

## Diagnosis of *Mycoplasma pneumoniae* Pneumonia with Measurement of Specific Antibody-Secreting Cells

*Mycoplasma pneumoniae* (*Mp*) is reported to be the most common bacterial cause of community-acquired pneumonia (CAP) in hospitalized U.S. children (1). However, current diagnostic tests, including PCR of upper respiratory tract (URT) specimens and serology, do not differentiate between *Mp* infection and carriage (2). *Mp* carriage in the URT is found in up to 56% of healthy children (2, 3). A  $\geq 4$ -fold increase in IgG levels is still used in most centers to confirm *Mp* infection but has low sensitivity (4) and is not helpful in acute clinical management (3). In the absence of an accurate diagnostic test, it is not surprising that studies and meta-analyses on the efficacy of antibiotics are inconclusive for *Mp* CAP in children (5, 6).

Circulating antibody-secreting cell (ASC) responses have been demonstrated to be more rapid and shorter-lived than antibody responses (7). We hypothesized that *Mp*-IgM-ASCs circulate in peripheral blood only for a few days or weeks after *Mp* infection, whereas *Mp*-DNA in the URT and serum antibodies persist for months. We aimed to evaluate the measurement of *Mp*-IgM-ASCs by enzyme-linked immunospot (ELISpot) assay as a new test for diagnosing *Mp* CAP.

### Methods

Pediatric patients with CAP ( $n = 152$ ) and control subjects ( $n = 156$ ) were enrolled from May 2016 to April 2017 after written informed consent. Inclusion criteria for patients with CAP were clinical diagnosis of pneumonia (fever  $>38.5^{\circ}\text{C}$  and tachypnea [8]) in previously healthy children aged 3–18 years. Children  $<3$  years were excluded because of a high probability of viral coexistence in the URT (8). Control individuals included healthy children (undergoing elective surgical procedures) and siblings of patients with CAP (with higher chance of being asymptomatic carriers) without recent ( $\leq 1$  wk) respiratory tract infections.

In all enrolled children, pharyngeal swabs were taken for *Mp* real-time PCR (9). If additional consent was given, blood samples also

were collected in control individuals and patients with CAP (before antibiotic treatment) to test for the presence of *Mp*-IgM-ASCs by ELISpot assay (detailed in the legend of Figure 1) (10) and *Mp*-IgM, *Mp*-IgG, and *Mp*-IgA by ELISA (2). Finally, we only included children with fresh (isolated  $\leq 4$  h) peripheral blood mononuclear cells to avoid poor ELISpot assay performance resulting from decreased ASC viability (in case of isolation  $>4$  h after sampling) or reduced ASC recovery (after a freeze–thaw cycle) (10). Samples and clinical data (using a standardized questionnaire) were collected at follow-up visits at  $<2$  weeks, 2 weeks to 2 months, and 2–6 months.

Assuming that 15% of pairs switch from PCR to IgM-ASC ELISpot assay (positive to negative) and 2% from IgM-ASC ELISpot assay to PCR (negative to positive), we calculated a sample size of 85 children (patients and control subjects) to achieve 80% power and 5% 2-sided significance. Dichotomous data were reported as percentages and compared with  $\chi^2$  or Fisher's exact test. *P* values are two tailed with significance at  $<0.05$  (R software environment, version 3.4.0).

### Results

*Mp*-DNA was detected by PCR in 29% ( $n = 44/152$ ) of patients with CAP and 8% ( $n = 12/156$ ) of control individuals ( $P < 0.001$ ). We were able to perform a complete diagnostic work-up for *Mp* in 63 patients with CAP and 21 control individuals ( $n = 12$  elective surgery;  $n = 9$  siblings), which included the *Mp*-IgM-ASC ELISpot assay of fresh peripheral blood mononuclear cells and *Mp*-IgM ELISA from serum samples. Chest X-rays were routinely performed in 60 (95%) of 63 included patients with CAP, and 98% ( $n = 59/60$ ) met the World Health Organization criteria for radiological pneumonia.

In the CAP series, *Mp*-DNA was detected by PCR in 32 (51%) patients, 29 (46%) of whom showed positive responses in the *Mp*-IgM-ASC ELISpot assay ( $P = 0.722$ ; Figure 1). In the three *Mp* PCR-positive patients with CAP who tested negative for *Mp*-IgM-ASCs, another pathogen was found based on the results of multiplex PCR from pharyngeal swab samples and specific serology (Table 1). All patients who were *Mp* PCR positive and *Mp*-IgM-ASC positive were also *Mp*-IgM seropositive, but *Mp*-IgM was also found in 3 (10%) patients with CAP who tested negative by *Mp* PCR and *Mp*-IgM-ASC ELISpot assay.

Pharyngeal swab and blood samples were collected at inclusion ( $n = 84$ ) and follow-up visits ( $n = 52$ , 41 patients with CAP and 11 control individuals) and resulted in more than two visits in 42 (81%) and more than three visits in 27 (52%) children, performed at  $<2$  weeks ( $n = 43$ ), 2 weeks to 2 months ( $n = 38$ ), and 2–6 months ( $n = 38$ ). In contrast to *Mp*-IgM-ASCs, which were found only within 6 weeks after symptom onset, *Mp*-DNA and/or *Mp*-IgM persisted  $\geq 4$  months in 7 (11%) patients with CAP. Only 10 (34%) *Mp*-IgM-ASC-positive patients showed a  $\geq 4$ -fold increase in *Mp*-IgG, whereas the remaining ( $n = 19$ , 66%) had significantly increased *Mp*-IgG already in first serum samples (median, 49 U/ml; range, 20–125 U/ml; cutoff, 15 U/ml), making a  $\geq 4$ -fold increase very unlikely.

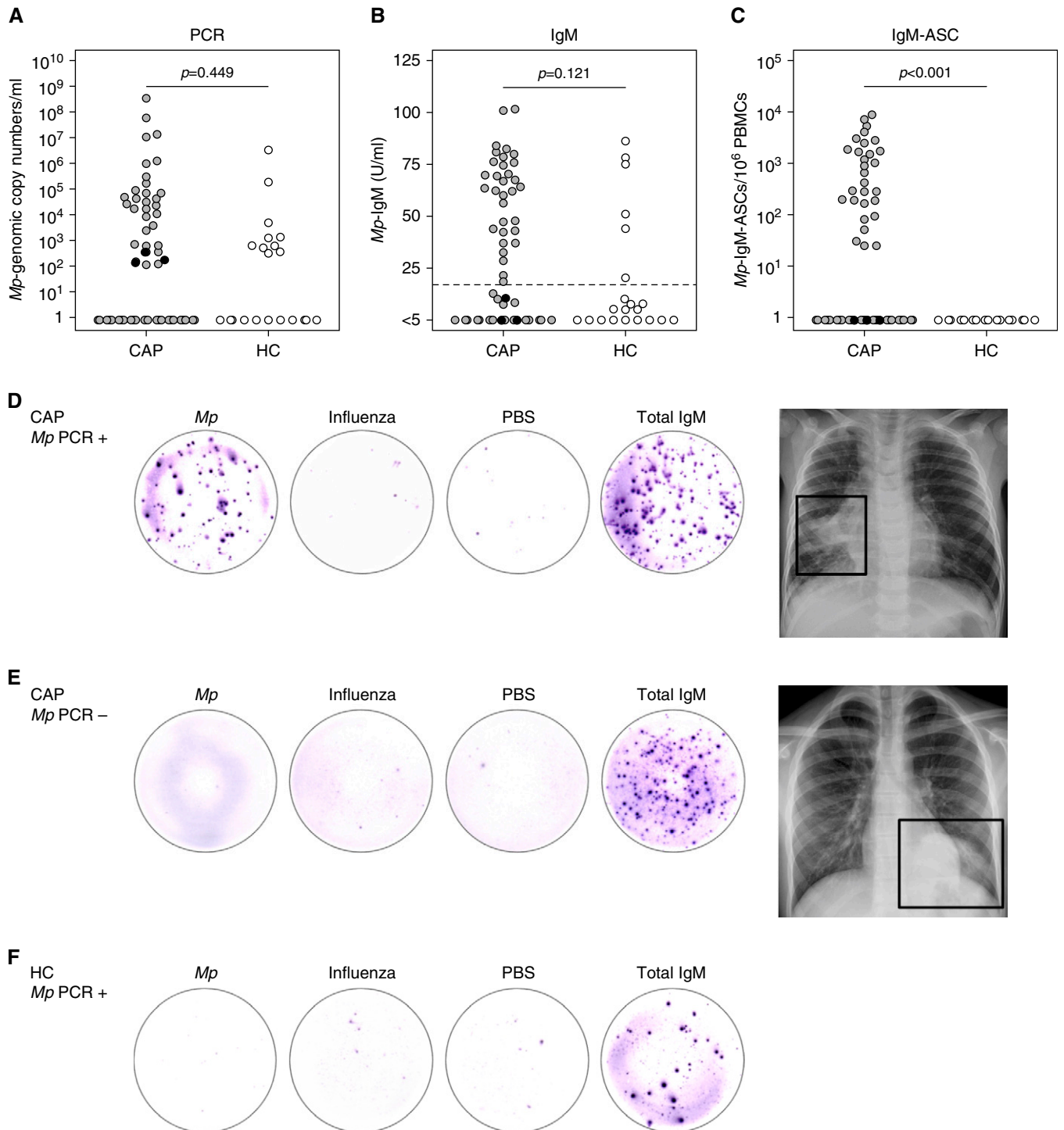
Among control subjects, *Mp*-DNA was detected by PCR in 10 (48%) children. All of these tested negative for *Mp*-IgM-ASCs ( $P < 0.001$ ; Figure 1). Six (29%) control individuals had positive *Mp*-IgM, of whom 1 (5%) showed a  $\geq 4$ -fold increase in *Mp*-IgG at

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**Figure 1.** (A–C) Comparison of diagnostic test results between patients with community-acquired pneumonia (CAP) ( $n=63$ ; median age, 6.0 yr; interquartile range [IQR], 4.4–10.2 yr) and control subjects ( $n=21$ ; median age, 6.1 yr; IQR, 4.9–7.9 yr). CAP samples were collected at disease presentation with a median of 12 days after onset of symptoms (IQR, 11–16; range, 2–29). PCR-positive patients with CAP testing negative for *Mycoplasma pneumoniae* (*Mp*)-IgM-antibody-secreting cells (ASCs) are indicated in black. Differences in medians are shown with the corresponding  $P$  value (Mann-Whitney  $U$  test). (A) *Mp*-DNA levels in pharyngeal swab samples. (B) *Mp*-IgM levels. The dashed line represents the cutoff for the test (17 U/ml), with a lower limit of quantification of 5 U/ml. (C) *Mp*-IgM-ASC responses. (D–F) *Mp*-IgM-ASC enzyme-linked immunospot (ELISpot) assay. Assays were performed as described previously (10) and were specific for the following antigens: *Mp* (detergent extract enriched for highly specific adhesion protein P1, 2  $\mu$ g/ml; Virion/Serion), influenza A and B virus (FluarixTetra quadrivalent influenza virus vaccine, 6  $\mu$ g/ml; GlaxoSmithKline), and total IgM (affinity-purified antibodies to human immunoglobulin light chains  $\lambda$  and  $\kappa$  as positive control, 10  $\mu$ g/ml; Southern Biotech). The negative control consisted of phosphate-buffered saline (PBS) only in uncoated wells.

**Table 1.** Diagnosis of Patients with CAP Who Are PCR Positive for *Mp* but Negative by *Mp*-IgM-ASC ELISpot Assay

	Patient 1			Patient 2		Patient 3	
Demographic characteristics							
Age, yr	4.5			5.9		3.4	
Sex	M			M		M	
Microbiological characteristics							
PCR							
<i>Mp</i> -genomic copy numbers/ml*	415			213		177	
Other pathogens detected by multiplex PCR†	Adenovirus Rhinovirus <i>Chlamydomphila pneumoniae</i>			Rhinovirus		RSV A Human bocavirus	
Serology‡							
Time point of serum sample collection after onset of symptoms, d	1	7	20	12	19	40	
<i>Mp</i> -specific antibodies	Negative	Negative	Negative	Negative	Negative	Negative	Negative
IgM, ≤17 U/ml	5	13	12	<5	<5	<5	<5
IgG, ≤15 U/ml	<3	3	<3	<3	<3	<3	<3
IgA, ≤14 U/ml	<2	<2	<2	<2	<2	<2	<2
<i>C. pneumoniae</i> -specific antibodies	Negative	Negative	Negative	—	—	—	—
IgM, <10 U/ml	5	8	9	—	—	—	—
IgG, <10 U/ml	<4	<4	<4	—	—	—	—
Adenovirus-specific antibodies	Positive	Positive	Positive	—	—	—	—
IgM, <1 Index	<1	<1	<1	—	—	—	—
IgG, <13 U/ml	19	23	24	—	—	—	—
RSV-specific antibodies	—	—	—	—	—	Positive	Positive
IgM, <1 Index	—	—	—	—	—	<1	<1
IgG, <15 U/ml	—	—	—	—	—	27	16
<b>Diagnosis</b>	<b>Adenovirus</b>			<b>Rhinovirus</b>		<b>RSV A</b>	

Definition of abbreviations: ASC = antibody-secreting cell; CAP = community-acquired pneumonia; *Mp* = *Mycoplasma pneumoniae*; RSV = respiratory syncytial virus.

Bold indicates the summary and conclusion of all testing in the table.

\*All three patients with CAP had significantly lower pharyngeal *Mp*-DNA levels than *Mp*-IgM-ASC ELISpot-positive patients with CAP (Figure 1).

†The multiplex PCR FTD Respiratory pathogens 21 (FTD21) assay (Fast-track Diagnostics) was used to test for respiratory pathogens other than *Mp* in these three patients. Notably, we are unable to provide information on cocolonization or coinfection in other patients with CAP and control individuals, as we did not systematically test for other pathogens. However, *Mp* was recently shown to frequently coexist with other bacterial and viral pathogens in the upper respiratory tract of both symptomatic and asymptomatic children (1, 2). Therefore, detection of other pathogens would likely not have changed the conclusions of this study.

‡Serum samples were tested with Serion ELISA classic tests (Virion/Serion). No serological assay was available for rhinovirus. It is important to note that reinfections are often characterized by weak or absent specific IgM antibody responses (3, 8).

follow-up. Although 4 (19%) control subjects were serologically or PCR positive for up to 2 months, *Mp*-IgM-ASC responses were undetectable during 6-month follow-up.

## Discussion

In this longitudinal observational study, the measurement of *Mp*-IgM-ASCs by ELISpot assay allowed a differentiation between infection and carriage. We detected *Mp*-IgM-ASCs as early as 2 days after symptom onset, with a peak at presentation of CAP at median 12 days. Another previous study about *Mp*-IgM-ASCs in 12 *Mp*-seropositive children with CAP corroborated these findings (11). The inclusion of asymptomatic carriers in our study was essential to assess the usefulness of

*Mp*-IgM-ASC detection as a diagnostic test that can distinguish between carriage and infection.

In the absence of a “gold standard” for *Mp* infection diagnosis, the discriminative potential of the *Mp*-IgM-ASC ELISpot assay could not be quantified by measures of diagnostic accuracy such as sensitivity and specificity (12). In fact, if a unanimously accepted reference standard is lacking, alternative study designs, as the longitudinal observational study design chosen in this study, may be more appropriate than test accuracy studies to determine the benefit of a new diagnostic test (12). However, it is important to note that our study population represents a convenience sample from a hypothesis-generating single-center study with small control group and longitudinal follow-up in only two-thirds of the children,

**Figure 1.** (Continued). Representative patterns of ELISpot wells with 10,000 peripheral blood mononuclear cells (PBMCs) per well are shown. Spots were counted by an ELISpot reader (AID) using predefined settings. The spots identified by the machine were manually inspected for the presence of artifacts. Antigen-specific spot counts were calculated as the mean of three wells minus the mean number of spots in PBS wells. Data were expressed as ASCs per 10<sup>6</sup> PBMCs (10). Corresponding chest X-rays of patients with CAP are shown on the right. The pulmonary infiltrate is indicated with a frame. (D) *Mp* PCR-positive CAP. (E) *Mp* PCR-negative CAP. (F) *Mp* PCR-positive healthy control (carrier). Notably, although the applied protocol has a rather long overall turnaround time (~24 h), alternative protocols were developed recently that suggest more rapid (~6–8 h) ASC detection (10). Optimizing such protocols in the future may help translate the *Mp*-IgM-ASC ELISpot assay into routine clinical care. HC = healthy control.

at pragmatically arranged visits instead of standardized weekly follow-ups. We thus cannot rule out that unintended selection bias occurred. A larger confirmatory study is needed, now that the potential for the *Mp*-IgM-ASC ELISpot assay has been shown. Improving the early diagnosis of *Mp* infection in patients with CAP by the *Mp*-IgM-ASC ELISpot assay may help future interventional studies assessing the effect of antimicrobial treatment in the management of *Mp* CAP (5, 6). ■

**Author disclosures** are available with the text of this letter at [www.atsjournals.org](http://www.atsjournals.org).

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## Understanding Hyperlactatemia in Sepsis: Are We There Yet?

To the Editor:

High plasma lactate is a useful indicator of shock, a canary in the coal mine, that is associated with increased mortality in sepsis.

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