Simultaneous PCR-Based Detection of Six Pathogens Inducing Sexually Transmitted Diseases

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Abstract: Background: Since many sexually transmitted diseases (STDs) show no noticeable symptoms immediately after the infections occur, their clinical documentation can be difficult. However, untreated infections with these pathogens may raise in time serious health problems. Conclusive identification of these pathogens is essential to limiting the spread of STD infections and other illness complications. Our study aims to test a molecular biology method based on Polymerase Chain Reaction (PCR) for the simultaneous determination in urine or vaginal samples of six pathogens that induce the most frequent STDs: Chlamydia trachomatis, Neisseria gonorrhoeae, Trichomonas vaginalis, Ureaplasma urealyticum, Mycoplasma hominis and Mycoplasma genitalium.

Methods: Urine (men) and urine or vaginal samples (women) were collected from individuals suspected of sexually transmitted diseases. DNA was extracted, purified and amplified via PCR for simultaneous detection of the above-mentioned pathogens, and further identified in the mix using a 2% agarose gel electrophoresis, with ethidium bromide as staining agent.

Results: Concentration and purity of the DNA extracted from all samples enabled proper identification of the pathogens causing STDs. Several double infections were noted.

Conclusions: The method tested is accurate for simultaneous diagnosis of multiple STDs. We propose this technique to be used in population screening on the STDs incidence.

Keywords: STD pathogens, simultaneous detection, PCR, DNA, gel electrophoresis.

1. INTRODUCTION

As most sexually transmitted diseases (STDs) are asymptomatic at debut, their clinical documentation is difficult. Prolonged, untreated infections with these pathogens may have serious consequences: they can induce anexitis, endometritis or pelvic peritonitis (in women) and prostatitis or epididymitis (in men).

Bacterial vaginosis is strongly implicated in female infertility and it probably is an underestimated cause of unexplained infertility. It is documented that screening and treatment of bacterial vaginosis during the course of infertility treatment have increased the pregnancy rate considerably [1]. Therefore, early diagnosis and treatment of STD may prove to be important in reducing infertility [2].

Further complications can cause symptoms similar to other pathogens, but antibiotic treatment may vary. These infections can affect the reproductive organs and may be transmitted from mother to newborn babies. Simultaneous detection of several STD pathogens is therefore important, particularly in pregnant women. STD detection in patients presenting with urinary- or pregnancy-related complaints needs to be improved and guidelines for empirical treatment of STD should be revised for pregnant patients to include more liberal policies for less symptomatic patient [3].

Chlamydia trachomatis is the most frequently pathogen agent reported for sexually transmitted infections in Europe [4]. Being mostly asymptomatic, it poses a challenge to both primary and secondary prevention. Urogenital C. trachomatis infections have a broad spectrum of clinical manifestations, including urethritis, cervicitis, and pelvic inflammatory disease [5]. C. trachomatis can persist a long time in the genital tract in a form resistant to immune destruction, and infection symptoms can go unnoticed in approximately 75-80% of women [6].

In spite of aggressive antibacterial control measures, incidence of C. trachomatis infections increases, generating serious public health concern due to its morbidity and socio-economic burden. Still, very little is known about the molecular bases underlying the phenotypic disparities observed among
C. trachomatis serovars in terms of tissue tropism (ocular conjunctiva, epithelial-genitalia and lymph nodes), virulence (disease outcomes) and ecological success. C. trachomatis is traditionally classified into 15 main serovars based on the differential serospecificity of the major outer membrane protein [7].

Neisseria gonorrhoeae is the etiological agent of gonorrhea, one of the most common sexually transmitted bacterial infections, which is characterized by purulent inflammation of the mucous membranes of the genitourinary system, each year producing more than 82 million new infections worldwide [8]. It primarily causes urogenital mucosal infections in men and women and is a serious cause of morbidity. In the human genitourinary tract, N. gonorrhoeae encounters an oxidative stress-rich environment [9]. The emergence and spread of multidrug-resistant N. gonorrhoeae strains and the absence of an effective vaccine are major problems worldwide. Some authors have recently reported a progressive increase in the incidence of gonorrhea in many countries. This may indicate a return to high-risk sexual behavior, contributing to the increase of STDs, and highlights the need for routine surveillance, prevention, and control measures [10].

Trichomoniasis is a common, worldwide, urogenital infection with Trichomonas vaginalis [11]. The protozoan was first described in purulent genital secretions by Donné in 1836. Trichomoniasis is a frequent cause of symptomatic vaginitis and a less common cause of non-gonococcal urethritis. Infected vaginal discharge contains 10 to 100 organisms/mL, with symptomatic women generally manifesting the largest numbers. T. vaginalis damages squamous epithelial cells through direct contact, and the process results in microulcerations and microscopic hemorrhages of the vaginal walls and exocervix. Columnar epithelium is not affected, and thus trichomoniasis presents with vaginitis but not with endocervicitis. The simultaneous presence of an endocervical discharge should alert the clinician to the possibility of a coincident infection with N. gonorrhoeae or C. trachomatis. T. vaginalis is isolated from the urethra in most infected women. Organisms can cause ulcerations beneath the prepuce [12]. The extracellular parasite T. vaginalis infects annually the urogenital tract of approximately 3% of the world population, and is thus the most widespread non-viral, sexually transmitted human parasite known [13]. Although the vast majority of T. vaginalis infections proceed without apparent symptoms, infection with the parasite decreases fertility, elevates the risk of prostate and cervical cancer and increases the risk of acquiring HIV [14].

Mycoplasma species are the smallest free-living cell wall-deficient microorganisms. Genital mycoplasmas represent species frequently found in the lower genitourinary tracts of sexually active healthy men and women. The most prevalent genital mycoplasmas are Mycoplasma hominis, Ureaplasma urealyticum and Mycoplasma genitalium. Their high prevalence among asymptomatic women must be taken into account in evaluating the role of these organisms in human diseases [15]. U. urealyticum and M. hominis are common inhabitants of the urogenital tracts of healthy adults. Colonization with U. urealyticum occurs in 10-50% of women, and M. hominis colonization in less than 20% [16]. The former two species are also thought to induce a wide spectrum of pathological conditions in both men and women, including unexplained chronic lower urinary tract symptoms, non-gonococcal urethritis, pelvic inflammatory disease, pyelonephritis, chronic prostatitis, and preterm labor and idiopathic abortion due to infections in the urogenital organs [17]. M. genitalium has been implicated as a causative agent in conditions with significant sequelae such as cervicitis, endometritis and pelvic inflammatory disease [18-21]. The prevalence of M. genitalium is similar to C. trachomatis, and infection may cause future risks. Most clinicians are unable to assess the potential risk of M. genitalium in adolescent and young adult patients. M. genitalium is difficult to culture because it is slow-growing, it has strict nutrient requirements and the proper culture medium is not widely available [22].

A reliable method for identifying STD pathogens is essential in limiting complications and controlling the spread of infections. In recent years numerous STD screening studies have been conducted in developed countries [23-26]. In our country, however, STDs incidence and dissemination in general population remains a topical issue. Without a reliable STDs detection method, treatment is largely inefficient.

Our study aims to test the effectiveness of simultaneous determination in urine or vaginal discharge of six STD pathogens (C. trachomatis, N. gonorrhoeae, T. vaginalis, U. urealyticum, M. hominis and M. genitalium) using a molecular biology method based on DNA extraction and PCR amplification.
2. MATERIALS AND METHODS

2.1. Samples

Genital/urinary specimens were collected from 21 persons from Cluj County, Romania, until November 2014. As the research involved human material, the approval of the Ethics Committee of the "Iuliu Hatieganu" University of Medicine and Pharmacy in Cluj-Napoca was obtained (no. 146/15.04.2014). 14 male urinal samples (10 to 30 mL of first void urine in the morning in sterile polypropylene containers) and 7 women urinal samples / vaginal secretion samples (in transport medium-containers endowed with a brush for scraping off the vaginal mucosa) were collected from persons with STD symptoms or asymptomatic individuals involved in unprotected sex with multiple partners. In addition, one woman also passed genital and urinary specimen, while another provided vaginal secretion on a wool swab, these enabling us to check possible variations in DNA concentration.

2.2. Pretreatment of Clinical Specimens and DNA Extraction

Urine and swab samples were refrigerated up to 7 days at 2-8°C and brought up to room temperature before DNA extraction. Urine samples were stored at room temperature (19-25°C) when the test was scheduled to be completed within 24 hours. Aliquots of urine samples were centrifuged for 15 minutes at 15,000 g and the pellet obtained was re-suspended in PBS by pulse-vortexing. Swab specimens in culture transport media or dilution media were vortexed and aliquots centrifuged for 10 minutes at 8,000 g, and the pellet being similarly re-suspended in PBS.

For DNA extraction, 150 μL aliquots of PBS-suspended samples were transferred into micro-centrifuge tubes with tissue and cell lysis solution containing protease K, and mixed thoroughly. The mix was incubated for 15 minutes at 65°C and then cooled to 37°C, when RN-ase A was added and mixed thoroughly. The sample was further incubated for 30 minutes at 37°C and then placed on ice for 5 minutes. MPC Protein Precipitation Reagent was added to the lysed sample and the mix was vortexed vigorously. The debris was pelleted through micro-centrifugation at 14,000 g for 10 minutes and the supernatant was transferred into a clean tube. Isopropyl alcohol was added for DNA precipitation and the tube was shook 30-40 times upside-down. The DNA pellet was separated by centrifugation while the isopropyl alcohol was poured off. Then, the DNA pellet was rinsed twice with 75% ethanol and re-suspended in TE buffer.

DNA concentration and purity were determined by nano-photometer readings against a reference TE buffer solution. When DNA concentration was not large enough, the DNA extraction was repeated from larger sample amounts. If purity was not appropriate, DNA purification sequence (DNA dilution in tissue and cell lysis solution, precipitation with isopropyl alcohol, elimination of the isopropyl alcohol and rinses with 75% ethanol, DNA pellet separation via centrifugation and re-suspension in TE buffer) was repeated.

2.3. Polymerase Chain Reaction (PCR)

The amplification reaction was carried out with Seeplex® STD6 ACE Detection (Seegene) in a total volume of 20 μl including 3 μl DNA extract and 17 μl PCR mix with 5x STD6ACE PM primers containing primer pairs for C. trachomatis, N. gonorrhoeae, T. vaginalis, U. urealyticum, M. hominis and M. genitalium, a primer pair for internal control and an internal control template.

The mixture was submitted to 40 amplification cycles on a DNA thermal-cycler. PCR was initiated with a 15 minutes denaturation step at 94°C and finished with a 10 minutes extension-step at 72°C. Each cycle included a denaturation step at 94°C for 30 seconds, an annealing step at 63°C for 90 seconds and a chain elongation step at 72°C for 90 seconds.

2.4. Agarose Gel Electrophoresis

A 2% agarose gel containing ethidium bromide was used with 0.5X Tris-borate 0.1 mM EDTA (TBE) as running buffer. The wells were loaded as follows: 5 μl negative control (NC), 5 μl of each sample, 5 μl positive control (PC), 5 μl STD6 ACE marker used to determine the approximate size of target products. With the electrophoresis source set at 100 V, the electric field was applied for 30 - 45 minutes. The PCR products were visualized using a UV transilluminator.

3. RESULTS AND DISCUSSIONS

3.1. Precision and Accuracy of DNA Extraction

The assay sensitivity may decrease if samples are repeatedly frozen/thawed or stored for long periods of
time because nucleic acids can easily be degraded. For urine samples the use of fresh specimens is recommended. To avoid sample alteration, specimens were transported to the laboratory as soon as possible, at indicated temperature. To avoid freezing, DNA extraction was carried out in the same week they were collected - during which time samples were stored at 2-8 °C. The importance of collecting the first void urine in the morning is well documented (patients should not urinate at least two hours prior to collection). Probability to remove the STD pathogens increases when a middle jet is collected and an actual infection, if not massive, could go unnoticed.

It was observed that the quantities of urine and/or transport/dilution medium of vaginal swabs needed for obtaining satisfactory DNA concentration (at least 20 \( \mu g/mL \)) depended on the microbial load. For high turbidity samples, amounts of 1 mL were sufficient, while low turbidity samples required larger amounts.

When initial DNA concentration was reduced, the extraction protocol was resumed with larger quantities of centrifuged samples. When DNA concentration exceeded 50 \( \mu g/mL \), it was diluted prior the amplification to 20-50 \( \mu g/mL \), thus avoiding the inhibition of the amplification reaction.

The A260/280 ratio (corresponding to absorbance values at 260 and 280 nm) and the A260/230 ratio were determined via spectrophotometric measurements and the Beer Lambert Law was used to convert optical density into DNA concentration, thus assessing DNA purity. In all samples the A260/A230 ratio was appropriate (above 1.5). Several samples exhibited an A260/A280 ratio below the optimal 1.8 to 2.0 range, indicative of protein contamination. Those samples required further purification according to the described protocol.

A huge difference in DNA concentration was seen between the two vaginal samples of the same patient when provided in the regular vaginal container endowed with a scraping brush and on a wool swab. This was probably due to the fact that a very large number of cells were brushed off the vaginal mucosa, resulting in an excess of genomic DNA. Optimal PCR amplification implied great dilution of the genomic DNA which could have caused a decrease in bacterial DNA concentration below detection limit. However, PCR reaction was appropriate in both cases (as shown on the internal control lane). We conclude that woolen (pharyngeal type) vaginal swabs could provide an option to avoid loading of extracted DNA with genomic DNA.

The other patient who offered two (urinal and vaginal) samples exhibited higher brushed-off DNA

<table>
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<th>No.</th>
<th>Sex</th>
<th>Sample</th>
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<td>urine</td>
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<td>urine</td>
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</tr>
<tr>
<td>8</td>
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</tr>
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concentration compared with the urinal DNA. Again the PCR reaction was appropriate in both cases, as it also was in the other urine samples collected from women. We conclude that urine samples collected from women present good sensitivity for PCR-based detection of STD pathogens. Evidently, they are more easily handled. We plan to conduct other studies to confirm these results.

3.2. Identification of the STD Pathogen Agents

5 (23.8 %) of the 21 patients enrolled in our study presented STD infections. We isolated four strains of *C. trachomatis* and two strains of *N. gonorrhoeae*, one of the patients having been found with a double infection. Isolation of the two most frequent STD pathogen agents is consistent with other literature studies [4, 8]. No STD pathogens were isolated from the asymptomatic patients.

Due to the small number of samples, no statistical conclusions can be drawn. However, the aim of our study was to develop a suitable DNA extraction method and check the technique deployed. The results we obtained in the simultaneous detection of the six STD pathogens are satisfactory as the PCR amplification reaction occurred (the presence of internal control lanes being observed in all samples, see Figure 1).

![Figure 1](image.png)

**Figure 1**: STD pathogens - agarose gel electrophoresis. NC: negative control, S: sample, PC: positive control, MK: marker, TV: *T. vaginalis*, MH: *M. hominis*, MG: *M. genitalium*, CT: *C. trachomatis*, NG: *N. gonorrhoeae*, UU: *U. urealyticum*.

The accuracy of the method employed was also satisfactory, as demonstrated by the two (negative and positive) controls: while the negative control showed there was no contamination on any band, the positive control exhibited bands corresponding to the six DNA fragments of known molecular weight in the STD6 ACE marker that we used to determine the size of the targeted products (Figure 1).

Our future goal is to use this method in the screening of population patients suspected of STD infections and asymptomatic, pregnant women or infertile couples as well.

The Seeplex® STD6 ACE Detection system has a high sensitivity and specificity for STD pathogens in clinical specimens from patients with single-pathogen or mixed infections. These results are consistent with other studies employing the PCR method in the detection of STD [27]. The technical ability to detect STD is clinically important. Furthermore, for both *M. genitalium* and *T. vaginalis*, PCR is the only method currently available for rapid laboratory diagnosis. The STD6 ACE Detection system uses pre-packaged reagents, which simplifies the analysis of PCR assays.

4. CONCLUSION

To obtain suitable DNA concentrations and purity for the six STD pathogens of interest, DNA extractions from urinal and vaginal samples require occasional volume adjustment and/or re-purification.

Wide scale application of the system based on simultaneous detection of these six pathogens inducing sexually transmitted diseases may facilitate diagnosis, treatment and care for STD patients and help in preventing the spreading of these infections.

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