Serodiagnosis of *M. pneumoniae* Infections by Enzyme-Linked Immunosorbent Assay (ELISA)

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The enzyme-linked immunosorbent assay (ELISA) has been used for detection of antibodies against *Mycoplasma pneumoniae*. The results of ELISA and its sensitivity compared with other serological methods, such as complement fixation (CF), metabolic inhibition (MI), mycoplasmacidal test (MC), and radioimmunoprecipitation (RIP) are reported. ELISA and MC showed greater sensitivity than CF and MI, while RIP showed serum titer two- to 16-fold higher. ELISA was specific as determined using other human mycoplasma. A simplified method based on the determination of ELISA antibody end-point titer by a single serum dilution has been proposed. ELISA presented several advantages: sensitivity, rapidity, and low cost and, if adequately standardized, could become a reliable method for the serodiagnosis of *M. pneumoniae* infection.

**MATERIALS AND METHODS**

*Mycoplasma*

*M. pneumoniae* (strain 10119) was kindly provided by the National Collection of Type Culture, Colindale, London, England. The culture conditions have been previously described [9].

*Human Sera*

Human sera were obtained from outpatients with suspected primary atypical pneumonia from the Pediatric Clinic of the University of Padua.

*Serological Methods*

The sera were examined with complement fixation (CF), metabolic inhibition (MI), mycoplasmacidal (MC), and radioimmunoprecipitation (RIP) methods as described in a previous paper [9].
Preparation of Antigen-Coated Well for ELISA

Linbro-type microtiter plates with flat-bottomed wells (Flow Laboratories) were used. Preliminary titrations were made to determine the optimal concentrations of *M. pneumoniae*. Mycoplasmas were either grown directly in microtiter wells or alternatively in flasks and after three washings with phosphate buffer solution (pH 7.2) 50 µl of suspension (A_{660} = 0.48) containing 25 µg of protein adsorbed onto microtiter wells and fixed in 10 percent formalin. A more detailed method is reported elsewhere [8].

Preparation of Conjugated Antibodies

Goat anti-human immunoglobulin G, M, and A combined (IgG-IgM-IgA), IgM and IgA antisera (Hyland, Costa Mesa, CA) were purified by gel filtration in Sephacryl S-200 superfine (Pharmacia, Uppsala, Sweden). Fractions were collected and the antibodies concentrated to 5 mg/ml and then they were coupled with alkaline phosphatase (Sigma type VII; specific activity: 1,000 U/mg of protein) by “one-step” procedure as described by Engvall and Perlmann [1]. Conjugated antibodies at 4°C, under sterile conditions, were stored for almost six months without any appreciable loss in enzymatic or immunological activity. We have found that for longer storage they were best preserved in 1 ml aliquots at -80°C. By using a working solution of 1:200 in PBS-Tween a hundred patients’ sera were examined with a milliliter of concentrated solution.

ELISA

ELISA was performed as previously described [8] with some modifications. The adsorbed antigen was washed with PBS containing 0.05 percent Tween 20, which prevented non-specific antibody adsorption to the plastic. Microtiter plates with 50 µl of different serum dilutions (in PBS-Tween), starting at 1:16, were incubated for one hour at 37°C. At the end of the incubation period, the plates were washed twice with PBS-Tween. Fifty µl of conjugated antibody solution, diluted 1:200 in PBS-Tween, were added to each well and the plates were incubated for one hour at 37°C. After three washings, 200 µl of p-nitrophenyl phosphate (1 mg/ml) solution in 0.05 M sodium carbonate buffer (pH 9.8) was added, and the plates were maintained for one hour at 37°C. The reaction was stopped with 50 µl of 3N NaOH. Absorbance at 405 nm was measured by a photometer instrument (Titertek, Multiskan, Flow Laboratories). Usually, ten patients’ sera were tested for each microtiter plate, along with one negative and one positive reference serum. The serum titer was expressed by the highest dilution giving absorbance values greater than 0.1.

RESULTS AND DISCUSSION

Figure 1 shows serum titers obtained by ELISA as compared with CF, MI, MC, and RIP. ELISA titers were in the range 1:16 to 1:2,048 and 43 were higher than those obtained with MI. Furthermore, 7/80 (8.75 percent) MI-negative sera were positive when examined by ELISA, indicating a greater sensitivity and giving the possibility of detecting low antibody levels in the early stages of *M. pneumoniae* infection. Statistical analysis showed that the values obtained with ELISA correlated well with those obtained by the MI method (p < 0.01). The CF method for detecting *M. pneumoniae* antibodies was of far lower sensitivity. Three of 65 sera tested which had a CF titer of 1:16 had a markedly higher titer in ELISA. MC and ELISA had similar sensitivities since most of the values were distributed in the prox-
FIG. 1. Comparison between the sensitivity of ELISA and other serological methods. These comparisons “two by two” were carried out using different sera. In some instances, the same serum was assayed by more than two methods. Each closed circle represents one patient’s serum.

Iminity of the baseline of the scattergram. ELISA did not reach the degree of sensitivity of RIP, which showed serum titers two- to 16-fold higher (up to 1:16,000). A comparative evaluation on the sensitivities of CF, MI, MC, and RIP was also reported in a previous paper [9].

The specificity of ELISA for serological diagnosis of *M. pneumoniae* infection was assessed using eight positive patients’ sera and other human mycoplasmas. In these experiments we used strains from the international collection such as *M. fermentans* NCTC 10117, *M. hominis* PG-21, *M. orale* 1 NIH (CH 19299), *M. orale* 2 NIH (NH 20247), *M. salivarium* PG-20, and *U. urealyticum* T-960. Of the eight sera taken at random from the seroteca, four had low titer (1:64) and four had higher titers (1:512). Only one out of 1:64 titer serum cross-reacted with *M. fermentans*. This may be attributed to the fact that the most important antigen of *M. pneumoniae* is a glycolipid and the major complement-fixing antigen of *M. fermentans* is also found in the lipid fractions and contains glycolipids and phospholipids [10]. This cross-reactivity did not limit the diagnostic value of the ELISA, since *M. fermentans* is not pathogenic for humans, and we have not found a significant rise in the antibody titer.

In our study, we have demonstrated that a fourfold rise in titer to *M. pneumoniae* occurred in only 50 percent of patients. Probably this is due to the insidious develop-
ment of the disease and the acute-phase sera already show high antibody levels when they are tested with ELISA.

In order to reduce the cost of the assay and to examine more sera in each microtiter plate, we tried to find a relationship between the absorbance value at the various sera dilutions and the serum end-point titer. In this way, an absorbance value determined on a single serum dilution (1:32, in our case) might be interpreted quantitatively to give the anti-*M. pneumoniae* end-point titer of that serum. One hundred and ten ELISA-positive sera were tested, using anti IgG-IgM-IgA conjugated antibodies. The regression line with the corresponding standard errors is shown in Fig. 2. To test the validity of this simplified method, an additional 48 sera were examined in ELISA at a single serum dilution. Only 10 percent of these sera showed a discrepancy in end-point titer determination. Therefore we suggest that the simplified method might be the test of choice for epidemiological studies where a positivity or negativity can be immediately ascertained.

A question arises on the kind of antibodies measured by the different serological methods. Later serum samples contain a predominance of IgG antibodies which are more effective in the MI [11], while in CF only IgM and some IgG subclasses are involved. On the other hand, the greater sensitivity of RIP and ELISA might depend on the ability of these methods to measure antibodies belonging to any molecular class and reacting not only with glycolipid antigen, but also with other antigens of the plasma membrane.

In addition, ELISA can be class-specific. Ninety percent of patients with *M. pneumoniae* infection had IgM while a lower percentage had IgA. We have found that IgM antibodies may persist long after one year following the onset of the disease. This finding was in agreement with the hypothesis that silent infections occur quite commonly [12] and that *M. pneumoniae* disease might result from reinfection of patients previously sensitized to the organism [13].

In this field, ELISA presents the advantages of rapidity, low cost, and avoidance of the problems which might be caused in the MI and MC by the presence of antibiotic in the serum. Furthermore, ELISA, if adequately standardized by its adaptability to automation, could become a reliable method for the serodiagnosis of *M. pneumoniae* infection.
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