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# Seroepidemiological study of *Pneumocystis jirovecii* infection in healthy infants in Chile using recombinant fragments of the *P. jirovecii* major surface glycoprotein

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SUMMARY

*Objectives:* To characterize the seroepidemiological features of *Pneumocystis jirovecii* infection in healthy Chilean children using overlapping fragments (A, B, C) of the *P. jirovecii* major surface glycoprotein (Msg).
*Methods:* Serum antibodies to MsgA, MsgB, and MsgC were measured every 2 months by enzyme-linked immunosorbent assay (ELISA) in 45 Chilean infants from about age 2 months to 2 years.
*Results:* Peak antibody levels (usually reached at age 6 months) and the force (or rate) of infection were somewhat greater for MsgC than for MsgA. Significant seasonal variation in antibody levels was only found with MsgA. Respiratory infections occurred in most children, but nasopharyngeal aspirates were of limited value in detecting the organism. In contrast, serological responses commonly occurred, and higher levels only to MsgC were significantly related to the number of infections.
*Conclusions:* Serological responses to recombinant Msg fragments provide new insights into the epidemiological and clinical features of *P. jirovecii* infection of early childhood. MsgA, the amino terminus fragment, is more sensitive in detecting seasonal influences on antibody levels, whereas MsgC is better able to detect changes in antibody levels in response to clinical infection.


## 1. Introduction

*Pneumocystis jirovecii* remains an important cause of serious pneumonia ('Pneumocystis pneumonia' or PcP) in HIV-infected patients and other immunocompromised hosts.[1] Serological studies have shown that primary *P. jirovecii* infection (as defined by the development of antibody responses to Pc antigens) is acquired in early childhood; so by 2–3 years of age, 70–90% of healthy children have serum antibodies to the organism.[2–5] It is thought that this infection is mild or asymptomatic.[4,6–8] One study found that the presence of *P. jirovecii* DNA detected by the polymerase chain reaction (PCR) was more commonly associated with upper respiratory tract infection (URI) symptoms than lower respiratory tract infection (LRI) symptoms.[9]

These serological studies mainly used antigens that consisted of crude extracts obtained from Pneumocystis-infected human or rodent lung.[10] Attention was focused on the time when antibodies were first detected and cumulative seropositivity over time. Little was known about the specific antigens that were recognized by serum antibodies, the rate or force of infection, or whether antibody levels varied over time.[10]

The development of recombinant *P. jirovecii* antigens has begun a new era in serology, with attention focused mainly on two moieties: the major surface glycoprotein (Msg) and kexin (Kex1).[11,12] We selected Msg as our target antigen because it elicits a strong immune response, contains protective epitopes, and plays a major role in the interaction of Pneumocystis with its mammalian host.[1,13–15] We developed three overlapping recombinant fragments (Msg A, B, and C) that span the entire length of a single Msg isoform: MsgA, the amino terminus, which is quite variable; MsgB, the mid portion; MsgC, the carboxyl terminus, which is the most conserved fragment. We analyzed reactivity with serum antibodies in prevalence studies and in HIV patients and other adult populations, and found that MsgC was best able to distinguish (1) HIV patients hospitalized with PcP from patients with pneumonia due to other causes, (2) HIV patients who had previous PcP from patients who never had PcP, and (3) healthcare workers who had clinical contact with patients from workers who did not.[16–18] We then developed variants (Msg C3, C8, and C9) of the parent MsgC (C1) in order to better delineate the reactivity of this antigen.[19–21]

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Similar to the prominent surface proteins of other eukaryotic pathogens, such as the variant surface antigen (VSA) of Plasmodium and the variant surface glycoprotein (VSG) of Trypanosoma,[22,23] Msg is encoded by multiple genes and is thus capable of antigenic variation.[24] Serological surveys of young children, who experience most of the malaria fatalities, and of adults in an endemic area provide information about the host immune response to VSA, which is important for vaccine development.[25–27] Analysis of serum antibody responses of populations in different geographic areas, climates, and seasons can enhance understanding of the host and environmental effects on the expression of VSA epitopes and their recognition by host antibodies.[28–30] Our studies so far have revealed geographic, but not seasonal differences in the serological antibody responses to Msg in adults.[21]

One of us (SLV) previously followed healthy infants during the first 2 years of life with regular visits and with nasopharyngeal aspirates (NPAs) taken when there were respiratory symptoms.[4] *P. jirovecii* DNA was detected in NPAs in 32% of these episodes, and at a significantly younger age than the NPAs that were negative for *P. jirovecii*. Yet, detection of *P. jirovecii* did not identify a specific pattern of symptoms. Serum antibodies to *Pneumocystis murina* extracted from the mouse with PcP, developed in 53% of the infants at 8 months of age and in 85% of the infants by 20 months of age; seroconversion occurred in the presence of respiratory symptoms in 79% of the subjects. Thus, *P. jirovecii* or an immune response to the organism could be frequently detected in healthy infants with mild respiratory disease.

Little is known about the serological responses of infants and young children to recombinant *P. jirovecii* antigens. In the present study, we examined the sequential serum antibody responses to Msg A, B, and C over a 2-year period in 45 infants from this pediatric cohort. We wanted to compare the serological responses to these antigens for their value in determining the force or rate of infection; in looking for seasonal differences in antibody levels; and in examining the host immune response to respiratory infections.

## 2. Materials and methods

### 2.1. Patient population

In 1997, a total of 107 healthy infants were enrolled by 1 month of age in a 2-year prospective cohort study in Santiago, Chile.[4] Follow-up occurred monthly at a well-baby clinic and serum specimens were obtained every 2 months. Parents were asked to report infections via the telephone or to bring the child to the clinic. NPAs were obtained for *P. jirovecii* amplification when a respiratory tract infection was diagnosed, especially when cough was reported. An infection was diagnosed as a URI if a cold (rhinitis), nasopharyngitis, or pharyngitis were present. An LRI consisted of bronchitis, bronchiolitis, or pneumonia. Other manifestations such as tonsillitis, tonsillopharyngitis, or otitis media were considered separate disorders. Serum specimens and NPAs (which were mixed with saline) that were obtained were stored at −70 °C. Chest radiographs were obtained when clinically indicated. An informed consent form for study participation was signed by both parents and approval for the study was given by the University of Chile Ethics Commission.

The 45 subjects in the present report were chosen because they had many sequential serum specimens taken, reliable clinical data, and exhibited a broad range of antibody responses to *P. murina* extracted-purified antigen in the previous study. Serum samples from the previous study were available from 2 to 24 months of age. However, for the analysis of antibody peaks, rate or force of infection, seasonal variation, and episodes of respiratory infection, only serum specimens obtained at or after 6 months of age were used in order to control for maternal antibodies.

### 2.2. DNA amplification

NPAs were examined for *P. jirovecii* by nested-DNA amplification of the large subunit mitochondrial ribosomal RNA gene of *P. jirovecii*.[4] NPA samples were digested with proteinase K (20 mg/ml) at 60 °C in the presence of 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.5% sodium dodecyl sulfate (SDS). Total DNA was purified and concentrated with use of the Pharma-Gen 'clean up' system (Pharma-Gen) and recovered in a volume of 50 μl. Five microliters of this DNA preparation were used for DNA amplification with *P. jirovecii* oligonucleotide primers pAZ102-E (5′-GATGGCTG-TAGG-3′) and pAZ102-H (5′-GTGTACGTTGC-AAAGTACTC-3′), which are internal to the first set of primers and specific for *P. jirovecii*, in a second round of PCR take 1 μl from the first-round PCR product . Amplified *P. jirovecii* had a predicted length of 267 bp. Negative controls with no added template DNA were included after each sample to monitor for cross-contamination.

### 2.3. ELISA

Serum antibody levels to the recombinant Msg fragments were analyzed in a blinded manner by an ELISA.[16,17] We later made slight modifications to the ELISA.[19–21] Analysis of the same specimens by both methods showed good correlation of the results. The small amounts of serum in the specimens available to us precluded repeating the analysis of the specimens in this study by the new method. For similar reasons, we were only able to measure IgG antibodies in this study.

All serum specimens and the standard reference serum were diluted 1/100 and tested in duplicate wells of a 96-well plate against the following antigens: MsgA, MsgB, and MsgC; *Escherichia coli* extract expressing the pET vector without insert (vector control); phosphate buffered saline (PBS) without antigen (negative control); and tetanus toxoid (TT) (positive control). The plates were washed, followed by the addition of horseradish peroxidase (HRP)-labeled goat anti-human IgG; the plates were washed again and tetramethylbenzidine (TMB) substrate was added. The reaction was stopped by the addition of 0.18 M $H_2SO_4$ and the plates were read at a wavelength of 450 nm. The reference serum specimen was obtained from a single individual with known reactivity to Msg. This serum was run each day as an additional control. HRP-labeled S-protein was used as a positive control and to correct for antigen loading. The antibody level or reactivity of each serum specimen to Msg was expressed as the ratio of reactivity: (mean OD $Msg_{test\ serum}$ − the mean OD $PBS_{test\ serum}$)/(mean OD $pET_{test\ serum}$ − mean OD $PBS_{test\ serum}$).

### 2.4. Statistics

Antibody levels to Msg A, B, and C (C1) across time were evaluated for each child. The median age at first visit and median duration of follow-up were calculated. Analysis of variance (ANOVA) was performed to determine the differences in serum

**Table 1**
Percentiles (%) of the distributions of maximum peak antibody levels to MsgA and MsgC and the age (months) when these levels were reached

| | No. of peaks | Median ($25^{th}$, $75^{th}$ percentiles) | Age ($25^{th}$, $75^{th}$ percentiles) |
|---|---|---|---|
| MsgA | 37 | 21.2 (11.0, 34.8) | 12 (8, 17) |
| MsgC | 42 | 48.5 (12.3, 110.7) | 10 (8, 14) |