a region of France in nasal swabs [14] in which mixed infections of *M. pneumoniae* with RSV, influenza A and B were recorded each year and *M. pneumoniae* was found to be the second most prevalent pathogen under test (to influenza A). Over a five year period *M. pneumoniae* detection in a region of France varied from 2.0% to 10.1%, proposed to be attributed to epidemic and sporadic circulating strains. In our study detection rates were lower per annum (0.9 to 3.0%), perhaps reflecting a sporadic circulation of strains as opposed to an epidemic year due to a strain of *M. pneumoniae*.

Symptomatic patients were only examined during September to April in this study and therefore the annual rate cannot be calculated and seasonal differences cannot be inferred.

Recently Nilsson et al. [16] demonstrated that oropharyngeal secretions from hospitalized patients contained higher detectable loads of *M. pneumoniae* than outpatients. In this study of patients presenting to GPs, those with LRT clinical signs had higher loads than patients with URT clinical signs or patients with both URT and LRT

clinical signs. The DNA load was also higher in patients with fever, without LRT or URT, although a statistical difference was not found in either comparison.

Asymptomatic carriage has been reported to occur within the community and up to 13% of healthy adults may act as carriers of *M. pneumoniae* during epidemic periods [9, 10]. In total 1.7% (65/3987, 95% CI, 1.3-2.1) samples from patients presenting to general practitioners with influenza like illness and 0.2% (1/428, 95% CI 0.01-1.5) community subjects were positive by PCR, giving an indication of the level of carriage of *M. pneumoniae* in the population outside a *M. pneumoniae* epidemic period. It would be interesting to determine the level of carriage in asymptomatic patients with *M. pneumoniae* in the population during a *M. pneumoniae* epidemic period, however such samples were not available in this study. Symptomatic patients were more likely to have detectable *M. pneumoniae* DNA than community subjects (Fishers Exact test $p=0.02$). The length of colonisation in asymptomatic individuals, the rate of transmission to others and the proportion that go on to develop clinical signs of *M. pneumoniae* infection remains unknown.

Focussing epidemiological studies on children only may result in the detection of outbreaks and individuals most susceptible to severe infection whilst decreasing the costs of large scale screening. Defilippi et al. [4] found that as many as 11% of children hospitalised with lower respiratory signs have *M. pneumoniae* detectable by PCR. This fact, combined with the data shown here which indicate that 1 in every 15 children aged 5-14 in winter 2005/06 attending general practitioners with clinical respiratory signs had detectable *M. pneumoniae* in a non-invasive sample signifies that an increase in testing of children may be beneficial. This study establishes the feasibility of mass screening for *Mycoplasma pneumoniae* detection that can help inform GPs and hospital clinicians particularly paediatricians of likely epidemic years.

To conclude, this study describes detection of *M. pneumoniae* infection by PCR in England and Wales linked to a well established virological surveillance programmes. Overall *M. pneumoniae* was detected in 1.7% patients presenting to GPs with respiratory symptoms/influenza-like illness and in 0.2% community subjects. Mixed

236 infections with influenza viruses were detected in 6.2% of all *M. pneumoniae* positive
237 cases. In 2005-2006 6.0% 5-14 year olds presenting to GPs with respiratory
238 symptoms/influenza-like illness had detectable *M. pneumoniae* infection.

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300 FIGURE LEGENDS

301 Fig. 1.

302 Laboratory reports of *M. pneumoniae* to HPA Centre for Infections in England and Wales
303 by date of report (1990-2010) including serological molecular and culture test results

304

305 Fig. 2.

306 Percentage positive *M. pneumoniae* patients per year and age (error bars indicate 95%
307 confidence intervals) During 2008-2009 only children less than 15 years old were
308 included in the study

309 Fig. 3.

310 Percentage positive *M. pneumoniae* and respiratory virus patients per week 2005-39 to
311 2009-07 (dotted error bars indicate 95% confidence intervals) HMPV testing was only
312 performed in the 2007-2009 seasons

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315 Fig. 1. *M. pneumoniae* Laboratory reports to HPA Centre for Infections in England and Wales by
316 date of report, 1990-2010.

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319 Fig. 2. Percentage positive *M. pneumoniae* patients per week 2005-39 to 2009-07.

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321 Fig. 3. Percentage positive *M. pneumoniae* and respiratory virus patients per week 2005-39 to
 322 2009-07.

324 Table 1 *Mycoplasma pneumoniae* positive cases, sex, age, *M. pneumoniae* DNA load and clinical signs.

	Influenza like illness	Fever/myalgia	LRT Infection	URT Infection ±otitis media	Total (3987)
	<i>Mpn</i> pos(P)/neg(N) (%, 95%CI)	P/N (%, 95%CI)	P/N (%, 95%CI)	P/N (%, 95%CI)	P/N (%, 95%CI)
Total	43/2217 (19.8,1.3-3.0)	35/1868 (1.9,1.3-2.6)	31/1924 (1.6,1.1-2.3)	35/2565 (1.4,0.9-2.2)	65/3922 (1.7,1.3-2.1)
Male	18/1179 (1.5,0.9-2.4)	16/888 (1.8,1.0-2.9)	17/797 (2.1,1.3-3.4)	17/1081 (1.6,0.9-2.5)	32/1695 (1.9,1.6-2.7)
Female	24/1478 (1.6,1.1-2.4)	17/1086 (1.6,0.9-2.5)	13/1069 (1.2,0.7-2.1)	16/1423 (1.2,0.7-2.1)	31/2134 (1.5,0.9-2.4)
Age years P/N (mean±SD)	26.1±17.1/33.8±19.5	29.5±17.6/33.7±19.6	27.7±22.1/34.8±21.8	24.9±19.4/31.1±20.6	26.7±19.5/32.1±21.3
Age <5	2/165 (1.2,0.05-4.6)	1/152 (0.7,0.01-4.0)	4/249 (1.6,0.5-4.2)	4/321 (1.3,0.4-3.3)	7/482 (1.5,0.6-3.0)
2005-2006	1/42 (2.4, 0.01-13.4)	0/42 (0,0-9.9)	2/56 (3.6,0.3-12.8)	0/67 (0,0-6.5)	2/111 (1.8, 0.1-6.6)
2006-2007	0/50 (0,0-8.5)	0/47 (0,0-9.2)	1/97 (1.0,0-6.2)	1/103(1.0,0-5.8)	1/155 (0.6, 0-3.9)
2007-2008	1/33 (3.0,0-16.5)	1/33 (3.0,0-16.5)	0/49 (0,0-8.7)	0/73 (0,0-6.0)	1/104 (1.0, 0-5.7)
2008-2009	0/40 (0,0-10.4)	0/30 (0, 0-13.5)	1/47 (2.1,0-12.1)	3/78 (3.9,0.9-11.2)	3/112 (2.6, 0.7-7.9)
Age 5-14	12/304 (4.0,2.2-6.8)	8/260 (3.1,1.5-6.1)	9/226 (4.0,2.0-7.5)	11/410 (2.7,1.5-4.8)	17/564 (3.0,1.9-4.8)
2005-2006	8/113 (7.1,3.4-13.5)	5/95 (5.3,1.9-12.0)	4/53 (4.6,2.5-18.4)	9/131 (6.9,3.5-12.7)	12/188 (6.0, 3.4-10.3)
2006-2007	2/70 (2.9,0.2-10.4)	1/67 (1.5,0-8.8)	3/82 (3.7,0.8-10.7)	2/103 (1.9,0.1-7.2)	3/138 (2.2, 0.5-6.4)
2007-2008	2/49 (4.1,3.5-14.5)	2/38 (5.3,0.5-18.2)	2/35 (5.7,0.6-19.6)	0/54 (0,0-7.9)	2/87 (2.2, 0.1-8.3)
2008-2009	0/72 (0,0-6.1)	0/60 (0,0-7.2)	0/56 (0,0-7.7)	0/122 (0,0-3.7)	0/151 (0, 0-2.9)

Age 15-44	23/1072 (2.1,1.4-3.2)	19/944 (2.0,1.3-3.1)	10/797 (1.2,0.6-2.3)	13/1146 (1.1,0.6-1.9)	29/1707 (1.7,1.2-2.4)
2005-2006	7/311 (2.3,1.0-4.7)	6/266 (2.3, 0.9-5.1)	3/189 (1.6, 0.3-4.8)	8/328 (2.4,1.2-4.8)	12/479 (2.4, 1.4-4.4)
2006-2007	10/385 (2.6,1.4-4.5)	9/346 (2.6,1.3-4.9)	4/375 (1.1,0.3-2.8)	4/426 (0.9,0.3-2.5)	10/630 (1.6, 0.8-2.9)
2007-2008	6/376 (1.6,0.7-3.5)	4/332 (1.2,0.4-3.2)	3/233 (1.3,0.3-3.9)	1/392 (0.3,0-1.6)	7/598 (1.2, 0.05-2.4)
Age 45-64	5/538 (0.9,0.3-2.2)	5/436 (1.1, 0.4-2.7)	5/461 (1.1,0.4-2.6)	5/521 (0.9,0.01-4.3)	10/865 (1.1,0.5-2.1)
2005-2006	5/142 (3.5,1.3-8.2)	4/123 (3.3,1.0-8.3)	4/101 (4.0,1.2-10.7)	2/141 (1.4,0.06-5.4)	6/227 (2.6, 1.1-5.6)
2006-2007	0/196 (0,0-2.3)	1/165 (0.6,0-3.7)	1/200 (0.5,0-3.1)	2/198 (1.0,0.04-3.9)	3/322 (0.9, 0.2-2.8)
2007-2008	0/200 (0,0-2.3)	0/148 (0,0-3.0)	0/160 (0,0-2.8)	1/182 (0.6,0-3.4)	1/316 (0.3, 0.2-6.2)
Age >65	0/111 (0,0-4.0)	1/106 (0.9,0.01-5.6)	2/172 (1.2,0.1-4.4)	1/142 (0.7,0.01-4.3)	2/264 (0.8,0.03-2.9)
2005-2006	0/32 (0,0.12.7)	0/26 (0,0-15.2)	1/41 (2.4,0-13.7)	0/27 (0,0-14.8)	1/59 (1.7, 0-9.7)
2006-2007	0/44 (0,0.9.6)	1/44 (2.3,0-12.9)	1/74 (0,0-8.0)	1/63 (1.6,0-9.3)	1/116 (0.9, 0-5.2)
2007-2008	0/35 (0,0-11.8)	0/36 (0,0-11.5)	0/57 (0,0-7.6)	0/52 (0,0-8.2)	0/89 (0, 0-4.9)
MPN DNA load	7.7x10 ³ (1.9x10 ⁴),	9.0x10 ³ (2.2x10 ⁴),	7.4x10 ³ (2.0x10 ⁴),	4.3x10 ³ (6.7x10 ³),	6.8x10 ³ (1.7x10 ⁴),
Mean(±SD), range	2.6x10 ¹ -1.1x10 ⁵	2.6x10 ¹ -1.1x10 ⁵	1.4x10 ² -1.1x10 ⁵	2.6x10 ¹ -3.1x10 ⁴	2.6x10 ¹ -1.1x10 ⁵