NATURAL HISTORY

A serological study of the role of *Mycoplasma genitalium* in pelvic inflammatory disease and ectopic pregnancy

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Objectives: Establishing a connection between the emerging urogenital tract pathogen *Mycoplasma genitalium* and upper genital tract infection in women would be of major importance. The aim of this study was to evaluate the association between *M genitalium* antibodies and pelvic inflammatory disease (PID) and ectopic pregnancy (EP) using a lipid-associated membrane protein-enzyme immunoassay (LAMP-EIA) method.

Methods: The LAMP-EIA was used to analyse sera obtained from patients with clinical PID and EP collected in Sweden between February 1984 and April 1986. Sera from healthy pregnant women (Ctrl) collected during approximately the same period were used as controls. Evidence of chlamydial infection was investigated using a commercial anti-*Chlamydia trachomatis* EIA assay.

Results: The LAMP-EIA was specific as determined by a lack of cross-reactivity with other Mycoplasma species. The LAMP-EIA showed that 17% (33/193) of the PID patients were *M genitalium* positive as compared to 18% (15/82) of the EP patients and 15% (36/246) of the Ctrl women. No significant association could be demonstrated between *M genitalium* antibodies and PID or EP in crude or adjusted logistic regression. Antibodies against *C trachomatis* were demonstrated in 54% of the PID and 57% of the EP patients, and also in 37% of the Ctrl women, showing a statistically significant association.

Conclusion: No statistically significant association between PID or EP and *M genitalium* antibodies could be found using the LAMP-EIA, although a slight tendency toward association was found when focusing on younger individuals.

Pelvic inflammatory disease (PID) almost exclusively affects sexually active fertile women, who then face an increased risk of infertility, ectopic pregnancy (EP) and chronic pelvic pain. The best known causative agents of PID are *Neisseria gonorrhoeae* and *Chlamydia trachomatis*. Some studies have indicated that non-gonococcal rather than gonococcal infections of the upper genital tract are more likely to result in infertility and EP.[1–3] Although many cases are caused by *C trachomatis*, some studies have also shown an association between *Mycoplasma genitalium* infection and PID and infertility.[4–7]

M genitalium was discovered in 1981 when it was isolated from the urethra of two men with non-gonococcal urethritis.[8] The infection is sexually transmitted, but the complete pattern of diseases caused by *M genitalium* has not yet been completely elucidated. The infections tend to run a chronic course and are often asymptomatic.[9 10] Experiments with female monkeys where *M genitalium* was inoculated into the oviducts, resulted in salpingitis followed by a specific antibody response.[11] The cross-reactions between *M pneumoniae* and *M genitalium*[12 13] have made it difficult to use serology for diagnosis and epidemiological studies, but Wang *et al* developed and evaluated a Triton X-114 extracted lipid-associated membrane protein (LAMP) assay and found no cross-reactions.[9 14] In the present study we have used sera obtained in the 1980s from women hospitalised with a clinical diagnosis of PID or EP when the incidence of PID in Sweden was high.[2]

The purpose of the present study was to evaluate the association between *M genitalium* antibodies and PID and EP using a LAMP-enzyme immunoassay (EIA) method.

MATERIALS AND METHODS

Patients and controls

A total of 303 sera from 194 women with a clinical diagnosis of PID, who were inpatients at the Department of Obstetrics and Gynecology, Örebro University Hospital, Örebro, Sweden, were obtained during a 25-month period from February 1984 to April 1986. The PID diagnosis was based on clinical criteria, that is, pain in the lower abdomen for less than 3 weeks with a palpable adnexal mass and/or motion tenderness, fever >38.0℃ and objective signs of lower genital tract infection; 60–65% of these patients underwent laparoscopy for direct visual diagnosis of acute salpingitis.[15] Unfortunately, the laparoscopy data and serum specimens could not be linked, as the sera were made anonymous after the end of the initial study.

In addition, 104 sera were obtained from 83 women with a clinical diagnosis of EP who were inpatients at the same department at the same time. Some of the EP patients had a previous history of PID or EP, and when clinical evidence was uncertain the diagnosis was confirmed by laparoscopy.[3] Sixty eight of the PID and 16 of the EP patients provided sera from both the acute and convalescence phases. Sixteen of the PID patients were seen twice at the same department during the study, but only data from the first visit were used in the evaluation. The median age was 23 (range 15–50) years for the PID patients and 29 (range 18–42) years for the EP patients. One serum sample from each group of patients was missing so these patients were excluded.

Sera from healthy pregnant women (Ctrl) were obtained in 1988 for rubella screening. From these stored serum samples, three samples for each EP case were matched for age and used as control material (n = 246). A second control group consisting of 150 serum samples obtained in 2002 from female blood

Abbreviations: CI, confidence interval; EP, ectopic pregnancy; LAMP-EIA, lipid-associated membrane protein-enzyme immunoassay; OR, odds ratio; PBST, PBS+0.05% Tween 20; PID, pelvic inflammatory disease; RT, room temperature; SD, standard deviation

donors (age 18–50 years) was used as a negative control group (Cb). All sera were stored at −20°C.

The ethics committee in Uppsala, Sweden, approved the study.

Enzyme immunoassays (EIA)

A LAMP-EIA method was adapted using two different strains of *M genitalium* as antigen, in order to represent different antigenic variants of *M genitalium*. The *M genitalium* cells were Triton X-114 extracted as previously described,[9 14 16] and the purified fraction containing the lipid-associated membrane protein (LAMP) of *M genitalium* was used as antigen in the EIA assay.

The LAMP-EIA was performed in 96-well microtitre plates (Maxisorb; Nunc, Roskilde, Denmark), coated with a mixture (100 µl) of the two antigen preparations (1 µg/ml) and incubated overnight at room temperature (RT). After the wells were blocked with 5% non-fat milk (Bio-rad, Hercules, CA, USA) in PBS+0.05% Tween 20 (PBST) for 2 h at RT, 100 µl of sera diluted 1/50 in blocking buffer was added to each well in duplicate and incubated for 2 h at RT. The plates were washed four times in PBST after each step. Peroxidase-conjugated goat-anti-human IgG (Fc-fragment, A0170; Sigma, St Louis, MO, USA) was added (100 µl) and incubated for another 2 h at RT, washed six times and followed by 30-min development with 100 µl SIGMA *FAST* OPD (o-phenylenediamine dihydrochloride; Sigma). The reaction was stopped with 50 µl 1.5 M HCl and the optical density was measured at 492 nm. Serum from a patient with a *Mycoplasma genitalium* PCR positive result in the urogenital tract was used as a positive control, and pooled sera from blood donors were used as a negative control in each run. Rabbit antisera against different Mycoplasma species, and sera from patients with *M hominis*- or *Ureaplasma urealyticum* positive cultures from urogenital tract specimens[17] and 10 human sera from adults with high antibody titres against *M pneumoniae* in a complement fixation test, were used for validation of the assay.

Serological evidence of chlamydial infection was detected using a well-documented[18–20] commercial anti-*Chlamydia trachomatis* EIA assay (Ani Labsystems, Oy, Finland), in order to control for confounding from *C trachomatis* infection in PID and EP.

Statistical methods

Logistic regression was used with outcomes PID or EP versus Ctrl. As explanatory variables, LAMP (cut-off 0.3), *Chlamydia trachomatis* (CT; yes/no) and age on a continuous scale were used. Crude statistical analyses were carried out, with age also included, and adjusted with all three variables. Data were stratified according to age (15–30 years and 31–50 years). The effect parameter was expressed as an odds ratio (OR) and its 95% confidence interval (CI). All statistical analyses were done in STATA (release 9.2; Stata, College Station, TX, USA).

RESULTS

The *M genitalium* LAMP-EIA was specific as determined by a lack of cross-reactivity with other Mycoplasma species. The negative cut-off level was set at 0.3 OD_{492} and was determined as 3 standard deviations (SD) above the negative control mean.

PID patients

Among the PID patients, 17% (33/193) were *M genitalium* LAMP-EIA seropositive. Sixty eight patients provided sera from both acute and convalescence phases. A seroconversion was seen in one patient (no. 184; table 1), but in most cases sera from both the acute and convalescence phases were negative (not shown). In two cases seroconversion occurred between the onset of the first PID and the onset of the second PID (patient nos. 87 and 188). When analysing the different age groups, 18% (26/143) of patients in the 15–30-year-old age group and 14% (7/50) of those in the 31–50-year-old age group were LAMP-EIA positive (table 2, fig 1A).

Antibodies against *C trachomatis* were found in the sera of 54% (104/193) of the PID patients; sera from seven patients were equivocal in the CT-EIA. No differences were found between the two age groups (table 2, fig 1B).

EP patients

Of the EP patients, 18% (15/82) were *M genitalium* LAMP-EIA seropositive. Sixteen patients provided sera from both the acute

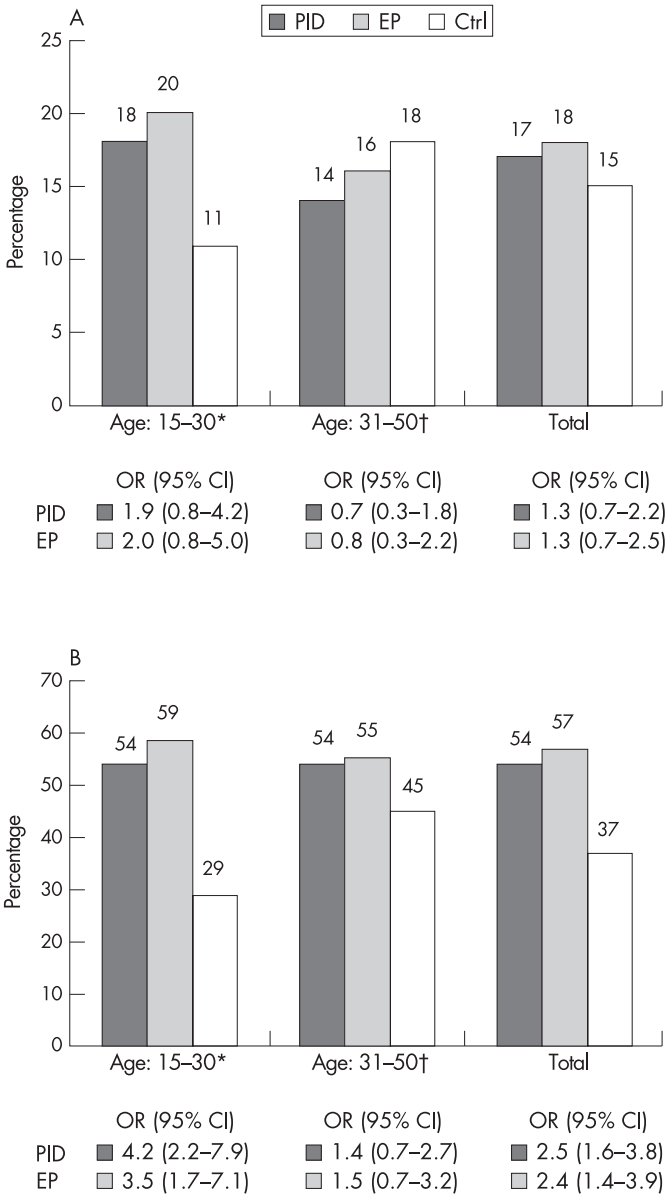

Figure 1 (A) *M genitalium* LAMP-EIA results in percentages (%). Seropositive pelvic inflammatory disease (PID) patients (n = 194) and ectopic pregnancy (EP) patients (n = 83) are compared to the control group (Ctrl) of healthy pregnant women (n = 246). Statistical analyses were carried out by logistic regression and crude results are shown as the odds ratios (OR) and their 95% confidence intervals (CI). (B) *Chlamydia trachomatis* IgG EIA results in percentages (%). Seropositive pelvic inflammatory disease (PID) patients (n = 194) and ectopic pregnancy (EP) patients (n = 83) are compared to the control group (Ctrl) of healthy pregnant women (n = 246). Statistical analyses were carried out by logistic regression and crude results are shown as the odds ratios (OR) and their 95% confidence intervals (CI).
*The age is 18–30 years for the EP patients and Ctrl.
†The age is 31–42 for the EP patients and Ctrl.

Table 1 Results for seropositive pelvic inflammatory disease patients

PID patient no.	First PID onset				Second PID onset			
	LAMP* acute phase (date)	CT†	LAMP conv phase (date)	CT	LAMP acute phase (date)	CT	LAMP conv phase (date)	CT
60	0.48 (14/09/84)	Pos	ND‡	ND	0.22 (28/02/86)	Pos	ND	ND
80	0.32 (06/03/86)	Pos	0.32 (18/03/86)	Pos	ND	ND	ND	ND
87	0.02 (05/11/84)	Pos	ND	ND	1.29 (06/03/86)	Pos	ND	ND
107	0.43 (14/01/85)	Pos	0.46 (27/01/85)	Pos	ND	ND	ND	ND
127	1.08 (08/08/84)	Neg	ND	ND	1.13 (16/04/85)	Neg	1.20 (26/04/85)	Neg
140	0.87 (17/02/86)	Pos	0.86 (25/02/86)	Pos	ND	ND	ND	ND
146	0.34 (13/08/84)	Pos	0.63 (23/08/84)	Pos	ND	ND	ND	ND
154	2.10 (28/09/84)	Pos	2.61 (05/10/84)	Pos	ND	ND	ND	ND
184	0.24 (19/08/85)	Pos	0.92 (06/09/85)	Pos	ND	ND	ND	ND
188	0.24 (26/09/85)	Neg	ND	ND	0.88 (20/03/86)	Neg	ND	ND

Results from the *M genitalium* lipid-associated membrane protein (LAMP)-EIA and the *Chlamydia trachomatis* IgG EIA (CT) are shown. Some seropositive pelvic inflammatory disease (PID) patients provided sera from both the acute and convalescence (conv) phases, and some were seen twice at the same department with verified PID during the study period (first and/or second PID onset). Results from seronegative PID patients are not shown.
*Optical density measured at 492 nm in the LAMP-EIA, cut off 0.3; †CT, *Chlamydia trachomatis* EIA results; ‡ND, not done (because no sera were obtained).

and convalescence phases, and in no case was there any difference in the antibody level. One patient provided five sera specimens from December 1984 to 31 March 1986, and the OD value was constant (about 0.45). Twenty per cent (9/44) of the EP patients in the 18–30-year-old age group and 16% (6/38) of the 31–42-year-old age group were LAMP-EIA positive (table 2, fig 1A).

Antibodies against *C trachomatis* were found in the sera of 57% (47/82) of the EP patients, while five were equivocal in the CT-EIA. In the 18–30-year-old age group, 59% (26/44) were seropositive, while 55% (21/38) were CT-EIA positive in the 31–42-year-old age group (table 2, fig 1B).

Control material

Fifteen per cent (36/246) of the control sera (n = 246) from healthy pregnant women (Ctrl) were *M genitalium* LAMP-EIA seropositive: 11% (15/132) in the 18–30-year-old age group and 18% (21/114) in the 31–42-year-old age group.

Antibodies against *C trachomatis* were found in 37% (90/246) of the Ctrl sera: 29% (39/132) in the 18–30-year-old age group and 45% (51/114) in the 31–42-year-old age group were CT-EIA positive.

In the control group of 150 female blood donors (Cb), five (3%) were *M genitalium* LAMP-EIA seropositive: 2% (1/50) were seropositive in the 18–30-year-old age group and 4% (4/99) in the 31–50-year-old age group.

In the CT-EIA, 9% (13/150) of the Cb group were positive: 2% (1/51) in the 18–30-year-old age group and 12% (12/99) in the 31–50-year-old age group. However, since these sera were collected at a later date (2002), no statistical comparisons were made.

Statistical analysis

No statistically significant association between PID and *M genitalium* antibodies in the LAMP-EIA was seen in the crude logistic regression (OR 1.3, p = 0.387) or in the adjusted regression analysis (table 2, fig 1A). However, *C trachomatis* antibodies were significantly associated with PID in both crude and adjusted analyses. The associations were more pronounced in the younger individuals aged 15–30 years, both for *M genitalium* LAMP antibodies (OR 1.9, p = 0.123) and antibodies against *C trachomatis* (OR 4.2, p<0.001) (table 2, fig 1B). When only the CT-EIA negative sera from the PID patients were considered, the OR was 1.3 (95% CI 0.5 to 3.5, p = 0.562) for *M genitalium* infection.

Also for the EP patients, no association with *M genitalium* LAMP antibodies could be found in the crude analysis (OR 1.3, p = 0.429), and even less so in the adjusted analysis. *C trachomatis* antibodies were statistically significantly associated with EP in both the crude and adjusted analyses. There was a slight trend towards an association between EP and *M genitalium* LAMP antibodies in the younger 18–30-year-old

Table 2 Results for pelvic inflammatory disease (n = 194) and ectopic pregnancy patients (n = 83) compared to the control group

Age group	PID patients (%)	EP patients (%)	Pregnant (Ctrl) (%)	PID vs Ctrl		EP vs Ctrl	
				Crude OR (95% CI)	Adjusted OR (95% CI)	Crude OR (95% CI)	Adjusted OR (95% CI)
15–30 years*							
LAMP	18 (26/143)	20 (9/44)	11 (15/132)	1.9 (0.8 to 4.2)	1.3 (0.6 to 3.0)	2.0 (0.8 to 5.0)	1.6 (0.6 to 4.0)
CT	54 (77/143)	59 (26/44)	29 (39/132)	4.2 (2.2 to 7.8)	4.0 (2.1 to 7.6)	3.5 (1.7 to 7.1)	3.3 (1.6 to 6.8)
31–50 years†							
LAMP	14 (7/50)	16 (6/38)	18 (21/114)	0.7 (0.3 to 1.8)	0.6 (0.2 to 1.6)	0.8 (0.3 to 2.2)	0.7 (0.2 to 1.9)
CT	54 (27/50)	55 (21/38)	45 (51/114)	1.4 (0.7 to 2.7)	1.6 (0.8 to 3.2)	1.5 (0.7 to 3.2)	1.6 (0.8 to 3.6)
Total‡							
LAMP	17 (33/193)	18 (15/82)	15 (36/246)	1.3 (0.7 to 2.2)	1.0 (0.6 to 1.7)	1.3 (0.7 to 2.5)	1.0 (0.5 to 2.0)
CT	54§ (104/193)	57§ (47/82)	37§ (90/246)	2.5 (1.6 to 3.7)	2.5 (1.6 to 3.8)	2.4 (1.4 to 3.9)	2.3 (1.4 to 4.0)

Results for pelvic inflammatory disease (PID) patients (n = 194) and ectopic pregnancy (EP) patients (n = 83) seropositive in the *M genitalium* LAMP-EIA and *Chlamydia trachomatis* IgG EIA (CT) as compared to results for the control group (Ctrl) of healthy pregnant women (three for each EP case). Statistical analyses were carried out by logistic regression. The effect parameter is expressed as the odds ratio (OR) and its 95% confidence interval (CI). Crude OR with variable age and adjusted OR with the variables age, LAMP and CT were included in the model. EP, ectopic pregnancy; PID, pelvic inflammatory disease.
*The age group for the EP patients and the control group is 18–30 years.
†The age group for the EP patients and the control group is 31–42 years.
‡One serum sample from one patients in each patient material was not found.
§Results were equivocal in the CT EIA for 7 PID patients, 5 EP patients, and 5 in the control group of pregnant women.

age group in both crude (OR 2.0, p = 0.133) and adjusted analyses, although this was not statistically significant. When only the CT–EIA negative sera were considered, there was a slight but insignificant trend towards an association (OR 2.3, 95% CI 0.7 to 7.0, p = 0.161) among the EP patients.

Combined antibody response

In the group of PID patients, 23 (12%) were seropositive both in the *M genitalium* LAMP-EIA and in the CT-EIA, while 10 (5%) were *M genitalium* LAMP antigen seropositive only. In the group of EP patients, 10 (12%) had antibodies against both pathogens, while five (6%) were *M genitalium* LAMP antigen seropositive only, compared to 4% of the Ctrl women.

DISCUSSION

Findings from serological and genetic studies on the association between *M genitalium* and PID have been controversial. A serological study by Lind *et al*[21] and a PCR study by Cohen *et al*[22] were unable to show evidence for an association between *M genitalium* and PID, whereas a serological study by Moller *et al*[4] and a PCR study on patients with endometritis by Cohen *et al*[7] did show an association.

In this study we successfully adapted a Triton X-114 extracted LAMP-EIA to detect antibodies against *M genitalium* with no cross-reactivity with other Mycoplasma species.

The LAMP antigen preparation described by Wang *et al*[9 14] and used in this study seems to cover the antigenic variation of the different genotypes of *M genitalium.*

Using the LAMP-EIA, we analysed sera from the 1980s from women with a clinical diagnosis of PID or EP. There was a trend toward an association between PID and *M genitalium* antibodies in the 15–30-year-old age group, where 18% of the patients were LAMP-EIA positive as compared to 11% of the Ctrl women (fig 1A). There was also a trend towards an association for the EP patients in the 18–30-year-old age group, where 20% of the EP patients were LAMP-EIA seropositive compared to the Ctrl women. Surprisingly, we found that the older pregnant women (31–42-year-old age group) had *M genitalium* antibodies more often than the younger age group. This was also the case for antibodies against *C trachomatis*, where a trend towards a higher seroprevalence was seen among the older women in the Ctrl group (fig 1B). These results may be due to the fact that the older women had a longer exposure time for the two pathogens. However, attention should also focus on behavioural differences between the groups. In the present study it was not possible to control for differences in the number of lifetime sexual partners, previous STIs and other factors since this information was not available. Future studies should address these issues. The seroprevalence for both *M genitalium* (3%) and *C trachomatis* (9%) was low in the blood donor population collected in 2002, and these findings strongly emphasises the need for careful selection of control material collected at the same time as the study material.

In Sweden, it has been mandatory to report gonorrhoea and syphilis since 1919 under the Communicable Diseases Act, and genital infection with *C trachomatis* since April 1988. The sera in this study were thus obtained before the start of mandatory contact tracing, screening and treatment of asymptomatic men and women, which may explain the high percentage of women with antibodies against *C trachomatis* among the patients and the Ctrl women in our study. These figures are in good agreement with those reported by other Scandinavian groups from the period.[23–25]

Dual infection with both *M genitalium* and *C trachomatis* as diagnosed by PCR, has been reported in several studies,[10 22 26–29] but in most investigations the two bacteria have more often been found alone than together, indicating that they may act as separate causes of disease. In the present study, some PID and EP patients had antibodies against both *M genitalium* and *C trachomatis* simultaneously, which may reflect successive infections in a population with a high burden of STIs.

In summary, we successfully adapted a LAMP- EIA to detect antibodies against *M genitalium*. Our findings did not indicate a connection between PID or EP and the presence of *M genitalium* antibodies. However, there was a slight trend towards an association between PID and EP and *M genitalium* LAMP antibodies in the younger group, although this was not statistically significant.

Further, preferably prospective, studies where serology and PCR are performed in parallel are required.

Key messages

- Establishing a connection between *Mycoplasma genitalium* and upper genital tract infection in women would be of major importance. In this study we adapted a lipid-associated membrane protein-enzyme immunoassay (LAMP-EIA) method that was specific as determined by a lack of cross-reactivity with other Mycoplasma species.
- The LAMP-EIA was used to analyse sera obtained from inpatients with clinical pelvic inflammatory disease (PID) and ectopic pregnancy (EP) collected in Sweden during the 1980s.
- No statistically significant association between PID or EP and *M genitalium* antibodies in the LAMP-EIA could be found compared to a control group of healthy pregnant women collected at approximately the same date.
- A slight tendency toward association was found in younger individuals.

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AUTHORS' CONTRIBUTION TO THE MANUSCRIPT

Margaretha Jurstrand initiated the study, adapted the LAMP-EIA, collected all data and wrote the first draft of the manuscript. Jørgen Skov Jensen provided antigen and control sera for the LAMP-EIA and contributed to the design of the study. Anders Magnusson carried out the statistical analyses, and Francis Kamwendo examined and sampled most of the patients. Hans Fredlund contributed to the design of the study and provided the serum samples from the PID and EP patients and from the control groups.

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Competing interests: None.

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