THE HIDDEN-Chlamydia TEST®

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The determination of endometrial status by testing menstruation tissue is an internationally patented clinical test (European Patent 1395670). LOCUS MEDICUS S.A., Athens, Greece is the sole clinical laboratory authorized by the proprietor Dr. Vassilis Tsilivakos for its use.

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1. What is the Hidden-C Test ®

The Hidden-C (Chlamydia) Test® is the molecular detection of Chlamydia by real-time PCR in menstruation material or "blood". The detection of Chlamydia in menstruation material allows the diagnosis of a Chlamydial infection even when it is contained ("hidden") in the female upper genital tract and consequently it is difficult to detect by conventional methods of testing.

The Hidden Chlamydia Hypothesis:

During the last 15 years, in the subfertility clinic of LOCUS MEDICUS SA, the correlation between infectious agents and infertility is being investigated through a wide array of cutting edge diagnostic tests including reproductive immunology and molecular microbiology techniques (Real-Time PCR, Microarrays, DNA sequencing, Flow cytometry etc.). Since 1996 and after more than 15,000 cases of Chlamydia studied, among the results of this effort was an international patent for the investigation of the endometrial status through the study of shedding endometrium tissue. Part of this patent is the molecular detection of microorganisms like Chlamydia in menstruation material or "period blood" – the Hidden-C Test®.

The discovery of this novel test was based on the following clinical observations:

a) C. trachomatis infections can be localized in the upper female genital tract (i.e. the endometrium and the Fallopian tubes) in which case conventional testing using cervical/vaginal swab specimens may miss such an infection unless it is also present in the cervix or the vagina.
b) Cells of the shedding endometrium found in menstruation material ("blood") will contain Chlamydia originating from an upper tract infection as well as from the lower genital tract as the material flows through, so **a PCR detection test performed on menstruation blood specimens will detect a Chlamydial (or any other clinically relevant microorganism) infection of both the upper and the lower genital tract**.

The above observation also led to the assumption that the use of this new test would reveal a much higher Chlamydia prevalence which up until its development was concealed due to false negative results. Indeed **this new diagnostic test revealed higher positivity rates for Chlamydia both in Greece and the UK than that indicated by conventional testing due to false negative results.**

A summary of the advantages of the Hidden-C Test® include:

- ✓ *More Sensitivity*
- ✓ *Economy*
- ✓ *Accessibility to the population*
- ✓ *A non - invasive, more acceptable specimen collection method*
- ✓ *Less susceptibility to inaccurate specimen collection*

2. Why chlamydia is a public health problem *(The following is adapted from ECDC TECHNICAL REPORT, Review of chlamydia control activities in EU countries, Stockholm, May 2008 and ECDC GUIDANCE, Chlamydia control in Europe, Stockholm, June 2009)*

Genital infection with Chlamydia trachomatis (commonly known as 'chlamydia') is the most common bacterial sexually transmitted infection in many European countries [1]. Rates in sexually active young people are commonly between 5 % and 10 % *(in the general population through conventional testing)*. The number of diagnosed cases is increasing, in part due to increased testing **and the use of more sensitive tests**. People with genital chlamydia may experience symptoms of genital tract inflammation including urethritis and cervicitis, but the majority remains asymptomatic. Chlamydia is a significant public health problem because untreated chlamydia may lead to pelvic inflammatory disease, **subfertility and poor reproductive outcomes** in some women. **The cost of treating subfertility due to chlamydia is high as it requires tubal surgery and in-vitro fertilization**. Although inexpensive and effective treatment is available, control of chlamydia is challenging since most people are asymptomatic. Although the infection often causes no symptoms, it can have severe long-term consequences in a proportion of cases. In women, C. trachomatis that ascends from the endocervix to the upper genital tract can cause pelvic inflammatory disease (PID), which can result in scarring and adhesions in the Fallopian tubes and adnexae. This increases the risk of ectopic pregnancy, tubal infertility and chronic pelvic pain [2]. In the UK it has been estimated that 64 000 cases of PID and 3 000 ectopic pregnancies each year are attributable to chlamydial infection, although the evidence for these statements is weak. These complications cause considerable distress to the individuals and, in the case of infertility, have major cost implications for health services [3]. **Chlamydia is likely to be the commonest preventable cause of such reproductive tract morbidity**. Chlamydia has been reported to account for up to two thirds of cases of tubal infertility and a third of ectopic pregnancies [4]. Infection during pregnancy is associated with premature rupture of the membranes, low birth weight and miscarriage[4]. Chlamydia can also be transmitted from mother to baby during labour, causing eye and respiratory infections [5]. In men, chlamydia can lead to acute genital inflammation (epididymitis, epididymo-orchitis) and occasionally to sexually-acquired reactive arthritis (SARA). In men and women chlamydia may produce proctitis. Individuals with chlamydia are at increased risk of acquiring or transmitting HIV [6].

Table 1: Complications of Chlamydia *(ECDC GUIDANCE, Chlamydia control in Europe, Stockholm, June 2009)*

Women (Infection at any time)	(Infection during pregnancy)	**Men**
Pelvic inflammatory disease	Miscarriage	Epididymitis
Tubal infertility	Premature rupture of membranes	Epididymo-orchitis
Ectopic pregnancy	Low birth weight	Sexually-acquired reactive arthritis
Sexually-acquired reactive arthritis	Transmission from mother to baby leading to ophthalmia neonatorum and atypical neonatal pneumonitis	

1. *Low N. Current status of chlamydia screening in Europe. Euro Surveill. 2004;8.*
2. *Cates W, Wasserheit JN. Genital chlamydial infections: epidemiology and reproductive sequelae. Am J Obstet Gynecol. 1991;164:1771-81.*
3. *Adams EJ, Turner KM, Edmunds WJ. The cost effectiveness of opportunistic chlamydia screening in England. Sex Transm Infect. 2007;83:267-74.*
4. *Peipart JF. Clinical practice. Genital chlamydial infections. NEJM. 2003;349:2424-30.*
5. *Hammerschlag MR. Chlamydial infections in infants and children. In: Holmes KK, Mardh PA, Sparling PF, editors. Sexually transmitted diseases. 3rd ed. New York: McGraw Hill; 1999. p. 593.*
6. *Fleming DT, Wasserheit JN. From epidemiological synergy to public health policy and practice: the contribution of other sexually transmitted diseases to sexual transmission of HIV infection. Sex Transm Infect. 1999;75:3-17.*

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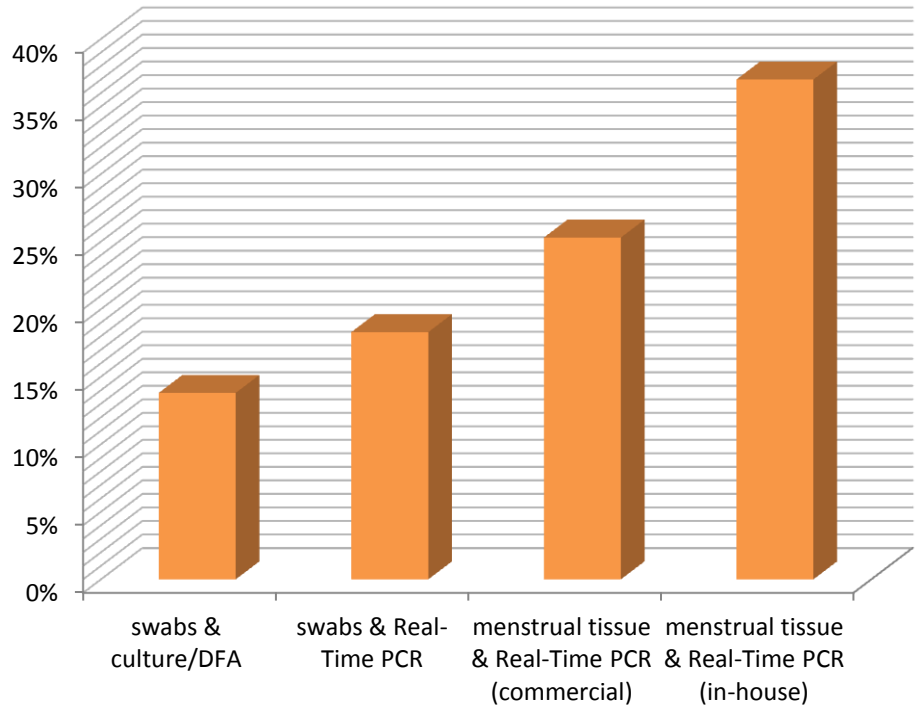
3. Scientific data supporting the HIDDEN-C Test®

In order to accurately estimate the prevalence of Chlamydia in a local as well as in an international level, the use of high sensitivity diagnostic methods for the detection of infection, is essential. This becomes even more important when the selection of candidates for screening is based on prior positive diagnosis such as in the case of partner notification (In this case, a false negative for a woman could potentially mean two or more misdiagnoses).

A recent relevant study by the author (Submitted for publication), compared the respective Chlamydia prevalence among 3 main methods used for C. trachomatis detection, in a group of women with a background of subfertility in Greece. The positivity for C. trachomatis in cervical-vaginal secretion specimens collected by swabs was 13.8% by culture/DFA (Immuno Fluorescence Antibody test), while positivity was 18.3% when the same specimens were analyzed using Real-Time PCR (Polymerase Chain Reaction) (Fig.1). The highest positivity however, was observed when the same women collected samples of menstrual material (period blood drops) by themselves at home, followed by specimen analysis by Real-Time PCR using a commercial C. trachomatis PCR detection kit (positivity~25%), or an in-house PCR protocol (positivity~37%) (Fig.1).

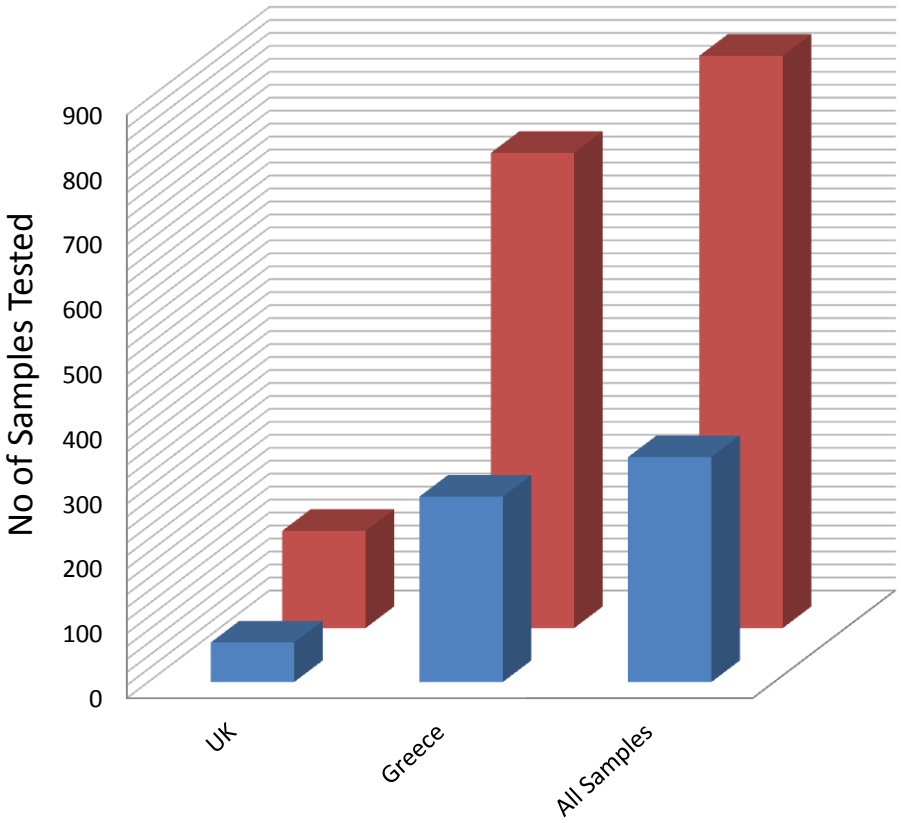
Fig.1 C. trachomatis positivity by different specimen collection and C. trachomatis detection methods in a group of subfertile women.



The positivity for C. trachomatis in cervical-vaginal swab specimens was 13% by culture/DFA or 18% for the same specimens analyzed by real-time PCR. The highest positivity was observed when the same women collected menstrual tissue samples themselves, followed by real-time PCR analysis using a commercial C. trachomatis detection kit (25%), or an in-house PCR protocol (37%).

Similar positivity (~39%) was observed when different groups of women (883 women in total) with subfertility backgrounds, were tested by the method described above, over a period spanning May 2010 to Feb 2011 (Fig.2). Furthermore, the same positivity was observed in menstrual tissue specimens collected from UK infertility clinics during the same time period (of the 883 specimens tested, the 150 were sent from the UK).

Fig.2 Number of UK and Greek samples positive for *C. trachomatis* infection compared to total number of samples tested, using the HIDDEN-C Test ®



Out of 883 UK and Greek women with subfertility backgrounds tested for C.trachomatis using PCR testing on menstruation tissue (HIDDEN-C Test®), 347 (39%) were found positive.

In my opinion, the detection of Chlamydia by PCR testing of menstrual tissue (Hidden-C Test ®) comprises a novel, alternative, non-invasive method for investigating infectious agents on specimens representing most of the genital tract (including the endometrium) which will contribute to the evaluation of silent Chlamydial infections, especially in the context of the ongoing, nation-wide UK screening programme for Chlamydia infections (including cases of non-sexually transmitted Chlamydial infections, mostly in non-sexually active women of young age).

Dr. Vassilis Tsilivakos MD, PhD
Immunologist and Pathologist
Subfertility Specialist

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4. The clinical significance of the Hidden-C Test ®.

According to recent data it gradually becomes clearer that people are hosts to infectious agents (microbes) that until now, were traditionally considered obligatory pathogens. It seems that our co-existence with Chlamydia, which under specific circumstances can create health problems, is closer that we thought and that people can get infected very easily in a non-sexually-transmitted way. Unfortunately, the health risk that a Chlamydia infection poses for each individual at a particular phase in his or her life has not been officially determined and the same is true for guidelines concerning the severity of the recommended treatment for each specific case. It is however safe to suggest, that Chlamydia testing should take place before any kind of gynaecological procedure or fallopian tubes permeability test.

Up until recently, there wasn't a satisfactory diagnostic test for the detection of Chlamydia. Furthermore, diagnostic approaches that implied the presence of Chlamydia such as the Pap test or other traditional, low sensitivity tests, misinformed to a large extend the general public for the prevalence of Chlamydia, due to false negative results. The method of study of the female genital tract that we proposed after 15 years of experience in the field of reproductive medicine is the Chlamydial DNA detection by PCR amplification in menstruation material ("blood") samples. This diagnostic test has considerable advantages over traditional ones. Some of its advantages are listed below:

- High content of upper genital tract tissue
- Easy and convenient sample collection that can be performed by the patient at home
- The sample can be kept at room temperature for a long time without affecting the result
- EXTREMELY HIGH SENSITIVITY AND SPECIFICITY

This new test has been greatly received by the general public both in Greece and internationally. Moreover, the Hidden-C Test ® appears to be the test of choice for young women for subfertility prevention (even before they become sexually active) as well as part of subfertility investigation testing for couples.

The Hidden-C Test ® reveals a positivity rate (positive diagnosis) for the presence of Chlamydial infection of about 37% in samples investigated for subfertility, in Greek and UK population.

For the treatment of Chlamydia, antibiotics are, of course, required. However, the theoretical objective of the treatment which is the total elimination of the infection is not always possible. In our experience, while some gynaecologists prescribe continuous antibiotic treatment for up to 8 months before the infection has been totally eliminated (according to our highly sensitive test), our opinion is that the correct way of dealing with a Chlamydial infection of the genital tract is prescribing a single course of antibiotics followed by retesting after 6 or 12 months.

IMPORTANT NOTE: To avoid back and forth transmission of Chlamydia between partners when their therapies are not synchronized, and also to care for the reproductive well-being of the male partner as well, we propose that men should also be tested for Chlamydia, as such infections can lead to chronic epididymitis, or to deterioration of sperm characteristics. In our opinion, the microbial factor should be tackled first during treatment of oligo-, astheno- or teratospermias,

especially prior to surgical intervention. For this reason it is imperative that men too should be tested through high sensitivity/high specificity methods.

In the specialized clinic for subfertility and reproductive medicine & immunology in LOCUS MEDICUS, led by our subfertility specialist, Dr. Tsilivakos (MD, PhD, Immunologist, Pathologist), the inventor of menstruation tissue testing and the Hidden-C Test ®, we provide specialized, highly sensitive diagnostic tests for viral and microbial detection in sperm, as well as information about sperm infections and their treatment.

5. The Hidden-C Test® Advantages:

a) High Sensitivity

It is particularly important that the method is characterized by high sensitivity and specificity, which minimize false negative and false positive results, respectively. Up until recently diagnostic approaches that indicated the presence of Chlamydia such as the Pap smear or other traditional, low sensitivity tests, misinformed to a large extend the general public for the prevalence of Chlamydia, due to false negative results. The sensitivity of culture/DFA a conventional method of detecting endometrial microbes is very low (Table 2). The otherwise highly sensitive methods such as NAATs, have also smaller chance of detecting **endometrial** microbes when performed on cervical fluid specimens (45-60%). **On the other hand, the correct specimen type used by the Hidden-C Test® combined with the extremely high sensitivity of state-of-the-art Real Time PCR, exceeds >95% sensitivity for screening the endometrium for the presence of microbes.**

Table 2: Advantages and disadvantages of Chlamydia detection methods*

Method	Advantages	Disadvantages
Diagnostic method		
• **Nucleic acid amplification tests (NAATs)** • **polymerase chain reaction (PCR)** • **Strand displacement amplification [SDA]** • **Transcription-mediated amplification [TDA]**	• **High sensitivity (90–95 %)** • **Can be used on urine samples and vulvovaginal swabs (including self administered tests)** • **Validated for extragenital sites, including rectum**	• **Expensive** • **False positive results may be a problem in some settings** • **Not licensed for extragenital sites**
• **EIA (enzyme-linked immunosorbent assay)**	• **Can be adapted for point-of-care tests** • **Cheap**	• **Low sensitivity (40–70 %)** • **Not appropriate for urine and self-collected swabs**
• **Cell culture**	• **Can be used on all specimen types** • **High specificity**	• **Low sensitivity (60–80 %)** • **Expensive – requires technical expertise and is labour intensive** • **Not suitable for large through-put**
• **Direct fluorescent antibody (DFA) tests**	• **Can be used on all specimen types** • **Rapid turnaround time**	• **Low sensitivity for urine** • **Labour intensive** • **Requires expertise**

**Table adapted from European Centre for Disease Prevention and Control (2009) ECDC Guidance: Chlamydia control in Europe, Stockholm, June 2009, ISBN 978-92-9193-165-1, doi 10.2900/11364*

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The Hidden-C Test® can also be extended to include detection of other medically relative microorganisms such as Mycoplasma Hominis and Ureaplasma urealyticum in a triple **C. trachomatis - M.hominis - U.urealyticum detection test**.

b) High quality, easy to collect *and* c) inexpensive to deliver samples

The Hidden–C Test® is a more sensitive method, compared to fine needle biopsy samples due to the fact that menstruation tissue is much more representative of the endometrium and it also contains cervical and vaginal material as it courses through the female genital tract. It is also very easy to extract sufficient quantity of material. In this aspect, this method is superior compared to other invasive techniques. Women, avoiding any gynecological examination stress can collect the sample themselves at home and repeat it if the first attempt fails. Obviously, a very important advantage of our method is that it can be easily repeated and thus antibiotic therapy results monitored.

Method	Advantages	Disadvantages
Specimen collection		
• **Clinician-obtained**	• **Ability to obtain good quality sample, e.g. endocervical swab which may increase sensitivity**	• **Less acceptable to some patients** • **More expensive in staff time**
• **Self-collected**	• **More acceptable to some patients** • **Less clinical facilities required**	• **May be less sensitive**

**Table adapted from European Centre for Disease Prevention and Control (2009) ECDC Guidance: Chlamydia control in Europe, Stockholm, June 2009, ISBN 978-92-9193-165-1, doi 10.2900/11364*

Method	Advantages	Disadvantages
Delivery		
• **Point-of-care tests (administered by healthcare professionals)**	• **Treatment can be offered at same time as diagnosis, so no need for patient recall**	• **Currently all EIA-based, therefore less sensitive than NAATs**
• **Over-the-counter, self-administered tests**	• **May be more acceptable and accessible for some groups**	• **Reliability of method needs to be assured — currently few quality controls**
• **Postal tests**	• **Patients can take samples in their homes** • **Tests are carried out by laboratory**	• **Good regulation and quality control required** • **Need to be linked to access to treatment**

**Table adapted from European Centre for Disease Prevention and Control (2009) ECDC Guidance: Chlamydia control in Europe, Stockholm, June 2009, ISBN 978-92-9193-165-1, doi 10.2900/11364*

Specimens for the Hidden-C Test® are self-collected by patients and are then sent to the laboratory by post. This provides the most accessible and acceptable method for the patient, while it does not require expensive facilities or trained staff. Furthermore, specimen collection is completely passive (the blood drips into the container), always guaranteeing a good quality sample (the patient cannot make a mistake collecting the sample) and ensuring reproducibility of results.

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The Hidden-C Test ® is the only test that combines Sensitivity, Economy, Ease & Convenience, Speed and Zero Rejection due to specimen inadequacy.

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LOCUS MEDICUS S.A. operates a variety of specialized medical clinics including Obstetrics & Gynecology, Molecular Pathology & Genetics, and Subfertility & Reproductive Medicine. Furthermore, LOCUS MEDICUS S.A. includes high-end clinical laboratories in the fields of Microbiology, Biochemistry, Hematology, Histopathology, Cytogenetics, Immunology, Cellular Biology, Molecular Pathology, Genetics and Reproductive Immunology, in which, during the last 15 years, more than 500.000 specialized medical diagnostic tests have been performed while more than 20 international scientific papers have been published (for more details on our research please visit our website).

To ensure high quality service LOCUS MEDICUS S.A. participates in external quality control programs including WHO global HPV, CMV, EBV Laboratory Network, European HPV Test External Quality Assurance Scheme, Cystic Fibrosis European Network, College of American Pathologists, Instand-Eurogentest External Quality Assessment Scheme, UK NEQAS for Molecular Diagnosis of Mycobacteria, UK NEQAS for Leucocyte immunophenotyping, UK NEQAS for Molecular Microbiology.

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