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Detection of Antibodies to *Ureaplasma urealyticum* in Pregnant Women by Enzyme-Linked Immunosorbent Assay Using Membrane Antigen and Investigation of the Significance of the Antibodies

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Optimal conditions of a microenzyme-linked immunosorbent assay using a group-specific membrane antigen of *Ureaplasma urealyticum* serotype 7 were established with rabbit antisera and applied for the evaluation of immunoglobulin M (IgM) and IgG antibodies in 139 serum specimens from pregnant women between 26 and 38 weeks of gestation, and the assay was compared with microorganism culture and investigated to determine the role of *U. urealyticum* in perinatal morbidity and mortality. *U. urealyticum* was isolated from 75 (54%) of 139 patients; 40 had a colonization $\geq 10^6$ cells per ml of swab (29%); 64 (85%) of 75 culture-positive patients had IgG antibodies (absorbance mean, 0.650), versus 4 (6%) of 64 culture-negative patients (absorbance mean, 0.103) ($P < 0.001$). There was no cross-reactivity with *Chlamydia trachomatis* infection from patients from whom no mycoplasmas were isolated, but this cross-reactivity occurred in 24% of patients with other mycoplasma infections. There was a good correlation between quantitative evaluation of *U. urealyticum* colonization and antibody level ($P < 0.05$). However, IgM antibody was found in 30% of culture-positive patients but also in 25% of the culture-negative group. Frequency of *U. urealyticum* colonization was greater in unmarried young women (<25 years old) with a history of genital infection, and a significantly greater frequency was detected in patients who smoked ($P < 0.01$) and had a lower socioeconomic status ($P < 0.001$). A lower infant birth weight was more associated with *U. urealyticum* colonization $\geq 10^5$ cells per ml. The enzyme-linked immunosorbent assay provided an additional means to diagnose and evaluate *U. urealyticum* infection in pregnant women.

Ureaplasma urealyticum has been associated with infection in pregnancy and perinatal disorders (11, 19). As it is difficult to distinguish between colonization and infection (19) and as *U. urealyticum* is sometimes associated with genitourinary infections by other mycoplasmas or by *Chlamydia trachomatis* (2, 7, 11, 13, 15), the measurement of the antibody response and the evaluation of the amount of *U. urealyticum* colonization per culture are two approaches for the diagnosis of infection in pregnant women (2, 4, 5, 7, 9, 12, 20). Antibody response has been measured by several methods, such as the metabolism inhibition test, immunofluorescence, and more recently by the enzyme-linked immunosorbent assay (ELISA) procedure (1, 3, 5, 12, 20). Immunoglobulin M (IgM) class antibody evaluation has been also proposed to permit the early diagnosis of acute infection (1, 20).

This paper presents the development of an ELISA with a membrane antigen of strain serotype 7 for evaluation of anti-IgM and anti-IgG class antibodies to *U. urealyticum* in sera from 139 pregnant women between 26 and 38 weeks of gestation. The results were compared with those from microorganism culture from lower genital tract specimens and were investigated to determine their relation with perinatal morbidity and mortality.

MATERIALS AND METHODS

Organisms. Pure culture of *U. urealyticum* was supplied by C. Bonissol (Institut Pasteur, Paris, France). The strain was serotyped by C. Bebear (Hôpital Pellegrin, Bordeaux, France) and was serotype 7.

Rabbit antiserum. Antiserum to the *U. urealyticum* strain was obtained by immunization of New Zealand White rabbits, with subcutaneous injection of 1 ml of whole washed organisms (0.5 mg) in complete Freund adjuvant, followed 15 days later by intravenous challenge (0.25 mg in 0.5 ml of aqueous solution). Rabbits were bled on day 25 and tested for reactivity by ELISA. If necessary, one to three booster injections were given, and rabbits were exsanguinated. Rabbit serum obtained before antigen injection served as the normal serum control. Sera were stored at −25°C until use to standardize the ELISA procedure.

Preparation of whole cell antigen. Strain suspension of *U. urealyticum* (5 ml) was inoculated into 30 ml of Shepard fluid medium U-9 modified to contain only 5% horse serum. After a first overnight incubation at 37°C, this culture was inoculated into 3 liters of the same growth medium and incubated for a further 18 to 20 h. The organisms were harvested by centrifugation at 48,000 × *g* for 40 min at 4°C. The pellet was washed three times with sterile phosphate-buffered saline (PBS) (pH 7.3). The washed pellet of cells was finally suspended in PBS (1 mg/ml) and used for rabbit immunization.

Preparation of membrane antigens. *U. urealyticum* cells were obtained by the procedure described above with the substitution of fetal calf serum (5%) for horse serum. Five milliliters of this washed pellet of cells was suspended in 50 ml of 0.05 M carbonate-bicarbonate buffer (pH ranged from 8 to 12) (6). Membranes were separated from the cytoplas-

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mic fractions by centrifugation at 100,000 × *g* for 2 h, washed three times in PBS, and suspended in PBS (pH 7.2) for ELISA use. The lysate protein content was determined by the method of Markwell et al. (14), and all antigens were stored at −70°C in aliquots. The lysed antigen was controlled by electron microscopy. Lysis optimal conditions were calculated by optical density measurement (500 nm) of the cell suspension. Percent lysis was calculated by the equation defined by Sayed and Kenny (17). Optimal lysis of cells was obtained in carbonate-bicarbonate buffer (pH 10.5), measured by optical density (82%), and controlled by electron microscopy.

Human sera. Positive control was prepared by pooling sera from 10 individuals known to be culture positive only for *U. urealyticum* (>10^3 cells per ml of swab) in the lower genital tract; no other genital mycoplasmas were isolated from these patients. A negative control was prepared by obtaining 10 serum specimens from culture-negative individuals. Sera from 75 *U. urealyticum* culture-positive (group 1) and 64 culture-negative (group 2) pregnant women from the obstetrics and gynecology clinic were used to determine *U. urealyticum*-specific IgG and IgM antibody levels and the correlation with *U. urealyticum* colonization and with clinical findings for mothers and neonates. Sera from 17 patients who were culture positive for *Mycoplasma hominis* ($n = 2$) or who had a significant *Mycoplasma pneumoniae* antibody level ($n = 15$) (group 3) and sera from 17 patients with proved *C. trachomatis* infection (group 4) were used to study the specificity of the reaction to the membrane antigen.

ELISA procedure. Incubation times and concentrations of antigen, conjugate, and rabbit and human antisera were determined by checkerboard titration. Membrane and control antigens diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6) were added in 100-μl amounts to the appropriate wells of the microtiter plates (Dynatech Laboratories, Inc.). The plates were incubated overnight at 37°C and washed three times with PBS containing 0.05% Tween 20 and 1% bovine serum albumin (PBS-TB).

The rabbit antisera were diluted in PBS-TB, and 100-μl amounts of each dilution (10^{-1} to 10^{-5}) were added to the appropriate wells at 37°C for 90 min. The plates were washed as before and incubated at 37°C for 60 min with 100 μl of alkaline phosphatase-labeled goat anti-rabbit immunoglobulin (Biosys) per well. The enzyme substrate, consisting of paranitrophenyl phosphate (1 mg/ml) (Sigma Chemical Co.), was dissolved in diethanolamine buffer (pH 10). The plates were washed three times, and 100 μl of substrate solution was added to each well at 37°C in darkness for 50 min. The reaction was stopped by the addition of 100 μl of 0.1 N NaOH, and the A_{400} was determined on ELISA Processor II (Hoechst-Behring).

For human sera, samples were diluted to 1/100 and alkaline phosphatase-labeled goat anti-human IgG or IgM (Behring), diluted to 1/70 and 1/60, respectively, in PBS-TB at 37°C, was used and samples were diluted in Rheumatoid Sorbent (Behring) to remove rheumatoid factors before IgM antibody determination.

A sample was considered to be positive if its optical density value was greater than or equal to 0.3 (standard deviation, ±0.1).

Other determinations. For *U. urealyticum* isolation, lower genital tract swabs were cultured in the medium described above, supplemented with lincomycin (20 mg/ml), which inhibits *M. hominis*; for *M. hominis* isolation, isolation medium, supplemented with erythromycin (20 mg/ml) to inhibit *U. urealyticum*, was used. Microorganisms were identified by their biochemical properties (arginine and urease degradation).

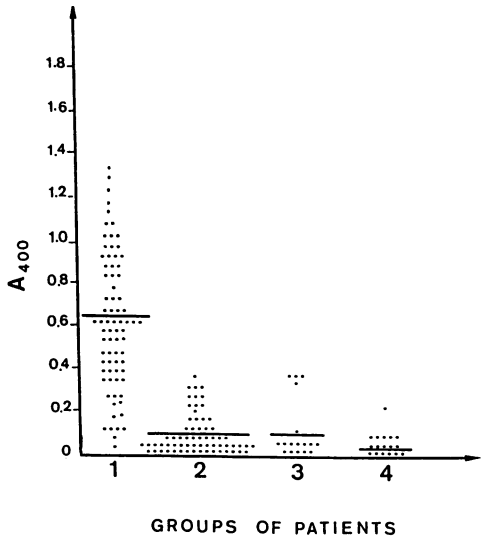

FIG. 1. Levels of IgG antibodies in patients with positive culture for *U. urealyticum* (group 1, $n = 75$), with negative culture (group 2, $n = 64$), with other mycoplasma infections (group 3, $n = 17$), and with *C. trachomatis* infection (group 4, $n = 17$). The mean value for each group is indicated by a solid line (group 1, 0.650; group 2, 0.103; group 3, 0.126; group 4, 0.062). Serum antibody levels in group 1 were significantly higher than in the other groups ($P < 0.001$).

Quantitative evaluation of *U. urealyticum* colonization of the genital tract was determined by making dilutions of 10^{-1} to 10^{-8} of the initial culture in fluid medium.

C. trachomatis was cultured in McCoy cells and identified by immunofluorescence.

Serum antibodies to *M. pneumoniae* were determined by the complement-fixing test by standard microtechnique with Behring antigen, and antibodies to *C. trachomatis* were determined by indirect immunofluorescence.

Statistical analysis. Results were analyzed by the Student *t* test.

RESULTS

Culture results. *U. urealyticum* was recovered by culture of the lower genital tract of 75 (54%) of the 139 patients. A significant active infection was defined as ≥1,000 microorganisms per ml of swab. Culture-positive patients were divided into three groups by a quantitative evaluation of *U. urealyticum* colonization: group A, <10^3 cells per ml ($n = 11$); group B, ≥10^3 and <10^6 cells per ml ($n = 24$); and group C, ≥10^6 cells per ml ($n = 40$).

Antibody evaluation. Intra-assay variation was determined by assaying 10 times for rabbit antiserum and pooled human antiserum in any one experiment; the variation coefficients were, respectively, 5.6% and 5.1%.

Interassay variations were assayed on 2 specimens each, measured in 10 consecutive assays; the coefficient was 3%.

Results of the assay for IgG antibodies in the 173 patients studied are shown in Fig. 1. Of 75 culture-positive individuals, 64 had IgG antibodies against *U. urealyticum* with a mean A_{400} value of 0.650 (maximum, 1.650); the sensitivity was 85%. Of 64 culture-negative individuals, 60 did not have IgG antibodies. The mean A_{400} value was 0.103; the specificity of the test was 94% (Fig. 1).

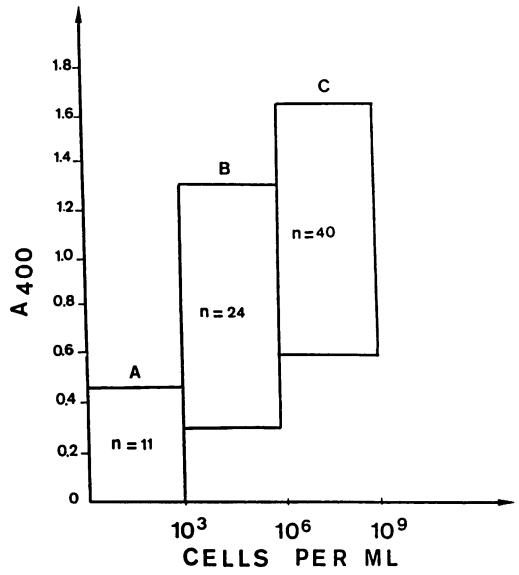

FIG. 2. Correlation between the amount of the colonization by *U. urealyticum* and the IgG antibody level. There was a significant difference between group A ($<10^3$ cells per ml), group B ($\geq 10^3$ and $<10^6$ cells per ml), group C ($\geq 10^6$ cells per ml), and serum antibody levels ($P < 0.05$).

Of the 17 patients who had *C. trachomatis* infection, 16 (94%) were seronegative (mean A_{400} value, 0.062).

Of 17 patients who presented other mycoplasma infections (*M. hominis*, *M. pneumoniae*), 13 (76%) were seronegative (mean A_{400} value, 0.126). These findings seemed to be due to the existence of prior infection with ureaplasma (Fig. 1). The mean A_{400} value in group 1 (culture positive) was significantly different from that in the other groups ($P \leq 0.001$). A good correlation was obtained between the quantitative evaluation of microorganisms by the dilution procedure and the level of IgG antibodies evaluated by optical density ($P \leq 0.005$) (Fig. 2).

IgM antibody determination was performed with the sera of groups 1 and 2. In group 1, 30.6% of the sera had IgM class antibodies and 25% of the sera in group 2 had IgM class antibodies; 15 of the culture-positive patients had IgM class antibodies without IgG class antibodies to *U. urealyticum*.

There was no significant difference between groups 1 and 2 concerning age, number of pregnancies, occupation, contraceptive use, and number of sexual partners. The frequency of *U. urealyticum* colonization in group 1 was greater in young unmarried women (<25 years old) with a history of genital infections, and *U. urealyticum* infection $\geq 10^5$ cells per ml was significantly more frequent in patients who smoked ($P < 0.01$) and who had a lower socioeconomic status ($P < 0.001$). There was no significant causative relationship between *U. urealyticum* colonization of the genital tract and pregnancy complications (abortion, amnionitis, and premature rupture of the membranes), duration of pregnancy (39 weeks mean), or incidence of prematurity. However, a lower infant birth weight was more frequently associated with *U. urealyticum* colonization of $\geq 10^5$ cells per ml of swab ($P < 0.1$).

DISCUSSION

The existence of 14 serotypes of *U. urealyticum* should be considered in choosing from available antigens. Current literature is divided on the fact that antibodies to ureaplasma could be measured by using an antigen of any serovar (1, 18, 20), but we have not yet carried out suitable experiments to test this possibility.

However, our present studies established the value of a serotype 7 membrane antigen for the detection of *U. urealyticum* antibodies in pregnant women by ELISA. ELISA was compared with culture and was found to be reliable and specific, as has been described previously (1). Moreover, we have demonstrated a significant correlation between the IgG antibody titer and the quantitative amount of *U. urealyticum* colonization ($P < 0.05$).

It has been suggested that a highly purified antigen was not required for the ELISA procedure (8) and that sonicated suspensions of organisms could give serotype-specific results while the cell lysis would give group-specific results (20). In our study, using ultrasonicated antigen, few differences were observed between positive and negative sera; e.g., the optical density difference between group A upper values and group C lower values was <0.2 (Fig. 2). This shows that an improvement of the antigenic preparation would be necessary. The use of purified antigen might permit better results. During the determination of optimal conditions for the ELISA procedure, a reaction was also obtained to antibodies to horse serum, developed in rabbits immunized by *U. urealyticum* cultivated in medium supplemented with horse serum (8, 20); it was not found when we used fetal calf serum in the medium used to prepare ELISA antigen or when we diluted specimens in buffer containing 5% horse serum.

Under these conditions, background levels were low (≤ 0.1 A_{400}); the specificity and sensitivity obtained were, respectively, 85% and 94%, in agreement with previously published data (1, 3). The comparison of the ELISA with the metabolism inhibition test and immunofluorescence to detect *U. urealyticum* antibodies was performed and demonstrated that ELISA was a better and more sensitive, rapid, and reliable method (1, 8).

With the sera from patients infected only by *C. trachomatis*, reactivity occurred in 6% of cases, whereas with the sera from patients infected by other mycoplasmas (*M. hominis* or *M. pneumoniae*), reactivity occurred in 24% of cases. These findings were in agreement with the previous results of Brown et al. (1), Lind and Kristensen (13), and Sasaki et al. (16). In the literature, neither mycoplasma is known to cross-react with ureaplasma, and this is not surprising since they are quite distinct organisms. Prior infection with ureaplasma could explain such results.

IgM positivity was equally distributed between culture-positive and culture-negative patients. The demonstration of IgM antibodies did not seem to have diagnostic significance.

A history of genital infections associated with smoking and low socioeconomic status was significantly related to importance and frequency of *U. urealyticum* colonization (7). Comparison between *U. urealyticum* colonization and antibody detection and clinical findings during pregnancy or delivery and in neonates confirmed a low correlation. The *U. urealyticum* colonization of apparently healthy pregnant women was substantial, as was observed in young women (from 54 to 80%) (9, 15). However, when there was a high number of microorganisms in culture and a high titer of antibodies, the frequency of prematurity and of low infant birth weight (<2,500 g) was greater (11.7% versus 8.3%) ($P < 0.1$). These data suggested that the association was causal (10). More recent studies showed that *U. urealyticum* infec-

tions were associated with perinatal disorders (2, 4, 12), but the role of *U. urealyticum* still remains unclear (5).

In conclusion, it can be said that demonstration of IgG antibodies to *U. urealyticum* in serum by ELISA with membrane antigen is sensitive and provides an additional means for the diagnosis and evaluation of *U. urealyticum* infection in pregnant women. Refinement of the ELISA will be required to fully realize its potential as a tool in understanding ureaplasma infection. Perhaps the most obvious deficiency of the present ELISA is its inability to detect serotype-specific responses. Once the serotype-specific antigens are defined, these purified antigens would be ideal choices for use in the ELISA.

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