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Molecular investigation of menstrual tissue for the presence of *Chlamydia trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma hominis* collected by women with a history of infertility

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Abstract

Aim: At present, routine laboratory investigation of the infectious agents implicated in female genital infections is mainly based on culture/direct fluorescence antibody (DFA) (immunofluorescence antibody test) results of cervicovaginal secretions. In this study the use of the menstrual tissue is introduced for the molecular detection of pathogens which are implicated in female infertility.

Material and Methods: Cervicovaginal secretions and menstrual tissue samples of 87 women (mean age 34.07 ± 5.17) experiencing infertility problems were screened for *Chlamydia trachomatis, Ureaplasma urealyticum* and *Mycoplasma hominis* presence using polymerase chain reaction (PCR, light cycler-PCR). Cervicovaginal secretions were also tested by the culture/DFA technique. The results were compared using the binomial test.

Results: In the overall study group, the prevalence of *C. trachomatis* was 25.3%, 18.3%, and 13.8%, the prevalence of *U. urealyticum* was 18.3%, 16.09% and 12.6% and the prevalence of *M. hominis* was 13.7%, 19.5% and 8.0% in the menstrual tissue, cervicovaginal secretions using PCR and cervicovaginal secretions culture/DFA, respectively. A statistically significant difference was revealed between the two methods for all three microbes and between menstrual tissue and cervicovaginal secretions PCR for chlamydia.

Conclusions: The use of menstrual tissue along with the PCR method seems to be an effective and thus novel alternative for the investigation of the infectious agents lying in the genital tract. One of the main advantages of this technique compared to cervicovaginal secretions is that it is non-invasive and the sample can be collected at home, thus allowing the early detection and treatment of a condition that can otherwise lead to serious consequences, such as tubal obstruction, pelvic inflammatory disease, ectopic pregnancy, spontaneous abortions and unexplained infertility.

Key words: *Chlamydia trachomatis*, infertility, menstrual tissue, *Mycoplasma hominis*, sexually transmitted infection, *Ureaplasma urealyticum*.

Introduction

The increasing prevalence of sexually transmitted infections is a major health issue, especially those implicated in female infertility.[1–3] The detection of sexually transmitted microbes, which can lead to endometrial inflammation in women, is of great clinical importance, because if untreated it can lead to

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serious consequences, including miscarriages, tubal obstruction, ectopic pregnancies, preterm birth, chronic pelvic pains, unexplained infertility and even ovarian cancer.[4–6] Studies have indicated that common obstetric and gynecologic conditions can be attributed to the presence of undetected bacteria in the endometrium.[7] In particular, some studies have shown that women suffering from unexplained infertility have increased their pregnancy rates when both they and their partners received broad-spectrum antibiotics.[8,9]

The traditional laboratory approaches available, such as cervicovaginal secretions culture or immunologic examinations, offer a way for the diagnosis of endometrial infections or indirectly male partners' infections.[10] An alternative highly effective way of detecting sexually transmitted infections (STI) combining specificity and sensitivity is the method of polymerase chain reaction (PCR)[11] on cervicovaginal secretions. Nevertheless, there are a series of studies indicating the high detective limitations of these techniques, which in the latter method are mainly attributed to the starting material used.[12–14] For instance, according to the same studies, the inadequacy of the samples submitted for chlamydial infection investigation reaches 49%. Nowadays, the basic routine method for the detection of microbes is the cervicovaginal secretions culture, which is able to detect only 70–80% of the infections of the lower genital tract and as a consequence an even lower percentage of the upper genital tract.[15–17] An additional drawback is that the samples used routinely do not involve the endometrium, thus in most cases an infection is not identified and women go untreated with all of the consequences.

In the present study, *Chlamydia trachomatis*, *Ureaplasma urealyticum* and *Mycoplasma hominis*, the three microbes mainly implicated to female infertility, were investigated in the menstrual tissue. The menstrual tissue consists of the shed functionalis layer of the endometrium along with supplied peripheral blood cells and is one of the main areas where these microbes lie. Furthermore, the investigation of this layer is of particular clinical importance as it is directly related to the site where the embryo is implanted.

Methods

Patient groups

Eighty-seven women (single partner) aged an average of 34.04 ± 5.17 years, suffering from fertility problems were examined in the Infertility Department of Locus Medicus Center. Along with the routine tests (immunophenotypical investigation of lymphocytes, autoimmune markers etc.), molecular detection of the microbes *M. hominis*, *U. urealyticum* and *C. trachomatis* was performed on their menstrual tissue and to a cervical vaginal swab prior to menstruation.

Specimen collection

The detached endometrial tissue was collected by the women themselves on the day of the greatest menstrual tissue flow, in a 50-mL sterile tube (BD 352070), containing 0.4 mL of phosphate-buffered saline (pH 7.6), according to the following instructions: in a sitting position, the tube's mouth is touched at the posterior fourchette of the vulva, where the endometrial tissue flows in an involuntary manner. Due to the mucosal nature of the material, when it adheres at the sidewalls of the tube, women are advised to shift the buffer towards the material and blend it all together. The procedure is repeated until around 1 mL of material is collected. The samples were stored in a cooler until they were brought to the laboratory, where they were briefly vortexed and stored at 4°C for processing within 2 days of collection. None of the women reported any difficulty in collecting the menstrual tissue. In addition, in the same women prior to their menstruation, a cervicovaginal secretion culture and a PCR on their cervicovaginal secretions were performed for all three microbes. The cervicovaginal secretions were collected by the same physician as follows: a sterile speculum was placed in the vagina and the swab was taken from the posterior vaginal fornix and the endocervix.

Written consent was obtained from all women, following the guidelines of the Greek Bioethical Committee.

DNA extraction

Total DNA was extracted using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions and was stored at −20°C for further processing.

DNA integrity

The quality and quantity of the extracted DNA was tested using a spectrophotometer.

Light cycler PCR

For the detection of *C. trachomatis* and *U. urealyticum* the PrimerDesign kit was used. *M. hominis* was detected using a previously described assay[16] in a Light Cycler.

Precautions

In order to minimize the risk of contamination, DNA isolation and PCR setting were performed in dedicated spaces, always with the use of DNase-free equipment and aerosol-resistant pipette tips.

Conventional detection techniques

Chlamydia were identified using the direct fluorescence antibody (DFA) test, whereas for *M. hominis* and *U. urealyticum*, culture techniques were employed using Immumark-Mycotest kit, Mycoplasma Agar A7.

Statistical analysis of the results

In the present study the presence of *C. trachomatis*, *M. hominis* and *U. urealyticum* was tested with three different methods (PCR menstrual tissue, PCR cervicovaginal secretion and culture in cervicovaginal secretion). The three methods were compared pairwise, giving a total of nine comparisons. In each case, the method that yielded the higher number of positives was the golden standard (GSM) whereas the other method was the comparison method (CM). None of the comparisons generated false positives, thus all positives detected by CM were invariably detected by GSM, which in turn detected a higher number of positives. These last cases are apparently the false negatives and they evidently lower the value of negative predictive value and especially that of sensitivity. Accordingly, the false negative rate is increased. The binomial test was used to compare the methods and the *P*-value was calculated. The hypothesis tested in each case is the following: H_0 (null hypothesis): the two methods detect the same proportion of positives; and H_1 (alternative hypothesis): GSM detects a significantly higher proportion of positives than CM. *P*-values < 0.05 (and especially < 0.01) mean that the null hypothesis is rejected and the alternative hypothesis is accepted at the specific significance level. Rejection of the above hypothesis is equivalent to the statement that the false negative rate of CM in comparison to GSM is significantly greater than zero.

Results

In the overall study group, the prevalence of *C. trachomatis* was 25.3%, 18.3% and 13.8%, the prevalence of *U. urealyticum* was 18.3%, 16.1% and 12.6% and the prevalence of *M. hominis* was 13.7%, 19.5% and 8.0% in the menstrual tissue, vaginal cervical secretions using PCR and vaginal cervical secretions using culture, respectively. The results are presented (Table 1) in pairwise comparisons in nine rows, reporting in each row the absolute numbers of true positive, false positive (= 0), false negative and true negative, as well as the proportion and number of positives found by each method. The sensitivity and false negative rate of CM in comparison to GSM and the *P*-value (significance) of a statistical comparison using the binomial test is also shown.

Table 1 Pairwise comparisons of the three sampling and examination methods for the detection of *Chlamydia trachomatis*, *Mycoplasma hominis* and *Ureaplasma urealyticum* ($n = 87$)

GSM	CM	True positive	False positive	True negative	False negative	% (*n*) in GSM	% (*n*) in CM	Sensitivity	False negative rate	*P*-value (binomial)
					Chlamydia trachomatis					
PCR MT	Culture CVS	12	0	65	10	25.3% (22)	13.8% (12)	54.5%	45.5%	<0.01
PCR CVS	Culture CVS	12	0	71	4	18.4% (16)	13.8% (12)	75.0%	25.0%	NS
PCR MT	PCR CVS	16	0	65	6	25.3% (22)	18.4% (16)	72.7%	27.3%	<0.05
					Mycoplasma hominis					
PCR CVS	Culture CVS	7	0	70	10	19.5% (17)	8.0% (7)	41.2%	58.8%	<0.01
PCR MT	Culture CVS	7	0	75	5	13.8% (12)	8.0% (7)	58.3%	41.7%	<0.05
PCR CVS	PCR MT	12	0	70	5	19.5% (17)	13.8% (12)	70.6%	29.4%	<0.05
					Ureaplasma urealyticum					
PCR MT	Culture CVS	11	0	71	5	18.4% (16)	12.6% (11)	68.8%	31.2%	<0.05
PCR CVS	Culture CVS	11	0	73	3	16.1% (14)	12.6% (11)	78.6%	21.4%	NS
PCR MT	PCR CVS	14	0	71	2	18.4% (16)	16.1% (14)	87.5%	12.5%	NS
					At least one infection detected					
PCR	Culture	24	0	48	15	44.8% (39)	27.6% (24)	61.5%	38.5%	<0.001

CM, comparison method; CVS, cervicovaginal secretion; GSM, golden standard method; MT, menstrual tissue; NS, not significant; PCR, polymerase chain reaction.

Table 2 Microbial presence according to clinical history of the infertile women tested ($n = 87$)

	Chlamydia trachomatis	*Mycoplasma hominis*	*Ureaplasma urealyticum*
Infertility ($n = 35$)	14/35	8/35	7/35
Recurrent spontaneous aborters ($n = 14$)	8/14	6/14	6/14
Tubal factor ($n = 11$)	10/11	2/11	3/11
Male ($n = 25$)	10/25	6/25	3/25
Endocrine ($n = 13$)	4/13	1/13	0/13
Ovulation ($n = 2$)	0/2	1/2	0/2
Endometriosis ($n = 5$)	5/5	1/5	0/5
Ectopic ($n = 1$)	1/1	0/1	1/1
Unexplained ($n = 8$)	4/8	1/8	2/8

A statistically significant difference was observed in comparing the sensitivity difference between PCR on menstrual tissue and cervicovaginal secretion culture/DFA for all three infectious agents ($P < 0.01$ for *C. trachomatis* and $P < 0.05$ for *M. hominis* and *U. urealyticum*). Molecular investigation of menstrual tissue, compared to vaginal cervical swab, had a statistically significant sensitivity in the cases of chlamydia ($P < 0.05$), whereas it is interesting that cervicovaginal secretions sample chlamydia detection had no statistically significant difference between PCR and culture/DFA. PCR on vaginal cervical swab was more sensitive in *M. hominis* detection, whereas there was no significant statistical difference in *U. urealyticum* detection. The clinical history compared to the microbial presence is presented in Table 2.

Discussion

In the past few years, the occurrence of sexually transmitted infections and in particular *C. trachomatis*, which is the most prevalent in Europe as well as in the USA, has been progressively increasing.[18,19] The accurate detection, therefore, of STI becomes of great importance, especially when it comes to the investigation and treatment of both female and male infertility.[20] The necessity of a more effective treatment against inflammations of the upper genital tract in such cases has been clearly supported through epidemiological studies, performed in Sweden, where the frequency of secondary infertility has been significantly decreased with a proper STI treatment.[21,22]

The menstrual tissue of 25.3% of the examined women contained chlamydial genetic material, compared to 18.3% of vaginal cervical secretions. Vaginal cervical secretions using PCR and culture/DFA had no statistically significant difference on chlamydia detection, indicating that the starting sample is of importance as well. The above results are indicative of the microbial presence mainly in the upper genital tract, but still are not indicative of its activity status. However, even in the case where the microbial infection remains in a latent phase, the possibility of a long-term reactivation and of course its transmission potency remains an issue of consideration.[23] In another study, molecular investigation of fresh tissue specimens of endometrium, tube and ovaries detected chlamydial DNA in 56% of the cases of ectopic pregnancies and in 71% of the women having a tubal-obstruction related infertility,[24] thus further supporting the relation between chlamydia and infertility. The fact that chlamydial infection is the most common STI in teenagers[25] and has high prevalence rates in asymptomatic women[26] and that it is often detected only after it has caused pathologic conditions makes the investigation of the menstrual tissue in adolescent populations an important preventive strategy.

DNA from *M. hominis* and *U. urealyticum* was isolated in 13.7% and 18.3% in the menstrual tissue of women having fertility problems, respectively. According to Witkin and partners,[27] PCR on vaginal secretions of women undergoing *in vitro* fertilization indicated the presence of *U. urealyticum* in 17.2% of them and of *M. hominis* in 2.1%. The difference between the two studies could be attributed particularly to the different tested material and to the low number of women included in that study. The presence of *M. hominis* has been correlated with the development of pelvic inflammation, spontaneous abortions and infertility while *U. urealyticum* beside the aforementioned conditions has been associated with chorioamnionitis and preterm labor.[28–30] The preventive investigation of such agents in women along with a proper therapeutic management seems to be an important prerequisite, especially when planning a future pregnancy so as to avoid any complications. An interesting observation was that mycoplasma had higher detection rates in the vaginal secretion PCR, which can probably be attributed to the site of mycoplasma infection.[31]

Furthermore, the presence of such microbes in women can also be indicative of a respective infection in the male partner, potentially leading to serious alterations in the morphology, concentration and motility of the sperm, along with epididymitis, prostatitis, and non-gonococcal urethritis.[32,33]

The conventional culture technique of cervicovaginal secretions has produced significantly lower percentages of infected individuals in all three microbes, with very low sensitivities, 54.5% for chlamydia, 58.8% for *M. hominis* and 68.8% for *U. urealyticum*. In all cases there was a statistically significant difference between menstrual tissue and culture, but none was detected between PCR on cervicovaginal secretions and culture, for ureaplasma and chlamydia detection. Both the different starting material as well as limitations of the conventional detecting methods can account for this big discrepancy in the produced results. The increased sensitivity of PCR as compared to the culture technique has also been shown in a similar study where 36% of women with a negative culture test were PCR-positive for the presence of chlamydia.[23] In another study, PCR detected the presence of *U. urealyticum* in the amniotic fluid in 28% of women suffering from premature rupture of membranes, in contrast to culture of the same sample, which failed to detect its presence in 42% of positive sample.[34] Furthermore, the infections produced from these three microbes seem to have an upper genital tract tropism and often turn towards the pelvis, while the vagina and the cervix can still remain negative for their presence. In fact, Lucisano *et al*.[35] showed that 65% of women undergoing laparoscopy, with lower genital tract negative cultures, had a chlamydial infection in their upper genital tract alone.

In order to overcome collected specimens inadequacy, several studies suggest the use of modified sanitary napkins and tampons as alternatives for cervicovaginal secretions collection, which can in fact enhance the quality of the collected specimen; however, these still are indirect approaches and rather invasive.[36,37] Overall, the main advantage of using the menstrual tissue as the starting material in such cases is its direct relation with the exact location of the microbial activity, whereas approaching endometritis from cervical or vaginal materials is rather indirect. By this non-invasive technique, patients can easily collect their own sample at home and repeat this procedure as many times as required throughout their treatment. In fact, collection of samples at home is considered to be one of the preventive strategies against STI.[38,39] It also allows sample collection by virgin women as well, where obtaining material for investigation of non-sexually transmitted agents, even at the level of the cervix, is infeasible. A drawback of DNA amplification for this type of examination as compared to culture is its inability to carry out antibiotic sensitivity tests.

Finally, the use of PCR on menstrual tissue in everyday diagnostic practice can also be extended to other microbes, such as *Neisseria gonorrhoeae*, *Mycobacterium tuberculosis*[40,41] and viruses like cytomegalovirus, which actually does not grow on cultures,[42] and other herpes viruses[43] that are associated with infections of the genital tract.

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Patent

The use of menstrual tissue is patented by Vassilis Tsilivakos (European Patent 1395670 B1, Determining endometrial status by testing menstruation tissue: international patent pending).

Disclosure

There is no conflict of interest to disclosure.

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