Chapter 6

The Advantages of Using Multiplex PCR for the Simultaneous Detection of Six Sexually Transmitted Diseases

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Additional information is available at the end of the chapter

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Abstract

Sexually transmitted diseases (STDs) are among the most common infections. Their clinical identification is difficult because STDs are often asymptomatic. Untreated infections with these pathogens can in time lead to serious consequences. It is documented that isolation of some of these bacteria from cultures is very difficult. Because there is a large number of STD pathogens which can generate coinfections, their simultaneous detection in a unique sample is very important. Multiplex polymerase chain reaction (PCR) is an advanced method of molecular biology which allows for simultaneous detection of multiple pathogens in the same sample. The advantages of the multiplex PCR method were assessed by various researchers by comparing the diagnosis results obtained with different other conventional methods. The sensitivity and specificity of these methods were analyzed on different specimens in comparison to traditional methods, such as culture media or direct microscopic examination. These studies demonstrated beyond any doubt that the multiplex PCR system is highly effective in the detection of each of multiple STD pathogens depicted from a single specimen and argued for multiplex PCR superiority in terms of sensitivity and rapidity.

Keywords: STD pathogens, DNA, simultaneous detection, multiplex PCR

1. Introduction

Sexually transmitted diseases (STDs) are an issue of great interest as they are among the most common of all infections. It is documented that STDs are more prevalent in economically underdeveloped populations. Only a few countries outside Western Europe and

North America have implemented monitoring systems for these infections. In the rest of the world, epidemiological studies are based on the results of samples provided by certain population segments (mainly symptomatic patients or prenatal controls) not necessarily representative for the majority of the population. Usually, persons presenting obvious signs (secretions, lesions or pain) interrupt their sexual activity and seek medical help. Ironically, most often those who actually transmit these infections are asymptomatic or present mild symptoms [1].

It is well-known that vaginal bacterial infections can induce human infertility, yet they are underestimated in infertility testing. Population screening and treatment of individuals presenting unexplained infertility that are detected as STD-infected persons seem appropriate in this context [2].

No STD can be regarded as an isolated problem since multiple infections are rather common and STD infections generally denote a high-risk sexual behavior that often can be associated with more serious infections. It is therefore important to acknowledge that STDs are often asymptomatic or cause nonspecific symptoms and that periodical STD testing is crucial in limiting the risk of human immunodeficiency virus (HIV) infection. STD control could significantly reduce HIV incidence worldwide, although the impact of interventions may vary depending on local epidemiological contexts. Analysis of data from several studies has suggested that a better management of STD cases is more likely to reduce HIV incidence in the early stages of an epidemic, when HIV infections are concentrated in population groups with a high prevalence of other curable STDs [3].

A correct STD diagnosis is needed to prevent further spreading of such infections in the healthy population. Several techniques and laboratory methods for highlighting these diseases were developed in the past decades. For instance, because of their high sensitivity, specificity and suitability for different types of samples, nucleic acid amplification tests (NAATs) are suitable for the diagnosis of urogenital infections. Lately, however, polymerase chain reaction (PCR) techniques are increasingly employed in such cases as they allow direct, sensitive, automated and usable detection of STD-causing pathogens on all sample types and even the simultaneous detection of several STDs.

2. The most frequent causative agent of STDs

Chlamydia trachomatis is an intracellular human pathogen. *C. trachomatis* infection is the most common STD reported in Western Europe developed countries [4]. About 75% of all cases involve young people aged 15–24 years, being more common in women than in men. It infects the epithelial cells of the endocervix in women and the urethra in men. Infection with *C. trachomatis* is often asymptomatic, especially in women, but presents a high risk for complications. The treatment is rather simple if early detected. Of note, the bacteria can be passed from mother to newborn during delivery [5].

Urogenital infection with *C. trachomatis* shows a broad spectrum of clinical manifestations, including urethritis, cervicitis and pelvic inflammatory disease (PID). Intense mucosal

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inflammation is characterized by erythema, swelling and mucous secretions caused by mucopurulent cervicitis in women and nongonococcal urehtritis (NGU) in men. It is very important to diagnose these bacteria in early stages of infection and beginning treatment as soon as possible to prevent long-term complications. It can persist in the genital tract for a long time, in a form that is resistant to immunodestruction; such symptoms are unnoticed in approximately 75–80% of women [6]. *C. trachomatis* genital infection significantly increases the risk of sequelae in the reproductive tract in women, including tubal deterioration, ectopic pregnancy and miscarriage [7]. The role of the *C. trachomatis* infection in decreasing male fertility is also well-known. Given the severity of the complications, several countries (e.g., France) made recommendations for screening these bacteria in asymptomatic young subjects [8].

Neisseria gonorrhoeae is the etiologic agent of gonorrhea, one of the most common sexually transmitted bacterial infections, producing more than 82 million new infections worldwide each year [9]. *N. gonorrhoeae* or gonorrhea is an aerobic Gram-negative bacterium presenting itself in the form of diplococci. Along with *C. trachomatis,* it is the most prevalent sexually transmitted bacterial infection. It causes urogenital mucosa infections in men and women, being a serious cause of morbidity. The disease is characterized by purulent inflammation of the urogenital system mucosa. It is also presumed that gonorrhea infection may increase susceptibility to HIV and its transmission [10].

Neisseria gonorrhoeae has developed mechanisms to alter the epithelial barriers in order to reach subepithelial tissues and colonize in the host organism. Emergence and spread of multiresistant *N. gonorrhoeae* strains and the absence of an effective vaccine are major problems worldwide. Gonococcal endocarditis is a rare (1–2%) but serious disease that occurs in patients with disseminated gonococcal infections, which are also rare (0.3–5%) [11].

Several data indicate the involvement of the gonococci in miscarriages. Screening and medical management of the *N. gonorrhoeae* infection, including screening of women visiting abortion centers, have been proposed in order to identify associated risk factors and to assess the benefits of systematic screening in avoiding complications [12].

Trichomonas vaginalis is the causative agent of trichomonosis, one of the nonviral STDs. This protozoan was first described in 1836 by Donné. Infection with this parasite can give birth to serious complications, especially in women. The spectrum of clinical presentation range from asymptomatic or slightly symptomatic, particularly in men, to severe vaginitis with abundant vaginal secretions in women [13]. Infections with this protozoan can lead to serious health problems such as infertility, preterm delivery, low birth weight [14, 15], susceptibility to cervical cancer [15] or increased prevalence of high risk human papillomavirus (HPV) infection [16]. Moreover, there is a strong association between *T. vaginalis* infection and acquisition of HIV [17]. Because it is sexually transmitted, trichomoniasis is common in populations at higher risk for other sexually transmitted infections. Its presence in an individual is a marker for high-risk behaviors and coincident STDs should be sought after. This infection is detected in approximately three quarters of the infected women's male sex partners [18]. For better cure rates, sexual partners should be treated simultaneously even if the infection is asymptomatic.

Trichomonas vaginalis infects squamous epithelial cells through direct contact, producing micro-ulcerations and microscopic bleedings in the vaginal walls and endocervix. In most cases, men are asymptomatic, but they transmit the infection to women. As the women's columnar epithelium is not affected, trichomonosis is manifest as vaginitis, but not endocervicitis. The simultaneous presence of an endocervicitis should alert the physician to check possible coinfections with *C. trachomatis* or *N. gonorrhoeae*. Severe infections are usually treated with nitroimidazole derivatives, but the number of resistant strains is constantly growing [19, 20].

The term mycoplasma is used to designate organisms in the Mollicutes class, the smallest free-living cell-wall-deficient microorganisms, the most simple life forms capable of replication outside a host cell. *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma genitalium* and *Mycoplasma hominis* are the most common species of mycoplasmas in both men and women. Mycoplasma species (*U. urealiticum* and *M. hominis* in particular) are frequently detected in sexually active asymptomatic youngsters. However, they can induce a broad spectrum of pathological conditions in both women and men, including unexplained chronic symptoms of lower urinary tract, NGU, PID, pyelonephritis, chronic prostatitis, preterm labor and idiopathic abortion due to infection of the urogenital bodies [21].

Ureaplasma species are sometimes detected in the commensal bacteria of the lower genital tract, so their role among STD is still questioned. Some studies demonstrated that the association of *Ureaplasma* species with NGU depends on the detected species and that *U. urealiticum* is an etiologic agent of NGU, unlike *U. parvum*. In addition, it is reported that *U. urealyticum* can cause infections in the lower genital tract and is a pathogen agent of urethritis in males [22]. In assessing the role of these microorganisms in producing human diseases, their high prevalence among asymptomatic individuals should be taken into consideration.

M. genitalium is a microorganism associated with acute and chronic sexually transmitted nongonococcal urethritis in men. Data regarding infections in women suggest that *M. genitalium* is associated with urethritis, cervicitis and PID. According to some authors, individuals with clinically-significant urethritis, persistent PID or cervicitis should be tested for *M. genitalium* [23]. As in the case of *C. trachomatis*, *M. genitalium* infection is often asymptomatic.

Several authors have reported that these organisms are engaged in women infertility, preterm delivery, premature rupture of membranes and chorioamnionitis [24, 25]. In addition, some studies present mycoplasma as causative agents of male infertility. It has been reported that these infections change various sperm characteristics, such as motility, density or morphology, and that antibiotic treatment improves the quality of the sperm [26]. A recent study found out that detection rates of *M. hominis* and *U. urealiticum* in infertile couples were about two times higher than in fertile couples [27].

3. STD detection methods

Chlamydia trachomatis was first isolated from the female genital tract in 1959 [28]. Culture techniques for the isolation of these bacteria were developed since 1965. Microimmunofluorescence

tests for seroepidemiological studies and serotyping emerged later. Currently, detection of *C. trachomatis* is accomplished via bacteriological examination or enzyme-linked immunosorbent assay (ELISA) [29].

Due to technical difficulties resulting from the need to inoculate specimens immediately after sampling, culture assays present low sensitivity (50%) and are seldom used today in the microbiological detection of *C. trachomatis*.

It is thought that *Chlamydia* immunoglobulin (IgG) antibodies persist in the body for years and therefore are used as markers for *C. trachomatis* infections [30]. These markers can be useful if a previous infection could have produced changes in the reproductive tract, but the presence of antibodies is not indicative of an infection present in the body at the time of detection, so these markers cannot be used in diagnosis.

Today, there are numerous methods available for *C. trachomatis* diagnosis, but it should be underlined that the PCR direct approach is given great interest lately. Also, the quick and easy to use loop-mediated isothermal amplification (LAMP) is often employed in detecting *C. trachomatis* [31]. Molecular biology tests based on gene amplification improved diagnostic quality in terms of sensitivity and specificity and are gradually replacing all other techniques (cell cultures, antigen tests, molecular hybridization without amplification, etc.). These techniques confirmed the high frequency of asymptomatic infections, their prevalence in women and men and evaluation of the frequency of recurrent and/or persistent infections.

In detecting *N. gonorrhoeae* infections, bacteriological cultures are yet the gold standard for diagnosis as they allow further testing of antibiotic susceptibility. Culture assays using Thayer-Martin agar have long been seen as the standard method [32]. Due to difficulties in collecting, transferring and storing specimens, these tests exhibit low sensitivity and are unsuitable for screening. Although enriched agar cultivation is still widely used for the diagnosis of *N. gonorrhoeae* infections, recent years have seen extensive usage of NAATs, especially in detecting carriers. These genetic tests enable establishing a diagnosis in 2–3 days for gonococcal urethritis or cervicitis with a sensitivity of up to 99%. In contrast, cell cultures provide a sensitivity of 85–95% in acute urethral infections and below 50% in chronic forms in women. These figures largely depend on sampling conditions, as *N. gonorrhoeae* is a rather fragile organism [33].

Precise diagnosis of gonorrhea is needed to prevent severe complications and to control transmission, especially in the case of asymptomatic infections. Molecular approaches such as hybridization assays or nucleic acid amplification tests have revolutionized the diagnosis of gonococcal infection due to their increased accuracy compared to the culture media and their ability to simultaneously test multiple species. NAAT should be the technique of choice in the diagnosis of coinfections and screening. In addition to diagnostic, molecular approaches have been successfully applied for testing *N. gonorrhoeae* in tracking genetic diversity.

Suspicion of *T. vaginalis* is often considered by clinicians in symptomatic women presenting a combination of vaginal discharge, vulvar irritation and unpleasant smell. Unpleasant smell and low irritation is more likely to be correlated with bacterial vaginosis than with trichomonosis, the irritation being more prominent in the latter case. After completing the physical

examination, it is useful to determine the pH of the vaginal secretion. Vaginal pH is higher than 4.7 in most women presenting trichomonosis, but high pH values are also seen in most women with bacterial vaginosis, therefore further investigations are required for an accurate diagnosis. A final diagnosis is based on the positive detection of this parasite. This can be determined microscopically in saline serum, using a magnification of 400×, but the sensitivity of this method is about 60%. A crucial step in the infection process involves a dramatic morphological change in the parasite. Free ovoid cells that resemble the familiar image of a flagellated protozoan take an amoeboid shape in contact with the urogenital tract [34], making microscopic detection more difficult. Pap smears can detect *Trichomonas* infections, but the Gram staining is useless here. Microscopic evaluation is time consuming and standardization in the interpretation of positive results is rather ambiguous [18]. Another disadvantage is that the microscopic examination should be performed within the first 10 min after sample collection [35].

Microscopic examination of the smear and/or culture is presently the most commonly used method in the detection of *T. vaginalis*. Because of its very slow growth rate, strict requirements for nutrients and specific culture embodiments that are not widely available, *T. vaginalis* is difficult to be grown in culture media [36]. Not only are such media very expensive, they also present a low sensitivity compared to the PCR methods recently introduced and require microscopic evaluation. Another disadvantage is that in women *T. vaginalis* is undetectable several months after metronidazole therapy [37]. Therefore, a precise diagnosis in women and men based on cell cultures is rather difficult to be made and the newer molecular techniques are more and more brought into action [38]. In the last decade, several PCR assays were developed and studies comparing the two methods in the detection of *T. vaginalis* demonstrated a higher sensitivity and specificity on behalf of the PCR.

The contribution of flow cytometry technology in investigating adhesion of the extracellular parasite to human host cells has been clearly demonstrated. This methodology can be optimized and the test can be used in a format in which several different strains may be analyzed simultaneously [39]. It is, however, a difficult method for the diagnosis of trichomooasis. A recent study [40] indicated the advantages of using DNA in the detection of *T. vaginalis.*

As mentioned above, *U. urealyticum, U. parvum, M. hominis* and *M. genitalium* are the most common mycoplasma species in both men and women. While the first three can be grown in culture media, the latter cannot. However, growing on culture embodiments is difficult and lasts longer, up to 4 days for *U. urealyticum* [36]. Also, the technique requires biochemical identification to determine the species of mycoplasma involved. Mycoplasmas are hard to grow in normal laboratory conditions, their simplistic genome demanding more complex environments. Difficulties in their cultivation and identification in clinical samples that often contain other bacteria or fungi have further complicated causal diagnosis and their association to disease. Mycoplasma infections are often associated with the presence of asymptomatic carriers that may or may not develop the disease [41]. Thus, identification of the virulence factors in mycoplasma has been complicated by the difficulties encountered in isolating the strains.

M. genitalium detection using culture cells is no longer employed in routine clinical practice because its growth takes several weeks and presents technical difficulties [42]. According to some authors, *M. genitalium* requires a special medium and incubating for up to 8 weeks to be successfully detected [43]. Although many researchers would rather turn to serological methods when documenting an infection caused by these bacteria [44], such diagnosis approach is not appropriate due to cross-reactivity with *M. pneumoniae*. For a precise diagnosis, a direct identification is therefore recommended.

The safe detection of *M. genitalium* and other bacteria was for a long time hampered by the absence of a commercially available diagnostic test. In recent years, however, NAATs revolutionized the detection and study of mycoplasma. These techniques contributed greatly to the DNA-based improvement of the detection, identification and serotyping methods [45]. In addition, the DNA-based method enabled the identification as separate species of *U. urealyticum* and *U. parvum*, previously considered as biovariants (biovar 1 and biovar 2) of *U. urealyticum* [46].

Over 50 mycoplasma genome sequences are now available in public databases, revealing a genetic diversity more complex than first predicted. This growing set of data is extremely valuable in the study of organisms otherwise difficult to cultivate, offering new means for testing and molecular diagnostics [47].

4. Multiplex PCR methods as diagnostic tools for STDs

Multiplex PCR methods for the simultaneous diagnosis of several STDs are more and more often employed in recent years. These methods place a number of primer pairs corresponding to specific DNA sequences of various STD pathogens all in one reaction tube, such commercial ready to use kits being available. While some kits can simultaneously detect two commonly associated STDs, such as *C. trachomatis* and *N. gonorrhoeae* infections, others can manage three pathogen detections. However, kits containing the primer pairs needed for the simultaneous detection of 6–12 STD pathogens are gaining momentum on the market. The most effective and widely used kits are presently those simultaneously detecting the top six most frequent STD agents, namely *C. trachomatis*, *N. gonorrhoeae, T. vaginalis, U. urealyticum, M. hominis* and *M. genitalium*, agents which play hard to get for other detection methods and often exhibit multiple associations.

Another asset is that easily collectable urine samples are adequate DNA sources for these PCR techniques along with the urethral/vaginal discharge swabs. About 30–50 mL of first void urine (FVU) samples collected in sterile polypropylene containers early in the morning, at least 4 h after the previous urination, are more than enough for a successful analysis. FVU is definitely less invasive compared to the harvesting of vaginal or urethral secretions, an aspect reported to have influenced a significant number of subjects to avoid STD identification tests previously [48]. As urine specimens can be self-collected in intimacy, an increased number of patients tend to favor such option that also enhances the applicability of the screening programs [49]. The fact that urethral secretions were found to present increased sensitivity

and specificity compared to FVU in immunological analyzes for male subjects seems to hold lesser relevance, anyway differences have diminished since NAAT were first performed [46]. Concerning the female subjects, the use of urine specimens in STD identification produced similar results to vaginal or endocervical secretions [50]. It is known that assay sensitivity may decrease if samples are repeatedly frozen/thawed or stored for longer periods of time because nucleic acids can easily degrade. Urine samples hold another advantage as well: they can be stored at 4–8°C up to 7 days prior to the processing.

Thanks to this easy and noninvasive sampling, the multiplex PCR can be used to detect STDs in both symptomatic and asymptomatic individuals and could prove to be a useful screening tool for the general population.

A 15 min centrifugation at 15,000g and subsequent supernatant removal and pellet resuspension are needed as a successful DNA extraction requires concentrated pathogen suspensions. Both urethral/vaginal discharge swab specimens and urine samples should be brought to room temperature prior to the centrifugation. One can use up to 10 mL amounts of sample to increase the DNA extraction yield. Several commercially available kits for DNA extraction can be used according to the manufacturer's instructions, e.g., QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), AccuPrep Genomic DNA Extraction Kit (Bioneer, Seoul, Korea) or MasterPure™ Complete DNA and RNA Purification Kits (Epicentre Biotechnologies, USA).

In our marketing era, such new techniques and gadgets (kits) need to be accompanied by studies assessing their performance compared to other traditional methods.

One such study conducted in 2010 [36] compared the multiplex PCR assay for the simultaneous detection of *T. vaginalis*, *U. urealyticum* and *M. hominis* with data obtained using microscopic and culture techniques. Three cotton swabs were obtained from each of the 240 women enrolled in the study based on claims of lower genital tract infections. For the detection of *T. vaginalis,* swabs were inoculated in a modified Diamond's Trypticase-yeast medium including fetal bovine serum, streptomycin, penicillin G and amphotericin B and a wet mount was prepared for its detection by direct microscopic examination. The samples were incubated at 37°C in carbon dioxide atmosphere and observed for a week under a light microscope. The presence of *M. hominis* and *U. urealyticum* was assessed by culture detection using the Mycoplasma Duo kit (Sanofi Diagnostic). A number of different primer sets were synthesized, PCR amplifications and ethidium bromide detections in 1% agarose gels were performed for a multiplex PCR to be designed for the simultaneous detection of *T. vaginalis, M. hominis* and *U. urealyticum*. Results were then favorably compared to more conventional methods for the detection of *T. vaginalis, M. hominis* and *U. urealyticum*. When tested on vaginal swabs, the multiplex PCR correctly detected 14 out of 14 *T. vaginalis*-positives, 95 of 98 *U. urealyticum*-positives and 22 out of 22 *M. hominis*-positive samples. In contrast, the microscopic detection of *T. vaginalis* exhibited a 28.6% sensitivity, whereas culture methods identified 71.4% of the trichomonad infections documented with multiplex PCR. While the kit's sensitivity in the detection of *U. urealyticum* was 96.9%, a value comparable to culture test sensitivity (91.8%), identification of *M. hominis* was greatly improved (100 vs. 36.3% sensitivity). The study argued for multiplex PCR superiority in terms of sensitivity and rapidity (hours vs. days/weeks).

Another study [43] in 2011 compared the results of 113 STD patients tested for six sexually transmitted microorganisms (*C. trachomatis, M. hominis, M. genitalium, N. gonorrhoeae, T. vaginalis* and genital *Ureoplasma*) with an automated Seeplex® (Seegene, Korea) multiplex PCR-based STD6B autocapillary electrophoresis (ACE) system vs. culture and conventional PCR-based tests on genital and urinary specimens. *C. trachomatis* was cultured on cycloheximide-treated McCoy cells, while A7, arginine broth and agar media were used for *M. hominis* and *Ureaplasma*. Conventional culture media were employed for *N. gonorrhoeae. N. gonorrhoeae* and *C. trachomatis* was also tested with the PCR-based COBAS Amplicor. On the other hand, in-house validated PCR assays were performed for *T. vaginalis, N. gonorrhoeae* and *C. trachomatis*, while a Hy-Mycoplasma PCR Detection Kit (Hy-Labs, Israel) enabled the detection of *M. hominis*, *M. genitalium* and *Ureaplasma*. All six pathogens (*T. vaginalis, M. hominis, M. genitalium, C. trachomatis, N. gonorrhoeae* and *Ureaplasma*) were subjected to a multiplex PCR amplification and the products were separated and detected via automated capillary gel electrophoresis. The sensitivity of the STD6B ACE kit was 100% in case of *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *T. vaginalis* and *M. hominis*, and 98% for the *Ureaplasma*. Specificity was also 100% for *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium* and *T. vaginalis*, while for *M. hominis* and *Ureaplasma* figures were 99 and 97%, respectively. These results prove beyond any doubt that the integrated STD6B ACE system is highly effective in the detection of each of the six STD pathogens depicted from a single specimen.

Seeplex® STD6 ACE Detection kit (Seegene, Korea) was evaluated in yet another Korean study, completed in 2012, which was conducted on 739 subjects [51]. Cervical swabs were collected from the women enrolled, while men were requested to provide 30–40 mL of FVU. The six pairs of dual priming oligonucleotide primers specifically targeting the *C. trachomatis, M. hominis, M. genitalium, N. gonorrhoeae, T. vaginalis* and *U. urealyticum* genes were tested against a combined monoplex PCR. All specimens were tested with the multiplex PCR for the six pathogens and amplification products were separated with a LabChip® DX Seeplex® assay system (Caliper, USA). All specimens were retested with six monoplex PCRs (one for each of the STD agents) and a duplex strand displacement amplification (SDA) for *C. trachomatis* and *N. gonorrhoeae* was accomplished with a BD ProbeTec™ (Becton–Dickinson Microbiology System, USA). An absolute correlation between the multiplex and monoplex PCRs was found in terms of both sensitivity and specificity. The results of the multiplex PCR and duplex SDA were 99.7% concordant in the case of *C. trachomatis* and 100% for *N. gonorrhoeae*. The results confirmed that the simultaneous detection of several species in one sample was feasible and that the clinical sensitivity provided by the multiplex PCR was on an equal footing with the monoplex PCR and duplex SDA, proving to be fully adequate for routine detection of several STDs while holding an upper hand in terms of cost-effectiveness and rapidity.

Anyplex™ II STI-7 Detection Kit (Seegene, Korea) was tested to the limit in a 2013 Korean study [46] aiming to investigate the accuracy and reliability of a real-time multiplex PCR assay employed for the detection of seven STD agents (*C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium, M. hominis, U. urealyticum* and *U. parvum*) in clinical samples. A total of 897 specimens (696 FVU and 201 endocervical swabs) were collected from 365 symptomatic patients and 532 asymptomatic volunteers and five diagnostic methods were employed as the speci-

mens were subjected to parallel testing using four NAATs and one *Mycoplasma* detection kit. The RT-PCR amplification was performed for all seven microorganisms with Anyplex™ II STI-7 Detection Kit. PCR amplification was performed using the Seeplex® STD6 ACE Detection Kit, in accordance with the manufacturer's protocol. A battery of kits were deployed for detecting the pathogens: *C. trachomatis* and *N. gonorrhoeae*—SDA technology using BD ProbeTec™; *T. vaginalis* and *M. genitalium*—AmpliSens® (EPH PCR Kit-InterLabService Ltd, Russia); *M. hominis* and *U. urealyticum*—Mycoplasma IST 2 Kit (bioMérieux, France). Test sensitivity and specificity of each method for every STD agent were calculated in parallel and conclusions were that the multiplex real-time PCR (Anyplex™ II) demonstrated outstanding results compared with other diagnostic tools as it yielded 100% sensitivity and very high specificity in the detection of *C. trachomatis, N. gonorrhoeae, T. vaginalis, M. genitalium* and *M. hominis* and was also useful in discriminating between *U. urealyticum* and *U. parvum*. Despite the increased workload and higher costs due to the larger number of tests conducted, multiplex real-time PCR was found to be equivalent or superior to other assessment methods and in the future is expected to become a standard diagnostic tool.

We ourselves have used a Seeplex® STD6 ACE Detection kit in a study on 224 Romanian subjects including persons with STD symptoms and asymptomatic individuals involved in unprotected sex with multiple partners, couples experiencing unexplained infertility or others requesting STD screening for various reasons. Each individual contributed with 30–50 mL FVU samples and DNA extraction was accomplished with MasterPure™ Complete DNA and RNA Purification Kits (Epicentre Biotechnologies, USA). A 2% agarose gel containing ethidium bromide was used with 0.5× Tris-borate 0.1 mM EDTA (TBE) as running buffer. The PCR products were visualized using a UV transilluminator. An example of gel electrophoresis results can be visualized in **Figure 1**.

The accuracy of the method was demonstrated by the lack of contamination (no amplicons in the negative control band) and the presence of positive control bands corresponding to all six DNA fragments of known molecular weight included in the marker used to determine the size of the amplification products. Also, internal controls indicated proper PCR amplification in each microtube (see **Figure 1**).

About 74 (33.03%) of the 224 subjects, both symptomatic and asymptomatic, were positive for one or more STDs. All six pathogen agents were detected with the multiplex PCR kit, several double or triple infections demonstrating associative patterns. The following double associations were detected in the study: *C. trachomatis* and *N. gonorrhoeae*, *C. trachomatis* and *U. urealyticum*, *N. gonorrhoeae* and *U. urealyticum*, *C. trachomatis* and *M. hominis*. Several triple infections grouped *C. trachomatis*, *N. gonorrhoeae* and *U. urealyticum*.

The bacteria most frequently identified in the analyzed urine samples was *C. trachomatis*, found in 20.9% of the cases. Consequently, a number of associations between *C. trachomatis* and other STD agents (*N. gonorrhoeae*, *U. urealyticum* and *M. hominis*, respectively) were found. These results are consistent with studies conducted in other countries or international statistics indicating *C. trachomatis* as the most common STD agent [4, 52, 53]. Detection of *C. trachomatis* in several asymptomatic patients demonstrated again how easily this infection can pass unnoticed.

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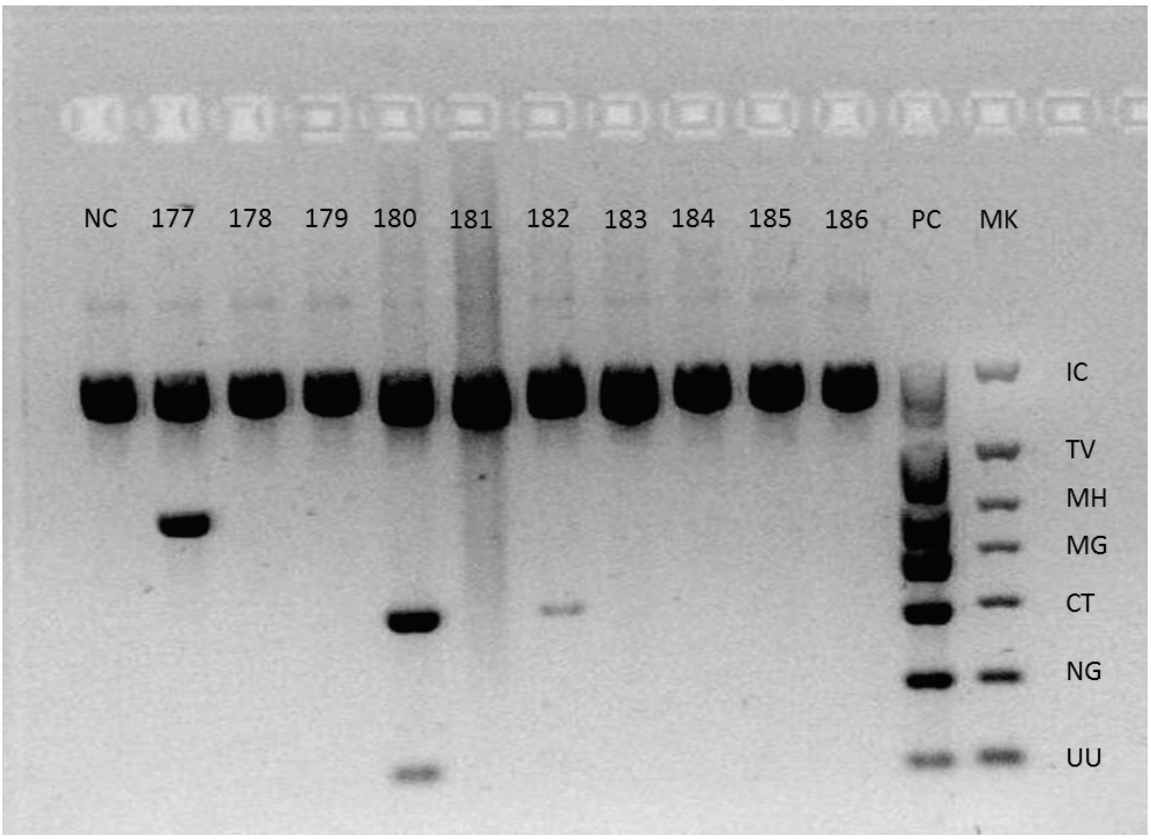

Figure 1. STD pathogens—agarose gel electrophoresis. NC: negative control, 177–186: samples, PC: positive control, MK: marker, TV: *T. vaginalis*, MH: *M. hominis*, MG: *M. genitalium*, CT: *C. trachomatis*, NG: *N. gonorrhoeae*, UU: *U. Urealyticum*.

Some studies recommended that all sexually active women should be tested, pregnant ones or those that have experienced miscarriages in particular, given the high prevalence of *C. trachomatis*/*N. gonorrhoeae* infections [54, 55]. In the US, concomitant empiric treatment of *C. trachomatis* and *N. gonorrhoeae* is recommended for all gonorrhea positives considering the frequent association between the two pathogens [56]. However, results indicate that several other associations (such as those between *C. trachomatis* and *U. urealyticum* or *M. hominis*) are often encountered, suggesting that a population screening for *C. trachomatis*/*N. gonorrhoeae* is not good enough and a multiplex method for the simultaneous detection of the most frequent six or more STD agents would be more appropriate, considering the different associations that may arise.

N. gonorrhoeae was detected in 7.1% of the cases, as was the case of *U. urealyticum*. These results are consistent with other studies indicating a high prevalence of both bacteria, the most frequent STD agents except for *C. trachomatis* [9, 57]. A notable finding is that we detected patients positive to *N. gonorrhoeae* that presented no or very atypical symptoms of gonococcal infection, which could have resulted in inadequate treatment without this DNA-based analysis that allowed us to identify the presence of the gonococcus. The *N. gonorrhoeae*/*U. urealyticum* coinfection we found was another confirmation that both *C. trachomatis* and *N. gonorrhoeae* can associate with other STD pathogens, an additional argument for the use of multiplex kits able to detect a large number of STDs in the same sample.

A recent study in Korea [58] found a 3.3% infection rate for *T. vaginalis* in the general population and argued that the rate was higher in women over 50 years of age. This followed a previous study documenting a 2.4% infection rate for *T. vaginalis* in the same area 15 years before and the significant increase lead researchers to recommend PCR-based testing of *T. vaginalis* in all women over 50 years old, irrespective of their symptomatology. Our study detected but one (0.44%) case of *T. vaginalis* infection, suggesting that in our area prevalence is much lower than the literature data based on other than NAAT determinations (e.g., physical and microscopy examinations or cell cultures) indicated. NAAT seems to be for now the only accurate method to diagnose this parasite. Our results are consistent with those conducted in other countries where *T. vaginalis* was detected in similarly low percentages compared to other STD [43, 51]. However, the incidence of trichomonosis has gradually decreased over the past 40 years. This may be due to the large amount of metronidazole and other imidazoles generally used in the treatment of bacterial vaginosis. Although the number of people infected with *T. vaginalis* is low, the analysis is justified considering its asymptomatic manifestation and the serious complications it produces.

In a study conducted in the United States, *M. hominis* and various *Ureaplasma* species were detected in 21–53% of the cases involving asymptomatic women and 40–80% of the sexually active women, respectively, their prevalence being slightly lower in men [59]. In our study, mycoplasma infections were detected in 25 individuals (a rate of 11.1%), the most commonly encountered being *U. urealyticum* (in 7.1% of the patients). Although *M. hominis* and *M. genitalium* were less represented (1.7 and 2.2% of the patients, respectively), these infections cannot be ignored. More so considering that *M. genitalium* was detected in an asymptomatic patient previously found negative for different STDs investigated on specific culture media (including ones for *M. genitalium*) in a number of different specimens (urine, urethral discharge). This last example reaffirmed the sensitivity of the multiplex PCR method over traditional alternatives.

5. Advantages of the multiplex PCR method

The advantages of the multiplex PCR method were assessed by various researchers comparing the diagnosis results obtained with different other conventional methods. The sensitivity and specificity of these methods were analyzed on different specimens in comparison to traditional methods, some of these considered to be the gold standard in diagnosis [36, 43, 46, 51]. Although this method can sometimes increase the workload, implying more protocol steps (DNA extraction, purification—if necessary, PCR amplification, electrophoresis), it still holds a huge advantage in terms of the exact diagnosis of STD. Except for special circumstances, such as when testing susceptibility to antibiotics of the various bacterial agents, NATTs have become the most sought after tests in STD detection. Regarding bacterial resistance to antibiotics, the DNA extracted and identified as belonging to such bacteria may be genetically tested to determine the genes that induce resistance to antibiotics or the different mutations that cause such resistance.

Although the symptoms are often similar, the treatment may differ depending on the STD agent detected. Without an accurate diagnosis, treatment is often ineffective. Clinicians often employ point-of-care (POC) tests to diagnose vaginal infections and STDs. For example, most

clinicians rely on Amsel's clinical criteria in defining bacterial vaginosis: increased vaginal pH, the presence of amines, clue cells observed on wet mounts and homogeneous vaginal secretions [60]. Although bacterial cultures and other methods are still widely used in STD detection, PCR tests are relevant, reproducible, sensitive and specific enough tools implying low costs and simplicity. In addition, the availability of commercial NAATs that include several STD pathogens allows more detailed studies regarding the relationship between such organisms in the etiology of these diseases. As well documented, all STDs occur in populations at high risk for other sexually transmitted infections. Their presence in a given individual is a marker for high-risk behaviors and coincidental infections should be sought after, as well as other more serious sexually transmitted diseases such as HIV.

An advantage of this method is its great sensitivity consisting in the detection of STD pathogens in individuals who have previously been tested with conventional methods and came out with negative results.

The greatest advantage of this method is the detection of double or multiple coinfections. This is an important argument for promoting multiplex testing in the same sample. These results could have important implications in epidemiology and treatment by improvements in the accuracy of determining the possible synergies and interactions between such microorganisms.

STD screening for certain categories of population (e.g., patients attending sexual health clinics, infertile persons or women who had miscarriages) is necessary not only to identify symptomatic persons in order to diagnose and treat their infection but also to identify asymptomatic individuals who serve as possible infection carriers in order to reduce morbidity and help controlling these STDs. The multiplex PCR method provides a good opportunity to argue for STD screening.

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