Chlamydia trachomatis and ectopic pregnancy: retrospective analysis of salpingectomy specimens, endometrial biopsies, and cervical smears

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Abstract

Aims—To examine the role of *Chlamydia trachomatis* in ectopic pregnancy by detection of DNA in archival salpingectomy specimens, and in their preceding cervical specimens and endometrial biopsies, by using the polymerase chain reaction (PCR).

Methods—Archival paraffin embedded salpingectomy tissues (n=48) from 37 women with ectopic pregnancy were examined for the presence of *C trachomatis* plasmid and *omp1* DNA by PCR. In addition, preceding cervical specimens (n=58) stored either as cervical cell suspensions or as archival cervical smears, and preceding endometrial biopsies (n=18), taken 0–5.8 years before the ectopic pregnancy, were examined by PCR for the presence of *C trachomatis*.

Results—*C trachomatis* DNA was detected in only one of the 48 salpingectomy specimens from 37 women. However, in six of the 37 women, *C trachomatis* DNA was detected in the genital specimens (cervix and/or endometrial) taken before salpingectomy. *C trachomatis* infections were mostly found in endometrial or cervical specimens taken more than three years before ectopic pregnancy. No chlamydial DNA was found in endometrial or cervical specimens taken at the same time of the ectopic pregnancy.

Conclusions—Although no *C trachomatis* DNA was found in salpingectomy specimens, several women with ectopic pregnancy had *C trachomatis* infections in endometrial and cervical specimens in the past. This suggests that at least in these cases the ectopic pregnancy is a late post-inflammatory complication of an ascending *C trachomatis* infection resulting in a scarred fallopian tube.

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Keywords: *Chlamydia trachomatis*, polymerase chain reaction, ectopic pregnancy.

The incidence of ectopic pregnancy has increased in many industrialised countries during the last two decades.^{1–5} About 98% of ectopic pregnancies are tubal, often as a result of tubal damage after one or more episodes of pelvic inflammatory disease.⁶ The increasing in-

cidence of ectopic pregnancy has led to many investigations of its microbial aetiology. *Chlamydia trachomatis*, one of the most common bacterial pathogens of sexually transmitted diseases, has been reported to be associated with ectopic pregnancy,^{7–9} as shown by the significantly increased incidence (two- to sevenfold) of antichlamydial IgG antibodies in sera from patients as compared with controls.^{7–15} Moreover, studies of the microbial aetiology of pelvic inflammatory disease have shown that *C trachomatis* was present in the fallopian tubes at the time of salpingitis.^{16–19} So far the mechanism by which *C trachomatis* induces tubal damage in ectopic pregnancy remains unclear. It is speculated that persistent *C trachomatis* infection may cause tubal damage directly, or the occlusion may otherwise be the indirect result of post inflammatory damage. However, isolation of *C trachomatis* by cell culture from the resected salpinx of ectopic pregnancies has not been successful to date.^{13,20–22} Since the existence of a non-cultivable form of *C trachomatis* has been suggested,^{23–25} the role of this microorganism at the time of the ectopic pregnancy is still unclear.

DNA detection by polymerase chain reaction (PCR) has been successfully applied to paraffin embedded tissues and archival cervical smears.^{26,27} This offers the possibility of detecting *C trachomatis* independently of its viability, and also allows retrospective studies to be carried out using archival materials. Therefore we looked for the presence of *C trachomatis* by PCR in archival salpingectomy specimens of women with ectopic pregnancy. To study a possible association between a previous period of ascending *C trachomatis* infection and the later occurrence of ectopic pregnancy, available preceding cervical specimens and endometrial biopsies from the corresponding cases were also examined for the presence of *C trachomatis* DNA by PCR.

Methods

MATERIALS

Forty eight archival paraffin embedded salpingectomy tissues from 37 women with ectopic pregnancy (mean age 33 years) were collected at the Free University hospital and the OLVG hospital, Amsterdam. Two paraffin blocks were tested from 11 of the 37 women. In eight of these women two blocks from the

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Table 1 Summary of the 37 cases tested for *C. trachomatis* (CT) DNA in salpingectomy specimens (■/□), and in preceding endometrial (▲/△) and cervical specimens (●/○) in relation to the time intervals (years) between the sampling^a

Case No.	Time intervals (years) before salpingectomy					
	0	<1	1-2	2-3	3-4	>4
1	■*	△(2x)				
2	□	●	○	○	○	▲
3	□		●			
4	□*			○		▲
5	□*			△	▲, ●	
6	□*		●			●
7	□		○	○, △	○(2x)	●, ○(2x)
8	□*			○		○
9	□**	○(2x)				
10	□** (2x)			○		
11	□** (2x)				○	
12	□		○			
13	□(2x)	○				○
14	□(2x), ○				○, △	
15	□, ○	○				
16	□					
17	□, ○					
18	□				○	
19	□** (2x)				○	
20	□	○				
21	□(2x), ○					
22	□			○		
23	□	○				
24	□	△		△, ○		□*, ○
25	□, △					
26	□		△			
27	□*			○	○	
28	□*		△			
29	□*				○	
30	□		○		○(2x)	○
31	□*		○	○		○
32	□*	○		○		
33	□	○, △	□ ^b , △	○, △	○	
34	□	□ ^b	○(2x), △(2x)			
35	□*(2x), ○					
36	□*(2x)	○				
37	□	○				

^a The cases are presented in order of CT positive (■, ▲, ●) and CT negative (□, △, ○) as determined by plasmid PCR.

^b Tubal biopsies.

* Tubal tissues with presence of plical conglutination and fibrosis.

** Tubal tissues with presence of fibrosis.

same salpingectomy were available, and the other three had tubal biopsies taken earlier before the salpingectomy. Case selection was based on the availability of preceding genital specimens—that is, cervical specimens or endometrial biopsies, or both—taken at different times before the ectopic pregnancy (see table 1) in patients from whom salpingectomy specimens were available in the archive. The genital specimens included stored cervical cell suspensions ($n = 19$), archival cervical smears ($n = 39$) used previously for Papanicolaou (PAP) classification, and endometrial biopsies ($n = 18$). The cervical smears were taken as part of a routine screening programme for cervical neoplasia, and the endometrial biopsies were taken routinely during infertility investigation. The time intervals between the sampling of different specimens are listed in table 1.

SAMPLE PREPARATION

The samples were processed for PCR as follows. Paraffin embedded tissues (salpingectomy tissues and endometrial biopsies) were serially cut in $4\mu\text{m}$ sections. Plain paraffin blocks were included between tissue blocks to check for cross contamination between the tissues during sectioning. The first and the last sections were stained with haematoxylin and eosin for histological re-examination, whereas the sections in between were used for PCR. Proportionally, 1 cm^2 of the $4\mu\text{m}$ tissue section was directly digested in a $250\mu\text{l}$ proteinase K

buffer,²⁸ containing 500 pg/ml proteinase K, 0.45% Tween 20, 0.45% Nonidet P-40, $31.25\mu\text{l}$ of the PCR buffer ($10\times:15\text{ mM}$ MgCl_2 , 100 mM Tris-HCl (pH 8.3), and 500 mM KCl), at 37°C for 24 h. The proteinase K was inactivated by boiling and a $40\mu\text{l}$ portion of supernatant was subjected directly to PCR amplification. The inserted plain paraffin sections were simultaneously processed for PCR. In cases of unsatisfactory tissue processing by this direct proteinase K digestion (as indicated by an unsuccessful human β globin amplification, see below), DNA was purified from these paraffin sections as follows: four or five sections were deparaffinised with xylene, washed twice in ethanol, and air dried. The dry pellets were digested in the proteinase K buffer described above for 2 h at 42°C on a shaker, and the supernatants were extracted twice with phenol/chloroform/isopropanol (vol/vol/vol: 24:25:1) and precipitated by ethanol. The purified DNA was resuspended in 10 mM Tris-HCl (pH 7.5). Approximately 100 ng DNA were used for PCR.

Cervical scrapes—These were collected with cervix brushes (Rover BV) in phosphate buffered saline. Samples were centrifuged for 10 min at 4000 g, and the pellets were resuspended in 1 ml Tris-HCl (0.01 M, pH 7.5) and stored at -80°C until use. Subsequently $10\mu\text{l}$ of this freeze thawed sample was used for PCR amplification.

Archival cell smears—Cells were scraped off the smears and DNA was isolated as described

Table 2 List of primers used for human β globin PCR and chlamydial PCRs

Primers and probes	Reference	Nucleotide sequences	Length of amplicon (bp)
Human β globin PCR			
BGPO3-1 (sense primer)	36	5'-ACACAACGTGTTCCTAGC-3'	209
BGPO5 (antisense primer)	*	5'-GAAACCAAGAGTCTTCTCT-3'	326
BGPO6 (antisense primer)	*	5'-CATCAGGAGTGGACAGATCC-3'	
<i>C. trachomatis</i> plasmid PCR	31, 32		201
CTP1 (sense primer)		5'-TAGTAACTGCCACTTCATCA-3'	
CTP2 (antisense primer)		5'-TTCCCCCTTGTAATTCGTTGC-3'	
PCTP5 (probe)		5'-ATCTCATTACCATGCATTAGCAGCTATCCA-3'	
<i>C. trachomatis omp1</i> PCR	34		320
CM2A (sense primer)		5'-CCAAGCCTTATGATCGACGG-3'	
CM3A (antisense primer)		5'-GAATACATCAAAACGATCCC-3'	

* Primers were selected by computer analysis with PC/gene software (release 6.7, EMBL35, uGenbank 77-35, IntelliGenetics Inc, Mountain View CA, USA).

previously.^{29,30} Briefly, the cover slip was removed by xylene in a sterile container and the cells were scraped off separately with sterile blades. Cells were washed once with xylene and twice with ethanol. The pellets were air dried and lysed in the lysate buffer containing 50 mM Tris-HCl (pH 6.4), 20 mM EDTA, 1.3% wt/vol Triton-X 100, and 5.25 M guanidinium thiocyanate for 2 h at room temperature. DNA released in the lysate was bound to activated silica (50 μ l Silica, Fluka, 1 g/ml suspension in 0.1 N HCl). After washing twice with wash buffer (50 mM Tris-HCl, pH 6.4, plus 5.25 M guanidinium thiocyanate) and once with 70% ethanol, the pellets were air dried and the DNA was recovered in 200 μ l Tris-HCl (pH 8.3) at 58°C. Ten microlitres were used for PCR.

POLYMERASE CHAIN REACTION

Each specimen was subjected to human β globin PCR to check for the suitability of the processed sample for PCR purposes. Two different β globin PCRs were used, amplifying either 209 bp or 326 bp DNA fragments equivalent to the fragment lengths generated by the chlamydial plasmid PCR³¹⁻³³ and *omp1* PCR,³⁴ respectively. The *omp1* PCR was performed in order to detect a possible plasmid-free *C. trachomatis* strain which is so far described as a laboratory strain.³⁵ The primer sequences and the fragment lengths of generated PCR products are listed in table 2. PCR was performed in a final volume of 50 μ l containing of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 200 μ M of each deoxynucleotide (dATP, dTTP, dGTP, and dCTP), 1.5 mM MgCl₂, 1 U of Taq DNA polymerase (Amplitaq, Perkin Elmer), and either 25 pmol of each β globin primer or 50 pmol for each chlamydial primer. The PCR amplification consisted of a DNA denaturation at 95°C for 4 min followed by 40 cycles of amplification which were performed with a thermocycler (Biomed). Each cycle consisted of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and chain elongation at 72°C for 1.5 min. The final elongation was extended for another 4 min. Human placental DNA (100 ng) was used as a positive control for β globin PCR. Dilution series of *C. trachomatis* serovar L2 DNA, corresponding to 0.01, 0.1, and 1 inclusion forming units, were used as positive

controls for chlamydial PCRs. An additional positive control using a paraffin embedded salpingectomy tissue derived from a patient with *C. trachomatis* salpingitis was used for PCR performed with paraffin embedded tissues. An additional *C. trachomatis* positive cervical smear was used for PCR performed with cervical smears. Distilled water instead of DNA was used as negative control. Finally, 10 μ l of the PCR products were analysed by 1.5% agarose gel electrophoresis. *C. trachomatis* plasmid PCR products were further analysed by Southern blot hybridisation using an internal oligonucleotide (table 1) end labelled with [γ -³²P]-ATP.³¹ Chlamydial *omp1* PCR products were hybridised with a cocktail probe consisting of random primer labelled with [α -³²P]-dCTP *omp1* PCR products derived from 15 *C. trachomatis* reference strains for detection of *C. trachomatis*.³²

Results

In the 48 salpingectomy specimens (from 37 women) which we examined, β globin PCR generated a 209 bp fragment in 45 samples processed by direct proteinase K digestion, while after DNA extraction the three remaining samples also became positive. *C. trachomatis* DNA was found by plasmid PCR in only one of these β globin PCR positive tubal tissues (table 1). The β globin PCR generating a 326 bp was successful in 33 of the 48 tissues examined. However, in these β globin positive (326 bp) samples no additional samples were found to be positive by the *omp1* PCR, amplifying a 320 bp fragment. Positive controls for chlamydial PCRs using a dilution series of *C. trachomatis* serovar L2 DNA had a sensitivity corresponding to 0.01 inclusion forming units after Southern blot hybridisation in both chlamydial PCRs. The positive control, using *C. trachomatis* positive salpingectomy tissue, was positive in both PCRs. In addition, plain paraffin sections cut between two cases and processed for PCR simultaneously remained negative.

All cervical specimens (cell suspensions and smears) were positive by β globin PCR generating a 209 bp fragment. Six of the 58 cervical specimens, derived from five women, were found to contain *C. trachomatis* DNA by plasmid PCR (table 1). In addition, 15 of the 19 cervical cell suspension and 35 of the 39 smears were

positive by 326 bp β globin PCR. However, no additional positive samples were found by *omp1* PCR (320 bp). The positive control using a *C trachomatis* positive cervical smear was found to be positive in all chlamydial PCRs performed with cervical smears.

All 18 endometrial biopsies examined were suitable for PCR amplification of a β globulin fragment of 209 bp using either direct proteinase K digestion ($n=13$) or DNA extraction ($n=5$). *C trachomatis* DNA was found by plasmid PCR in three biopsies from three different women (table 1). In addition, 13 of the 18 biopsies were positive for 326 bp β globin PCR, but no additional positive samples were found by *omp1* PCR (320 bp).

Table 1 summarises the results of 37 ectopic pregnancy cases in which the presence of *C trachomatis* DNA was sought by PCR in the salpingectomy tissues, the endometrial biopsies, or the cervical specimens in relation to the time intervals between the sampling.

The histological picture of the salpingectomy specimens was that of a mild round cell infiltrate consisting of lymphocytes and some plasma cells. In two of these cases there was admixture with eosinophilic granulocytes. In 19 of the tubal specimens, plical conglutination or fibrosis was present (table 2). The specimen that was positive for *C trachomatis* also showed plical conglutination, fibrosis, and some lymphocyte and plasma cell infiltration (table 2, case 1). All cervical smears showed PAP I or II cytology. No endometrial specimens showed any sign of inflammation.

Discussion

In this study we found that the PCR can be successfully applied for the detection of *C trachomatis* DNA in paraffin embedded tissues and archival cervical smears, indicating that this technique could be very useful for the retrospective analysis of the role of *C trachomatis* in late complications such as ectopic pregnancy. Because of damage to the DNA templates as a result of fixation or storage, the fragment length in PCR amplification is restricted. In this study, fragments of up to 326 bp could be efficiently amplified in the majority of the specimens. The incorporation of the β globin PCR was therefore important for a reliable interpretation of the chlamydial PCR data.

As shown in this study, *C trachomatis* DNA was not, or only sporadically (1/48), present in salpingectomy tissues at the time of ectopic pregnancy. These PCR results rule out the possibility of infection with a non-cultivable form of *C trachomatis*, as has been suggested by others,²³⁻²⁵ and are in agreement with those studies reporting that direct isolation of *C trachomatis* by cell culture from the salpingectomy specimens of ectopic pregnancies was not successful.¹³⁻²⁰⁻²² In addition, infections with plasmid-free *C trachomatis* strains, so far only described as a laboratory strain,³⁵ can also be ruled out, since no additional positivity was found by *omp1* PCR. The sporadic incidence of *C trachomatis* infection found in tubal tissues of ectopic pregnancy and the high seropositivity

found by others⁷⁻¹⁵ probably reflects episodes of *C trachomatis* infection in the past. Indeed, *C trachomatis* DNA was found in preceding genital specimens (cervical specimens or endometrial biopsies) in six of the 37 women with ectopic pregnancy (table 1). Although the numbers of *C trachomatis* positive specimens are small, the increasing incidence of *C trachomatis* infection is reflected in the increasing time span before ectopic pregnancy: in 31% of women (five of 16) *C trachomatis* DNA was detected in cervical or endometrial specimens taken more than three years before the salpingectomy, while it was detected in only 9% of the women (three of 33) whose specimens were taken within three years of the ectopic pregnancy (table 1). This may still underestimate the incidence of *C trachomatis* in earlier specimens, since only a limited number of specimens taken more than three years before the ectopic pregnancy were available for testing. Nevertheless, the women with ectopic pregnancy investigated in this study were mostly in their early thirties, and the high incidence of *C trachomatis* genital infection (31%) occurred while they were in their twenties. This 31% percent of women who had *C trachomatis* in one or more genital specimens in the past is more than three times as high as the prevalence rate of 8-9% found in a large group of age adjusted asymptomatic women and pregnant women (Lan J *et al*, manuscript in preparation). Furthermore, the women investigated in this study may already have had tubal damage caused by *C trachomatis* in their twenties, though it remained unnoticed for a long time because of postponed pregnancy. These data suggest that early *C trachomatis* infection makes an important contribution to the later development of ectopic pregnancy, as generally accepted on the basis of the serological data.⁷⁻¹⁵ It is of particular interest that *C trachomatis* positivity was found only in the endometrial biopsies (three of the four specimens) taken more than three years before salpingectomy (table 1). Although the study numbers are small, these data support the hypothesis that early *C trachomatis* infections ascending to the upper genital tract (endometrium and fallopian tubes) play an important role in the pathogenesis of a substantial number of ectopic pregnancies.

In conclusion, this study showed that in the vast majority of ectopic pregnancies *C trachomatis* was not present in the fallopian tubes at the time of the disease. However, the fact that a substantial number of women with ectopic pregnancy had cervical and endometrial *C trachomatis* infections in the past indicates that ectopic pregnancy is in part the result of a previous episode of *C trachomatis* infection, resulting in a scarred fallopian tube and subsequently leading to ectopic pregnancy.

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