# *Mycoplasma pneumoniae* infection is associated with subacute cough

*To the Editor:*

Cough, one of the most common reasons patients seek medical attention [1], is categorised according to duration. Acute cough lasts <3 weeks, subacute cough lasts 3–8 weeks and chronic cough >8 weeks [2, 3]. Acute cough is usually due to viral infection; it is transient and self-limiting [4]. Chronic cough has a variety of causes that can be established in 88–100% of patients using a diagnostic algorithm [3]. In contrast, the aetiology of subacute cough remains poorly defined, although post-infectious cough, sinusitis, post-nasal drip and bronchial asthma are listed as possible causes [2, 3, 5, 6]. As subacute cough is often refractory to recommended therapies, a better understanding of its aetiology is needed. The present work examined a possible role for *Mycoplasma pneumoniae* infection in subacute cough.

Between July 2010 and June 2011, patients who visited the Dept of Respiration at the Affiliated Hospital of Academy of Military and Medical Sciences (AHAMMS, Beijing, China) on their own initiative were screened and enrolled if they met the following criteria: cough was the only major complaint; cough was not associated with haemoptysis; cough had lasted for 3–8 weeks (determined by patient recall); chest radiography was normal; and age ⩾15 years. Exclusion criteria included inpatient status, a prior history of lung or other systemic disease that could account for cough, current or past smoking, taking angiotensin-converting enzyme inhibitors, known immunodeficiency or pregnancy. Control subjects were enrolled from healthy medical staff in the department, their family members and relatives of patients who accompanied the patients. Control subjects had no cough for ⩾1 month and were subject to the same exclusion criteria as patients. Efforts were made to match controls with patients by age, sex and time of enrolment. The study was approved by the ethics committee of AHAMMS (protocol #2010-08-99-1). Written informed consent was obtained from all patients and control subjects. Demographic data and medical history were documented using a pre-designed questionnaire. All symptoms and signs detected in a physical examination were recorded.

Oropharyngeal specimens were obtained from the posterior oropharynx using a sterile cotton swab from which *M. pneumoniae* was cultured [7]. A real-time quantitative PCR method was used to measure bacterial load by identifying 16S rDNA of *M. pneumoniae* using the following primer and probe sequences: forward 5′-GCAAGGGTTCATTATTTG-3′, reverse 5′-CGCCTGCGCTTGCTTTAC-3′ and probe 5′-6-carboxyfluorescein-AGGTAATGGCTAGAGTTTGACTG-tetramethylrhodamine-3′. PCR was performed in 25-μL reactions containing 2× Premix Ex Taq (Takara, Dalian, China), 10 μM of both primers, 3 μM of the TaqMan probe and 3 μL DNA template (prepared from swab agitate samples using QIAmp DNA mini kit (Qiagen China, Shanghai, China)) with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). PCR conditions were 93°C for 2 min, 10 cycles at 93°C for 45 s and 55°C for 60 s, followed by another 30 cycles at 93°C for 30 s and 55°C for 45 s. Pure *M. pneumoniae* DNA (Da'an Gene Co Ltd, Guangzhou, China) was used to construct a standard curve for quantification.

To confirm the presence of *M. pneumoniae*, positive isolates, identified by both bacterial culture and real-time PCR, were subjected to nested PCR using two sets of primers directed against the organism-specific *P1* gene sequence (*P1* gene-based strain typing) [8] using *M. pneumoniae* strain FH (ATCC 15531) as a reference. PCR amplicons of the *P1* gene were characterised by agarose gel electrophoresis and nucleotide sequence determination.

Isolates identified as positive by both bacterial culture and PCR methods were tested for susceptibility to erythromycin, azithromycin, midecamycin, ofloxacin, levofloxacin, moxifloxacin, tetracycline, minocycline, amikacin and etimicin. Minimum inhibitory concentrations were determined by broth microdilution [7].

Patient and control groups were compared for the presence of *M. pneumoniae* using Fisher's exact test. Subgroup comparisons of clinical characteristics were performed using t-tests or the Chi-squared test (SPSS for Windows, version 17.0; IBM, Armonk, NY, USA). $p<0.05$ was considered significant.

Out of 202 consecutive cough patients, 85 met the inclusion criteria. Two declined to participate. Excluded patients had the following illnesses: pneumonia (n=50), post-flu/cold cough for <2 weeks (n=31), interstitial lung disease (n=15), bronchiectasis (n=10), pulmonary hypersensitivity pneumonitis (n=6)

TABLE 1 Distribution of *Mycoplasma pneumoniae* detection in patients and healthy controls

| | Patients | Controls | p-value |
|---|---|---|---|
| **Subjects n** | 83 | 80 | |
| ***M. pneumoniae* detection** | | | <0.0001 |
| $Mp^+$ | 46 (55) | 4 (5) | |
| $Mp^-$ | 37 (45) | 76 (95) | |
| ***M. pneumoniae* genotype** | | | 0.715 |
| I | 38 (83) | 3 (75) | |
| II | 6 (13) | 1 (25) | |
| IIa | 2 (4) | 0 | |
| **Age years** | | | 0.615 |
| All subjects | 37.3±13.2 | 38.3±13.1 | |
| $Mp^+$ | 38.4±14.1 | 38.4±13.0 | |
| $Mp^-$ | 35.7±12.0 | 36.3±15.8 | |
| **Age group years** | | | 0.860 |
| 15–30 | 29 (35) | 26 (32) | |
| $Mp^+$ | 15 (33) | 2 (50) | |
| $Mp^-$ | 14 (38) | 24 (32) | |
| 31–50 | 41 (49) | 39 (49) | |
| $Mp^+$ | 24 (52) | 1 (25) | |
| $Mp^-$ | 17 (46) | 38 (50) | |
| ⩾51 | 13 (16) | 15 (19) | |
| $Mp^+$ | 7 (15) | 1 (25) | |
| $Mp^-$ | 6 (16) | 14 (18) | |
| **Sex** | | | 0.728 |
| Male | 31 (37) | 32 (40) | |
| $Mp^+$ | 15 (33) | 3 (75) | |
| $Mp^-$ | 16 (43) | 29 (38) | |
| Female | 52 (63) | 48 (60) | |
| $Mp^+$ | 31 (67) | 1 (25) | |
| $Mp^-$ | 21 (57) | 47 (62) | |
| **Season** | | | 0.673 |
| Spring | 33 (40) | 30 (38) | |
| $Mp^+$ | 14 (30) | 1 (25) | |
| $Mp^-$ | 19 (51) | 29 (38) | |
| Summer | 8 (10) | 10 (13) | |
| $Mp^+$ | 1 (2) | 0 | |
| $Mp^-$ | 7 (19) | 10 (13) | |
| Autumn | 16 (19) | 20 (25) | |
| $Mp^+$ | 10 (22) | 0 | |
| $Mp^-$ | 6 (16) | 20 (26) | |
| Winter | 26 (31) | 20 (25) | |
| $Mp^+$ | 21 (46) | 3 (75) | |
| $Mp^-$ | 5 (14) | 17 (22) | |

Data are presented as n (%) or mean±SD, unless otherwise stated. $Mp^+$: *M. pneumoniae*-positive subjects; $Mp^-$: *M. pneumoniae*-negative subjects.

and tuberculosis (n=5). 24 of the patients enrolled had prior consultations for cough; eight of them were prescribed a cephalosporin antibiotic for 3–5 days. 80 healthy controls were enrolled with a response rate of 100%. No significant difference existed between patient and control groups with respect to age, sex or seasonal variation (table 1).

Samples from 63 (76%) out of 83 patients were culture-positive for *M. pneumoniae*, and 61 (74%) out of 83 were found to be positive by real-time PCR (specificity ~80%). 46 (55%) samples from patients were positive by both culture and PCR methods, and all were confirmed as *M. pneumoniae* by *P1* gene-based strain typing. In contrast, samples from four (5%) out of 80 control subjects were culture-positive, and six (7.5%) were PCR-positive for *M. pneumoniae*. All four positive isolates identified by both culture and PCR were confirmed as *M. pneumoniae* by *P1* gene-based strain typing. The *M. pneumoniae* detection frequency among subacute cough patients was significantly greater than among healthy controls (55% *versus* 5%, p<0.0001). The difference would have been even more significant if either culture- or PCR-positive samples

were counted as *M. pneumoniae*-positive samples (94% *versus* 7.5%, $p<0.0001$), but such analysis would not exclude contribution from false-positives.

Quantitative real-time PCR revealed that 98% (45 out of 46) *M. pneumoniae*-positive samples from the patient group had a bacterial DNA load of $10^5$–$10^7$ gene copies per mL of swab agitate suspension; 50% (23 out of 46) of these samples exhibited very high bacterial load ($\geqslant 10^6$ gene copies per mL). In contrast, the four *M. pneumoniae*-positive control samples gave only $10^3$–$10^5$ gene copies per mL.

Of the 46 subacute cough patients identified as positive for *M. pneumoniae* by both culture and PCR methods, adolescents accounted for 33% (15 out of 46), middle-aged adults 52% (24 out of 46) and older adults 15% (seven out of 46). The 46 positive samples came from 15 males and 31 females. Seasonal effects were apparent: 21 (46%) positive cases in the patient group were detected during the winter season, 14 (30%) occurred in spring, 10 (22%) in autumn, and one (2%) in summer. Thus, infection with *M. pneumoniae* was significantly lower in summer ($p=0.01$).

Antimicrobial susceptibility data revealed that most isolates were resistant to macrolides and aminoglycosides. However, they were largely susceptible to tetracyclines and fluoroquinolones.

The present study showed, for the first time, that *M. pneumoniae* is highly prevalent in patients with subacute cough. Our conclusion is unlikely to be confounded by an *M. pneumoniae* outbreak, since an epidemiology study performed in the same area and overlapping most of our study duration revealed only 18% infection frequency among 500 community-acquired pneumonia patients [9]. Almost all samples from patients exhibited high *M. pneumoniae* DNA load ($\geqslant 10^5$ 16S gene copies per mL). Among the factors analysed, season is the only risk factor that significantly affected *M. pneumoniae* detection rate; prevalence was significantly lower in summer ($p=0.01$). Antimicrobial susceptibility assays indicated that most *M. pneumoniae* isolates were nonsusceptible to both macrolides and aminoglycosides but highly susceptible to tetracyclines and fluoroquinolones. Thus, *M. pneumoniae* infection may play a significant role in subacute cough, at least among Chinese populations in the Beijing area. Although the American College of Chest Physicians evidence-based clinical practice guidelines assert no role for antibiotic therapy [10], because subacute cough is not thought to arise from bacterial infection, our work encourages the investigation of treatment benefit with tetracyclines or fluoroquinolones.

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**Subacute cough is associated with the presence of *Mycoplasma pneumoniae*, thus antimicrobial therapy may be beneficial** http://ow.ly/sPAsX

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# Maternal 25-hydroxyvitamin D levels in relation to offspring respiratory symptoms and infections

*To the Editor:*

Recently, there has been an increasing interest in the immunomodulatory effects of vitamin D. Several studies have suggested detrimental effects of insufficient 25-hydroxyvitamin D (25(OH)D) levels on the innate and acquired immune system, which may contribute to the development of infections and atopic and allergic conditions [1–4]. Children and pregnant and lactating females have been identified as groups with a high risk of 25(OH)D insufficiency [5]. Low maternal serum 25(OH)D levels in pregnancy may contribute to increased risk of infections and atopic outcomes in offspring. Previous studies demonstrate inconsistency regarding relationships between maternal vitamin D intake, serum 25(OH)D levels and umbilical cord 25(OH)D levels with these outcomes in offspring [6–10]. We aimed to explore relationships between maternal serum 25(OH)D levels during late pregnancy and parent-reported respiratory tract symptoms and doctor-diagnosed lower respiratory tract infections (LRTI) in early childhood in a large cohort study.

The study sample consisted of 2025 mother–child pairs from the Southampton Women's Survey with maternal serum 25(OH)D measurement at 34 weeks' gestation (DiaSorin radioimmunoassay; Diasorin, Stillwater, MN, USA) [11]. Follow-up was at children's age 6 months (n=2025), 12 months (n=1946) and 2 years (n=1876). Parents were asked whether the child had suffered from any of the following since the last visit: one or more episodes of chest wheezing/whistling, waking at night coughing for three or more nights in a row (prolonged cough), one or more episodes of croup or a croupy cough, bouts of vomiting or diarrhoea $\geqslant$2 days, or a doctor-diagnosed chest infection, bronchitis, bronchiolitis, pneumonia and/or ear infection. Chest infection, bronchitis, bronchiolitis and pneumonia were combined into one variable labelled "LRTI". Binary variables were created for each outcome. Relative risks were calculated using Poisson regression with robust variance with serum 25(OH)D >75 nmol·L$^{-1}$ as the reference category [12]. All analyses were adjusted for child's sex, birthweight and gestational age, and for maternal age at childbirth, educational level, pre-pregnancy body mass index, parity, ethnicity, smoking in pregnancy and duration of breastfeeding. The study was approved by the Southampton and South West Hampshire Research Ethics Committee (276/97, 307/97, 089/99 and 06/Q1702/104). Consent was obtained before the inclusion of participants.

Median (interquartile range) late-pregnancy serum 25(OH)D level was 59.0 (40.6–84.3) nmol·L$^{-1}$. Lower late-pregnancy serum 25(OH)D levels were not associated with increased risk of parent-reported respiratory symptoms or infections in children aged 6 months, 12 months or 2 years. On the contrary, mothers with serum 25(OH)D levels <50 nmol·L$^{-1}$ reported fewer respiratory symptoms and doctor-diagnosed LRTI in their children aged 0–6 months than those with serum 25(OH)D levels >75 nmol·L$^{-1}$ (table 1). Additional adjustment for season of blood sampling (April to September *versus* October to March) did not alter our findings.

Our results do not support an association between low late-pregnancy serum 25(OH)D levels and increased risk of parent-reported offspring respiratory symptoms and infections in early childhood. The positive associations between serum 25(OH)D levels and self-reported respiratory symptoms and LRTI at 0–6 months may be attributable to residual confounding. Thus, health conscious females have higher serum