Chlamydia trachomatis detection in a population of asymptomatic and symptomatic women: correlation with the presence of serological markers for this infection

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SUMMARY

A study of 371 women (261 asymptomatic and 110 symptomatic subjects with clinical PID) was performed to detect the presence of *Chlamydia trachomatis* (C.t.) and to correlate the serological markers against this microrganism, such as antibody to chlamydial hsp60 (Ab-Chsp60) and different levels of IgG, IgM and IgA, with epidemiology, pathology, sexual habits, age, diagnostic methods in the groups of women with and without pelvic inflammatory disease (PID). We found a statistically significant difference between the asymptomatic and symptomatic women regarding the presence of C.t. (3.4% versus 20%; $p<0.0001$). This presence was affected by the age of women (more in the group ≤25 years old), by having sex with new partners mainly if they did not undergo an antibiotic treatment. The association of antibody Chsp60 with the presence of clinical PID was quite striking. We also found a strict correlation between the detection of Ab-Chsp60 and previous chlamydial infection as well as between Ab-Chsp60 and elevated serum chlamydial IgG or IgA levels. Due to these findings, we can say that the use of serological markers for C.t. in clinical practice may be an important tool for an early screening and diagnosis of women at high risk of chlamydial infection.

KEY WORDS: Chlamydia trachomatis, Genital infections, Serological markers

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INTRODUCTION

Chlamydiae are obligate intracellular bacteria and the species *Chlamydia trachomatis* (C.t.) is the most common sexually transmitted disease that generally promotes intense chronic inflammation causing tissue damage. This infection is very often asymptomatic and can persist for very long periods. Microbial persistence is a state during which the human immune response is not able to eliminate the pathogen that remains silent at focal sites of infection and continues to exercise its virulence, increasing the risk of tubal damage, tubal factor infertility, ectopic pregnancy and chronic abdominal pain.

Microrganism clearance depends on a normal immune response more than on antibiotic treatment.

However some women are not able to clear the pathogen adequately and become asymptomatic, functioning as a reservoir of C.t. infection.

Repeated infections by *Chlamydiae* can be even more damaging for women because they cause serious sequelae for their genital apparatus.

Chsp-60 protein (*Chlamydia* heat shock protein) has been shown to be an important target for the

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intense immune responses linked with complications.
Antibodies to Chsp60 (Ab-Chsp60) have been suggested as markers of chronic inflammation and may therefore be good predictors for the risk of tubal pathology.
For preventive as well as therapeutic measures, it is highly desirable to have sensitive screening markers for this infection
The aim of our work was to study two groups of women to assess the presence of *Chlamydia* for each group. Then we determined whether the presence of serological markers for this infection such as Antibody to Chsp-60 (Ab-Chsp60) and the detection of IgG, IgA and IgM differed in these two groups of population and consequently if they could be a sensitive screening marker for C.t. infection. This would serve to screen the population at high risk for PID (Pelvic Inflammatory Disease) and sterility. In fact if the correlation between high levels of serological markers and serious sequelae following infection is demonstrated, women resulting positive at these tests could be identified and appropriately treated.

MATERIALS AND METHODS

Study population

This study was conducted in a group of 371 women aged 15 to 30 years, attending the Infectious Disease Department and the Gynaecologic Clinic of Policlinic Umberto I - "La Sapienza" University of Rome - over an 18 - month period (January 2005-June 2006). The age distribution was as follows: 80 women in the group of 15-19 years, 125 in the group of 20-25 years and 166 in that aged 26-30.
Out of these 371 women available for study, 261 were apparently healthy, sexually active women who had presented for a general health check-up at the Department of Infectious Diseases. They were asked about any genital symptoms, sexual behaviour, number of partners in the last 3 months and oral contraceptive use. They were checked for genital chlamydial infection. Criteria of exclusion from the protocol were the following: recent antibiotic use, current genital infection, suspected pregnancy or post-partum state, immunocompromised state (i.e. treatment with steroids, bone marrow or organ transplant recipients, haematological malignancy and AIDS).
The other 110 women were recruited in a Gynaecologic Clinic where they presented for their genital symptoms. All patients had a standardized gynaecologic examination and only women with confirmed PID were included in our study. Acute PID was clinically diagnosed on the basis of findings of new onset of lower abdominal pain and uterine tenderness on bimanual examination according to recommended criteria (Kanh *et al.,* 1991). After examination, all women were submitted to trans-abdominal or trans-vaginal ultrasound to confirm the PID and tubal pathology. A small subset of high risk patients underwent laparoscopy or hysterosalpingography (HSG).
From each individual, a sample of serum and endocervical swab was examined. A sample of endocervical swab was tested to detect *Chlamydia trachomatis*. This sample is more suitable and more appropriate than urine. Even though it is easier for the patient to collect urine samples by herself, which is very useful in systematic screening programmes in a large population, they cannot be tested for detection of C.t. in cell culture. *Chlamydia trachomatis* occurrence in endocervical specimens was detected by cell culture and by testing of nucleic acid through methods based on amplification of genetic material (NAATs nucleic acid amplification tests).
A patient was considered positive for *Chlamydia* when either the cell culture or the NATTs was positive. To reduce the possibility of false positive NATTs results and to obtain reliable diagnosis, we performed 3 independent test runs for each specimen.
A sample of serum was used to test the presence of antibodies particularly those against Chsp-60 (*Chlamydia* heat shock protein).
All women with *Chlamydia* detection were treated with appropriate antibiotics (1 g of azithromycin once a day for 6 days).

LABORATORY METHODS

CELL culture

After removal of the mucus from the cervix, the swab was revolved in the endocervix to collect ep-

ithelial cells. The swab was then shaken vigorously with the glass beads in *Chlamydia* transit tubes and compressed toward the tube wall to free the specimen from the swab. The cervical specimens were processed for cell culture within 24 h from collection.
The culture was performed by inoculation of 500 mcl of the specimen in sucrose-phosphate medium onto a monolayer of McCoy cells in flat-bottom, plastic shell vials with coverslips. The specimens were centrifuged (3,000 x g) at 35° for 1h. The medium was then changed to a medium containing 1 mcg of cycloheximide per ml. After incubation for 3 days, the cells were stained with iodine and screened for inclusions. If one or more typical inclusions were seen, the specimen was interpreted as positive (number of EBs-elementary bodies-on the positive slides).

PCR Light Cycle Real-Time
The Light-Cycler Real-Time PCR system (ROCHE) is a new instrument for detection of nucleic acids amplification, allowing real time monitoring of amplicons accumulation during PCR. The PCR reaction is based on hybridization primers (Boel CH *et al.,* 2005).
Before the extraction of DNA, to eliminate the potential interference of SP (sucrose phosphate) included in the specimens, we performed several washings in physiological solution. The DNA extraction was carried out by incubation of 100 mcl of the specimen at 60°C for 2 hours in 100 mcl of a solution containing 10mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 0.2% SDS and 500 mg/ml of proteinase K. The DNA isolation was then performed by phenol and chloroform followed by precipitation in an equal volume of isopropanol in the presence of 0.1 volume of sodium acetate pH 5.2 for 60 min. at -20°C, and finally washing in 1 ml of ethanol 70%.
The primers and the probes used in this test were the following:
The mix of amplification was composed of the following reagents: 50 mM Tris-HCl pH 8.5, 250 mcg/ml of albumin bovine serum, 3.0 mM MgCl2, 200 mclM dNTPs (each one), 0.4 U Taq Polymerase, 500 nM primers 1 and 2, 200 nM probes 1 and 2, 1 mcl DNA template. The total volume was 10 mcl. 3 mcl of the complete solution were inoculated in the capillary, centrifuged and then submitted to amplification.
The PCR programme was as follows: first cycle at 95°C for 45", then 40 cycles including 3 steps: 1° at 95°C for 2"; 2° at 57°C for 3"; 3° at 72°C for 6". Data were collected during the annealing step (57°C) of each cycle.
After the amplification reaction, a programme of melting with temperatures ranging between 45°C and 75°C with a variation of 0.2°C/sec. and with a continuous reading of the fluorescence was performed.
In brief, the normal PCR primers, two sequence specific oligonucleotide probes, complementary to an internal sequence of the amplified fragment, were added to a PCR mix. One primer was labelled at the 5'- end with a Light-Cycler Red fluorophore whereas the other one was labelled at the 3'- end with fluorescein. Only after hybridization were the two primers in close proximity resulting in a fluorescence resonance energy transfer (FRET); the emitted fluorescence was measured and was proportional to the amount of specific target sequences in the reaction mix.
100 mcl of 2-SP samples were processed and submitted to PCR. An internal control included in the reagent mixture was used for every specimen to reveal polymerase inhibitors. This internal control is a sequence of plasmid DNA with primers and probes binding regions identical to those of the C.t. target sequence and a randomized internal sequence with a length and base composition similar to those of the C.t. target sequence. A unique probe binding region differentiates internal control from the target amplicon. The inter-

5'-TGGGGGCTAGTGTCAG-3'	Forward primer	6059-6074	16 bp	CG%=62.5	Tm=61.1
5'-GGCTAGGTGGAGTGTC-3'	Reverse primer	6301-6286	16 bp	CG%=62.5	Tm=59.5
5'-GTGTGGACCTAATTCCTGCATCA-3'	Probe 1	6155-6177	23 bp	CG%=47.8	Tm=64.7
5'-TTTATCCGAACAGTTTAGAGAATTGGATATTCA-3'	Probe 2	6180-6212	33 bp	CG%=30.3	Tm=64.3

Probe 1 was labelled with fluorescein, whereas probe 2 was labelled with Cy5.

nal control rules out false negative results by detecting any problems concerning the DNA extraction or the polymerase inhibitors.
The presence of false positive results can be reasonably excluded owing to high stringency conditions of the primers and probes annealing and to the presence of internal FRET probes with highly species-specific sequences of sufficiently high length so that it is possible to be nearly sure of the uniqueness of the target. Moreover, the analysis of the melting curve obtained after amplification through Real-Time PCR identifies the amplificate identity with certainty.

Detection of IgM, IgG and IgA
Serologic response to C.t. was determined using the indirect microimmunofluorescence (MIF) technique of Wang (Wang SP, 2000) with purified formalin-fixed EBs of different chlamydial serovars as antigen. Sera were screened at a dilution of 1:8 and, if positive, were tested at subsequent dilutions of 1:32, 1:64, 1:128 and 1:256. Positive reactions appear as bright apple-green fluorescent EBs with a background matrix of yolk sac.
The reciprocal of the highest serum dilution that gives definite apple-green fluorescence is termed the serum end-point titre.

Detection of serologic response to Chsp60 (heat shock protein)
This test was determined at 1:50 dilution of sera, using purified recombinant C.t.-hsp60 as antigen by an ELISA-method. The Chsp60-IgG-ELISA (International Medac) uses recombinant full length heat shock protein 60 from C.t. as antigen. The Chsp-60 was produced as a hexahistidine-tagged protein in *Escherichia coli*. It is suitable for the detection of IgG antibodies to hsp60 from C.t. In spite of the high homology among the *Chlamydia* species of more than 95% on protein level, this assay seems to detect predominantly antibodies which are directed toward hsp60 from *Chlamydia trachomatis*.
In brief, the Chsp60 protein was added to each well of a 96 - well microplate and allowed to absorb. 100 mcl of patient sera (1:50 dilution in PBS) were added to the wells and the plate was incubated for 60 min at 37°C. After washing the wells with PBS to remove unbound products, bound antibody was detected with horseradish-peroxidase-conjugated-anti-human IgG followed by the addition of its chromogenic substrate. The optical density (O.D.) of each well was read photometrically. A positive result was considered as an optical density of ›0.35 (Dreesbach K. *et al.,* 2004).

Statistical analysis
Categorical variables were analyzed by the chi-square test with Epi Info (Dean AD *et al.,* 1990). A p-value of <0.05 was considered statistically significant.

RESULTS

The presence of C.t. was found in 31 individuals: 9 in the group of asymptomatic women (9 of 261; 3,4%) and 22 in the group of symptomatic ones (22 of 110; 20%) (average value for both groups 8,3%). This difference was strongly significant (p<0.0001) (Table 1).
Out of 31 individuals with C.t., 12 had a positive cell-culture (39%) whereas 29 were positive for NAATs (93.5%) (Table 1).
The prevalence of C.t. infection, even if not statistically significant (p=0.31), was higher in the 15-25 years age group (~10%) than in the group of 26-30 years (6%).
With regard to risk of infection concerning the sexual behaviour risk factor, we found that having a new sexual partner in the 3 months before the test, was associated with C.t. infection: 17% (10 of 60) of women who had a new sex-partner compared with 7% (21 of 311) of women who did not (p=0.01).
The use of oral contraceptives did not seem to interfere with *Chlamydia*-positive subjects (10% versus 7.2% p=0.34) whereas failing partner treatment was identified as an important risk factor for infection: only 6.5% of partners of women with *Chlamydia* underwent an antibiotic therapy (2 of 31). In general, more than 90% of sex partners (29 of 31; 93.5%) were not submitted to therapy (p<0.0001).
The distribution of Chsp60 antibody as measured in optical density (O.D.) units at 1:50 dilution of serum among 371 women under study, is reported in Figure 1.
The break-point was considered at 0.35 O.D units: 236 (63.7%) women were identified as having antibody level <0.35 (negative) and 135 (36.3%) as having antibody levels >0.35 O.D. units (positive).

TABLE 1 - *Chlamydia trachomatis positive.*

Overall	31 (371)	8.3%	
Cell culture positive	12 (31)	39%	
NATTs positive	29 (31)	93.5%	
Symptoms			
Yes	22 (110)	20%	P<0.0001
No	9 (261)	3.4%	
Age group			
15-19	9 (80)	11.25%	
20-25	12 (125)	9.6%	P=0.31
26-30	10 (166)	6%	
New partners in the last 3 months			
Yes	10 (60)	17%	P=0.01
No	21 (311)	7%	
Treatment of partner			
Yes	2 (31)	6.5%	
No	29 (31)	93.5%	P<0.0001
Use of oral contraceptive			
Yes	15 (150)	10%	P=0.34
No	16 (221)	7.2%	

Data are n° of C.t. positive / n° total (%)

Among the group with Ab-levels>0.35 O.D., we principally found symptomatic women (68%). In the group of asymptomatic ones, these levels were found in 23% of subjects.

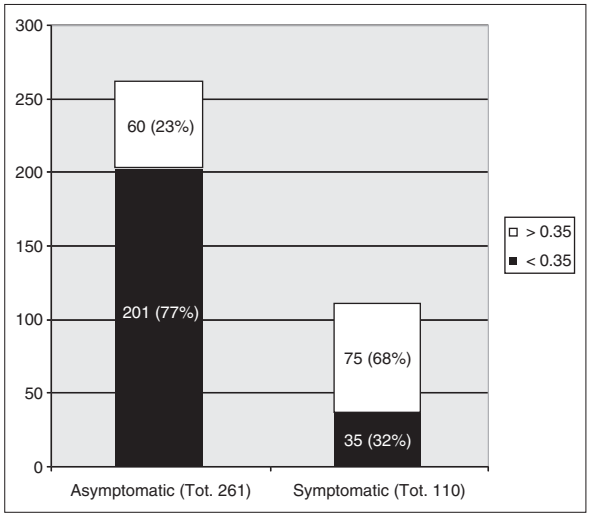

FIGURE 1 - *Distribution of Chsp-60 antibody as measured in optical density (OD) units at 1:50 dilutions of serum between the two groups of women tested (symptomatic and asymptomatic). The break-point is considered at 0.35 OD units (Dreesbach K. et al., 2004).*

The relationship between antibody to Chsp60, confirmed PID, *Chlamydia* serology results, culture positive and history of *Chlamydia* is reported in Table 2.

A statistically significant difference was noted between the two groups of women as far as the above parameters are concerned. The production of Ab-Chsp60 is statistically higher in symptomatic individuals than in asymptomatic ones (68%, 75 of 110 versus 23%, 60 of 261 respectively; p<0.0001); the presence of IgG at a titre >1:128 was significantly higher in the first group than in the latter [32% (35 of 110) and 6% (15 of 261) respectively (p<0.001)]; secretory IgA 1:10 was present in 22% (24 of 110) and 5% (13 of 261) respectively (p<0.0001). The prevalence of IgM >1:8 was slightly greater in the group of asymptomatic than in symptomatic women (not statistically significant: 3% versus 1.8%; p=0.49). C.t. by culture was detected in 8 and in 4 out of 31 women resulted positive in asymptomatic and symptomatic patients respectively (26% versus 13%).

A history of previous infections by C.t. or cervicitis was well correlated to symptoms (p<0.0001), to production of Ab-Chsp60, to high serum levels of IgG or IgA and to detection of the microrganism.

TABLE 2 - *Relationship between antibody to Chsp60, Clinical PID, Chlamydia serology, culture results and previous infections, in the groups of asymptomatic and symptomatic women.*

	Asymptomatic women (Total 261)	*Symptomatic women (Total 110)*
Detection of *C. trachomatis*	9 (3.4%)	22 (20%) P<0.0001
Clinical PID	0	110*
Ab-Chsp60 O.D. >0.35	60 (23%)**	75 (68%) P<0.0001
Serology		
IgM >1:8	8 (3%)	2 (1.8%) P=0.49
IgG ≤1:16	52 (20%)	11 (10%)
IgG (1:16> x <1:128)	47 (18%)	15 (14%)
IgG ≥1:128	15 (6%)	35 (32%) P<0.0001
Secretory IgA 1:10	13 (5%)	24 (22%) P<0.0001
Culture-cell	8/31 (26%)***	4/31 (13%)
History of *Chlamydia* or cervicitis	31 (12%)	66 (60%) P<0.0001

*Confirmed by gynaecologic examination, trans-abdominal/trans-vaginal echography or laparoscopy. **Data are n° (%). *** Data are n°/n° total (%)

DISCUSSION

The high prevalence of C.t. in the groups of women under study reflects the long and successful adaptation of these organisms to persist in their human host population.

Up to 70-80% of C.t. infections in women are asymptomatic having the possibility to be transmitted to sexual partners, thereby increasing the magnitude of the reservoir of chlamydial infections.

The screening of asymptomatic population can be a useful tool to identify the infected persons and to submit them to antibiotic treatment to avoid the sequelae that undetected infections can produce.

On the other hand, it is important to evaluate the occurrence of C.t. in women with genital symptoms to correlate the presence of this microrganism or its products or particular antibodies against it, with the presence of PID and the role played by *Chlamydia* in previous infections.

Several methods to assess the risk of C.t. infection are available. Laparoscopy, considered the reference standard, owing to its disadvantages, is only used in a small subset of high risk patients, more for treatment than for diagnosis (Chapron C *et al.,* 1998).

Serological screening tests have been developed for this purpose (Eckert *et al.,* 1997; Fiddelers *et al.,* 2005; Tiitinem *et al.,* 2006).

Since the association between *Chlamydia trachomatis*- IgG antibodies (CAT) and tubal pathology has been noted (Logan *et al.,* 2003), serum IgG detection has been introduced as a screening test for tubal pathology. The negative predictive value (NPV) of this test is reported to be very high (85-90%) in sterile women (Akande *et al.,* 2003; Land *et al.,* 2003; Molano *et al.,* 2005) whereas the positive predictive value (PPV) is lower and ranges from 30 to 65% (Mouton *et al.*, 2002). This means that the presence of tubal pathology in patients with absence of IgG is unlikely whereas a titre of IgG ≥64 by MIF is statistically associated with tubal pathology (Land *et al.,* 2003). Serum IgG antibodies are known to remain detectable for many years even after antibiotic treatment (Gijsen *et al.,* 2002). Consequently, the detection of IgG only means that there has been a contact with the pathogen in the past without detecting the infection status. Antibodies against heat shock protein 60 of C.t. correlated with serum levels of IgG, IgM and IgA, were found very useful for this purpose.

Chlamydia- hsp60 (Chsp60) is an extremely immunogenic genus specific protein called heat-shock protein or stress-protein, which strongly

stimulates the production of IgG. Antibodies to Chsp60 (Ab-Chsp60) have been suggested as markers of chronic inflammation and then of tubal pathology and sterility (den Hartog *et al.,* 2006; Tiitinem *et al.,* 2006). Chsp60 is mainly responsible for the development and progression of various immunopathological processes which may lead to chronic inflammatory diseases. At this stage Chsp60 is continuously over-expressed and consequently the human immune system permanently has to deal with this foreign protein. Cellular and immune responses are induced.

We found that C.t. detection was statistically higher in women with symptoms and in those whose partners had not been treated ($p<0.0001$) or in subjects who had had a new partner in the last three months ($p=0.01$). Although not statistically significant, the highest rate of prevalence was among the youngest age group (less than 25 years old).

The most significant differences between the two groups of women (without PID and with diagnosed PID) mainly concern the production of Ab-Chsp60 and the detection of high levels of IgG or of secretory IgA. IgM detection is more pronounced in asymptomatic than in symptomatic women supporting the finding that Ab-Chsp60 and IgG, but not IgM, are associated with confirmed PID and their sequelae.

Cell-culture was positive, above all in asymptomatic women. This may reflect the different status of chlamydial infection between the two groups. In fact, in our patients with PID (110 subjects), previous infections that could have turned into persistent infections with consequent reduced replication of the dormant pathogen, were more likely to be present (Veldhuijzen *et al.,* 2005). In these cases, bacterial growth can be prevented by the inflammatory reaction of the organism. C.t. are blocked at the phase of altered reticulate bodies carrying the Chsp60 protein of the external membrane, which is able to induce a long-term inflammatory reaction with continuous production of fibrin causing alteration of tubal mucosa, uterine tenderness, adhesions and Fallopian tube occlusion (Hamdad *et al.,* 2004).

In our study, a strong correlation existed between Chsp60 antibody production and symptoms. The risk of tubal pathology following pelvic inflammatory disease is reported to depend on the number of episodes and the severity of the disease. Westrom *et al.* (1992) reported that in women with PID diagnosed between 1960 and 1984, the risk of tubal factor subfertility was about 10% after one episode of PID, 20% after two episodes and 40% after three episodes. C.t. accounted for about 50% of all PID in this study.

den Hartog *et al.* (2006) reported that combining two tests, it is possible to increase the sensitivity, specificity or the positive and negative predictive values of each test. den Hartog *et al.* (2005) found that combining the IgG serum test which has a PPV of 62% and a NPV of 90% with the CRP test (protein C-reactive used as a marker of inflammation and as a predictive index of chronic infections) significantly increased the positive predictive value for tubal pathology (PPV 86%). Again the association between the IgG test and Chsp60-Antibody detection is said to be useful for increasing the predicting values for tubal pathology in subfertile women (den Hartog *et al.*, 2006).

A limitation of our study might be the small number of subjects which affects the generalization of the results and the extent to which statistical interferences can be made.

It is well known that cell-culture and NAATs are able to discriminate between a current infection from a past or persistent chlamydial infection. In fact in chronic infection, the level of the micro-organism is very low and bacteria are often not viable (cell-culture negative). Such infections could be characterized by continuous positive NAATs. The development of nucleic acid amplification tests has been a major advance in the field of chlamydial diagnosis, and the use of this method associated with serology tests, results in the best diagnostic technique (Hamidad *et al.,* 2004).

In conclusion the serological markers under study were to be crucial for this infection. The Chsp-60-IgG should be used for the diagnosis of the tubal status in the work-up to *in vitro* fertilization. In combination with C.t. serology, Chsp-60-IgG represents an additional marker for the detection of *Chlamydia trachomatis*-induced tubal damage. Our study showed that the detection of Ab-Chsp60 was critically important especially in asymptomatic women because this meant that such population could be at high risk of chlamydial infection and consequent tubal pathology and sterility. In this situation, women could be promptly identified and appropriately submitted to antibiotic treatment to avoid an eventual outbreak of PID and its complications.

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