ISSN : 2350-0743

www.ijramr.com

International Journal of Recent Advances in Multidisciplinary Research

Vol. 04, Issue 05, pp.2561-2565, May, 2017

RESEARCH ARTICLE

NEISSERIA GONORRHOEAE, CHLAMYDIA TRACHOMATIS, UREAPLASMA UREALYTICUM, MYCOPLASMA HOMINIS AND MYCOPLASMA GENITALIUM

[1,*]Danielle Alves Gomes Zauli, [2]Luiza de Miranda Lima, [3]Jordana Rodrigues Barbosa Fradico, [4]Carla Lisandre Paula de Menezes, [5]Claudio Galuppo Diniz, [5]Vânia Lúcia da Silva, [6]Renata Toscano Simões, [2]Maria Inês Lima, [1]Elvis Cristian Cueva Mateo and [1]Alessandro Clayton de Souza Ferreira

[1]Departamento de Pesquisa e Desenvolvimento, Instituto Hermes Pardini, Vespasiano, Brazil
[2]Faculdade de Medicina, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil
[3]Centro de Pesquisa René Rachou, Fundação Oswaldo Cruz, Belho Horizonte, Brazil
[4]Linhagen Produtos em Biotecnologia Ltda, Belo Horizonte, Brazil
[5]Departamento de Parasitologia, Microbiologia e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Juiz de Fora, Brazil
[6]Instituto de Ensino e Pesquisa da Santa Casa, Belo Horizonte, Brazil

ARTICLE INFO

Article History:
Received 26th February, 2017
Received in revised form
21st March, 2017
Accepted 18th April, 2017
Published online 30th May, 2017

Keywords:
Multiplex qPCR,
Sexually Transmitted Infections,
Diagnosis.

ABSTRACT

The identification of sexually transmitted pathogens by rapid and sensitive techniques is of interest due to the spread routes including the risks of acquisition and transmission. Our aim was to develop multiplex real-time PCR assays to simultaneously detect *M. hominis*, *M. genitalium*, *U. urealyticum*, *N. gonorrhoeae*, and *C. trachomatis*. Clinical samples from female patients with presumptive diagnosis of infection with these bacteria were examined. Two multiplex real-time PCRs were developed: MI: *N. gonorrhoeae*, *C. trachomatis*, *U. urealyticum*; MII: *M. hominis, M. genitalium, U. urealyticum*. The reactions were able to detect at least one of the agents, especially three or more microrganisms in a single clinical sample describing co-infections. The detection limits were 29.7 copies/µL for *N. gonorrhoeae*, 30.1 copies/µL for *U. urealyticum*, 29.9 copies/µL for *C. trachomatis*, 29.7 copies/µL for *M. hominis* and 30.4 copies/µL for *M. genitalium*. The multiplex tests developed in this study multiplex real-time PCRs provided a novel qualitative method to detect simultaneously sexually transmitted pathogens. Although additional studies with a greater number of clinical samples are needed, the results were notably encouraging, and these methods can be used as valuable tool in routine clinical laboratories.

INTRODUCTION

According to the World Health Organization (WHO), sexually transmitted infections (STIs) are transmitted from person to person through sexual contact. It is estimated that worldwide, 448 million new cases of STIs are diagnosed each year (Greer *et al*., 2008; McKechnie *et al*., 2009; Tucker *et al*., 2013). Untreated genital infections in women may lead to pelvic inflammatory disease, salpingitis, ovarian tube abscess, or perihepatitis, and may present with or without additional complications, such as chronic pelvic pain, ectopic pregnancy, and infertility (Lee *et al*., 2007). There are more than 30 different species of bacteria, viruses, and parasites that are sexually transmitted, and several of these agents can be transmitted during pregnancy and/or shortly after birth.

****Corresponding author: Danielle Alves Gomes Zauli,***
Departamento de Pesquisa e Desenvolvimento, Instituto Hermes Pardini, Vespasiano, Brazil.

Studies indicate that the high prevalence of chlamydia and neisserias, including the significant rates of asymptomatic infections (50 to 80% of men and women), and the potential for severe complications, are important factors that justify the need for routinely screening for these bacteria (Frias *et al*., 2001; Olshen *et al*., 2005; Nassar *et al*., 2008). Furthermore, *Mycoplasma* and *Ureaplasma* are transmissible by direct contact (i.e., by genital or oral-genital contact), or through vertical transmission between mother and fetus during birth. These bacteria have also been associated with a wide variety of infectious diseases in children and adults. *Ureaplasma urealyticum*, *Mycoplasma hominis,* and *Mycoplasma genitalium* are associated with genitourinary tract infections, such as pyelonephritis, pelvic inflammatory disease, chorioamnionitis, genital infections and even infertility (Gdoura *et al*., 2007; Imudia *et al*., 2008). Molecular assays have been described for the detection of pathogens, and multiplex real-time PCR (M-qPCR), which uses multiple primers and probes, is a sensitive, rapid, and high-throughput approach for qualitative analysis of

several infectious agents in the same reaction. This technique has become a mainstay of research and clinical diagnostic applications (Markoulatos *et al*., 2002; Fredlund *et al*., 2004).
In this regard, the widespread prevalence of STIs and the public health costs associated with STIs management have led to the need for fast and reliable methods for diagnosing STIs (Samra *et al*., 2011). However, because a number of pathogens can cause STIs, and because STIs can be caused by polymicrobial infection, methods to identify multiple pathogens in a single sample are necessary. The goal of this study was to develop and optimize a test for the simultaneous detection of five clinically important bacteria associated with STIs, including *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *M. hominis*, *M. genitalium,* and *U. urealyticum,* by M-qPCR.

MATERIALS AND METHODS

Ethical Aspects

The study was approved by the Ethical Committee of Research of the Federal University of Juiz de Fora and by the Ethical Committee of Research of the Santa Casa de Belo Horizonte (CAAE-0544.0.000 and CAAE- 22053313.5.0000.5138, respectively), and each patient signed a consent form. The inclusion criteria were female patients and the presumptive diagnosis of infections.

Clinical samples and controls DNA

A total of 136 cervical samples from asymptomatic patients were examined in this study. All clinical samples were collected in specimen transport medium and stored at – 80ºC. Control DNAs were obtained from Genekam Biotechnology AG (Duisburg, Germany), and included purified genomic DNA from *N. gonorrhoeae* (D0068), *C. trachomatis* (D0015), *M. hominis* (D0037), *M. genitalium* (D0035), *U. urealyticum* (D0020).

Primers and probes

The sequences of the primers and probes used in the M-qPCR assays are listed in Table 1. The primers and probes either were selected from the literature or were designed *in silico* using Primer Express Software (Applied Biosystems, Foster, CA, USA). The specificity of the primers and probes was confirmed *in silico* using the Basic Local Alignment Search Tool (BLAST) available from the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov/BLAST). The TaqMan® probes contained a single reporter dye [VIC (*C. trachomatis* and *M. hominis*), FAM (*N. gonorrohoeae* and *M. genitalium*), NED (*U. urealyticum*)] attached to the 5´ end and a "quencher" minor groove binder (MGB) attached to its 3' end. All of the primers and probes were synthesized by specialized companies.

DNA extraction

DNA was extracted from all positive controls and clinical samples as previously described (Sambrook *et al*., 1989). The concentration and quality of the DNA was determined by spectrophotometry (Nanovue GE Healthcare) and by amplification of a gene fragment coding for internal control (β-actin gene).

The DNA extracts were stored at -70ºC until use.

Standardization of a multiplex real time PCR assay

To optimize the PCRs conditions, primer and probe matrix experiments were conducted by selecting, for each gene, the primer and probe concentration that provided the lowest Ct and the highest ΔRn using a fixed amount of target template. The reactions were performed in a total volume of 25 µL with 12.5 µL of TaqMan Universal Master Mix buffer (Applied Biosystems, Foster CA, USA), each sense primer and antisense primer at 50, 300, or 900 nM, probes at 80, 125, 150 or 250 nM, and 1µL of template DNA. The reaction was performed in 7500 Real time PCR Systems (Applied Biosystems, Foster CA, USA) using universal conditions: 50°C for 2 min, 95°C for 10 min, 45 cycles of 95°C for 15s, 60°C for 1 min. Assays were performed in duplicate for all samples. The results were analyzed using the 7500 Fast Software v. 2.1 (Applied Biosystems, Foster CA, USA) and expressed as the number of copies/mL. The baseline and threshold values were adjusted automatically for each test.

Multiplex real time PCR assay

Two M-qPCRs were designed for this study according to the infectious agents: Multiplex I (M I): *N. gonorrhoeae*, *C. trachomatis*, *U. urealyticum*; Multiplex II (M II): *M. hominis, M. genitalium, U. urealyticum.* All the reactions were performed in a total volume of 25 µL with 12.5 µL of TaqMan Universal Master Mix buffer (Applied Biosystems, Foster CA, USA) in the 7500 Real Time PCR System (Applied Biosystems, California, USA). Assays were performed in duplicate for all genes, and the results were analyzed using the 7500 Fast Software v. 2.1 (Applied Biosystems, Foster CA, USA). All reactions were conducted with a no-template control (NTC) and the baseline and threshold values were adjusted automatically for each test. The cycling conditions were as follows: an initial denaturation and polymerase activation at 95ºC for 10 min, followed by 35 cycles of denaturation at 95ºC for 1 min, primer annealing at 60ºC for 1 min, and extension at 72ºC for 2 min. A final extension step of 10 min at 72ºC was used for the last cycle and the products were held at 4ºC indefinitely.

Limit of detection (LOD)

To determine the limit of detection, a standard curve was determined using a serial dilution of cloned plasmids containing the conserved region of each infectious agent in initial concentrations of 3.0×10^{10} copies/ µL, and ending in a total of eight points in the standard curve. The amplicons were cloned using the TOPO TA Cloning Kit (Invitrogen, Foster CA, USA), according to the manufacturer's protocols. The plasmid DNA was extracted using a Miniprep DNA Purification System kit (Promega Corporation, Madison, Wisconsin, USA) following the manufacturer's protocol. The concentration of recombinant plasmids was determined using a Qubit 2.0 fluorometer (Invitrogen, Foster CA, USA) with a Qubit dsDNA BR Assay kit (Invitrogen, Foster CA, USA) following the manufacturer's protocol. The reactions and cycling conditions were as described above. The limit of detection was determined by the lowest concentration of DNA yielding a positive detection rate of 90% or higher.

RESULTS

Selection and design of primers and probes for M-qPCR

The primers and probes selected in this study were specific for conserved regions of the genes encoding glyceraldehyde 3 phosphate dehydrogenase (GAP/GAPDH) (*M. hominis*), MgPa surface protein (*M. genitalium*), urease B subunit (UreB) (*U. urealyticum*), and outer membrane protein (OMP1) (*C. trachomatis*), or for unique sequences within the cryptic plasmid (pJD1) (*N. gonorrhoeae*). The selected primers were considered adequate and performed satisfactorily *in silico* PCR

Matrix experiments of primers and probes set

The concentrations of the primers and probes used in real-time PCR reactions were established in control assays using different concentrations of primers and/or probes in each reaction. The different concentrations of sense and antisense primers tested were 50, 300 and 900 nM, and of each probe were 80, 125, 150 and 250 nM. The results indicated that the optimal concentration was 50 nM for the forward primer and 300 nM for the reverse primer (data not shown). Our data demonstrated for all probes that the optimal concentration was 125 nM (data not shown).

Table 1. Sequences of primers and probes used in this study

Microorganisms	Targets genes	Sequence (5' -3')	Amplicon (base pair)	References
N. gonorrhoeae	PJD1	FW: AACTGCCGGGCGTTTATATCG RV: GACCTTCGAGCAGACATCACG Probe: FAM ACCGAAGCCGCCAGCATAGAGCAACA MGB NFQ	144	14
U. urealyticum	UreB	FW: GATCACATTTCCACTTATTTGAAACA RV: AAACGACGTCCATAAGCAACTTTA Probe: NED AAACGAAGACAAAGAAC MGB	100	3
C. trachomatis	OMP1	FW: GGTTTCGGCGGAGATCCT RV: AGTAACCAACACGCATGCTGAT Probe: VIC CTTGCACCACTTGGTGTGACGC MGB	70	15
M. hominis	GAPDH	FW: GGAAGATATGTAACAAAAGAAGGTGCTG RV: TTTATCTTCTGGCGTAATGATATCTTCG Probe: VIC AGCAGGTGCTAAAAAGGTGTTTATTACTGCTCC MGB	144	16
M. genitalium	MgPa	FW: GAGAAATACCTTGATGGTCAGCAA RV: GTTAATATCATATAAAGCTCTACCGTTGTTATC Sonda: FAM ACTTTGCAATCAGAAGGT MGB	78	17
Internal Control	β-actin	FW: CCGAGCGCGGCTACAGCTTCA RV: GGAAATCGTGCGTGACATTAAG Probe: VIC CACCACGGCCGAGC	59	This study

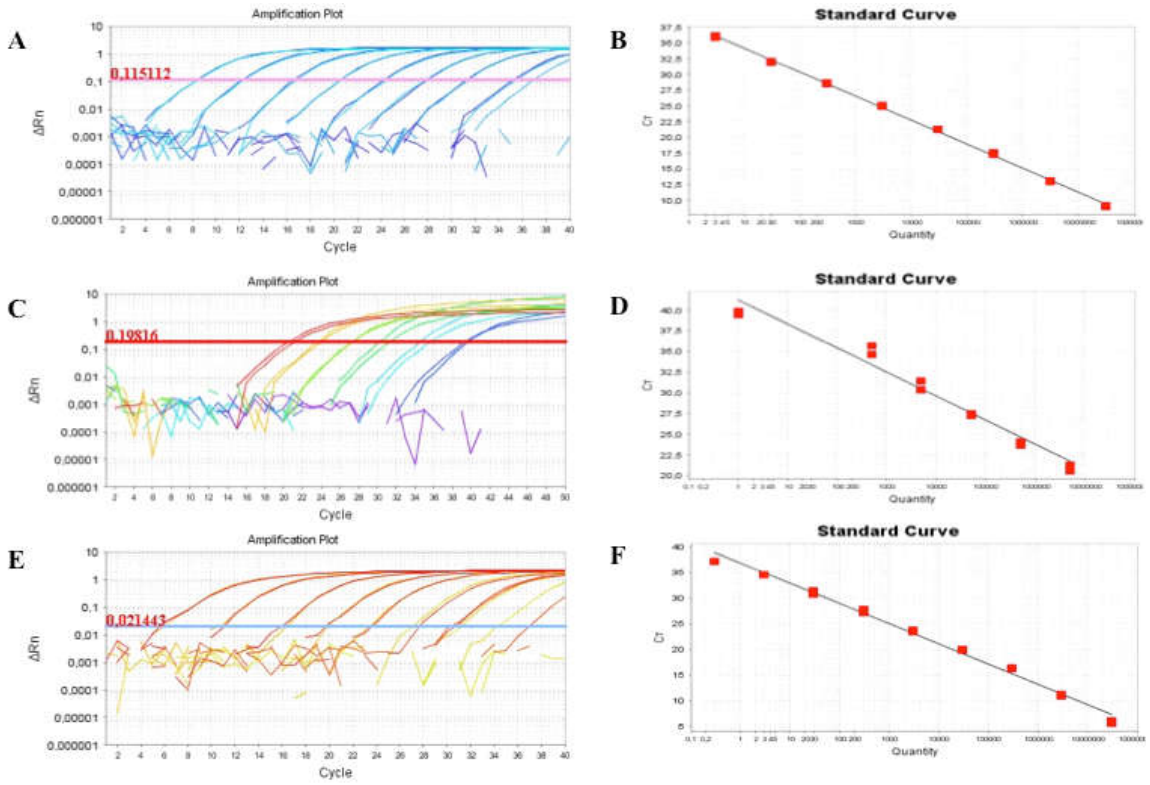

Fig. 1. Amplification plot and standard curve of *C. trachomatis* (A and B, respectively), *M. genitalium* (C and D, respectively) and *N. gonorrhoeae* (E and F, respectively) using standard plasmids. Dilution series of the standard plasmids ranged from 3.0 x 10^{10} to 3.0 x 10^{0} copies/µL. The results were expressed in copies/µL. The performance amplification was generated by the 7500 Fast Software v. 2.1 (Applied Biosystems, Foster CA, USA)

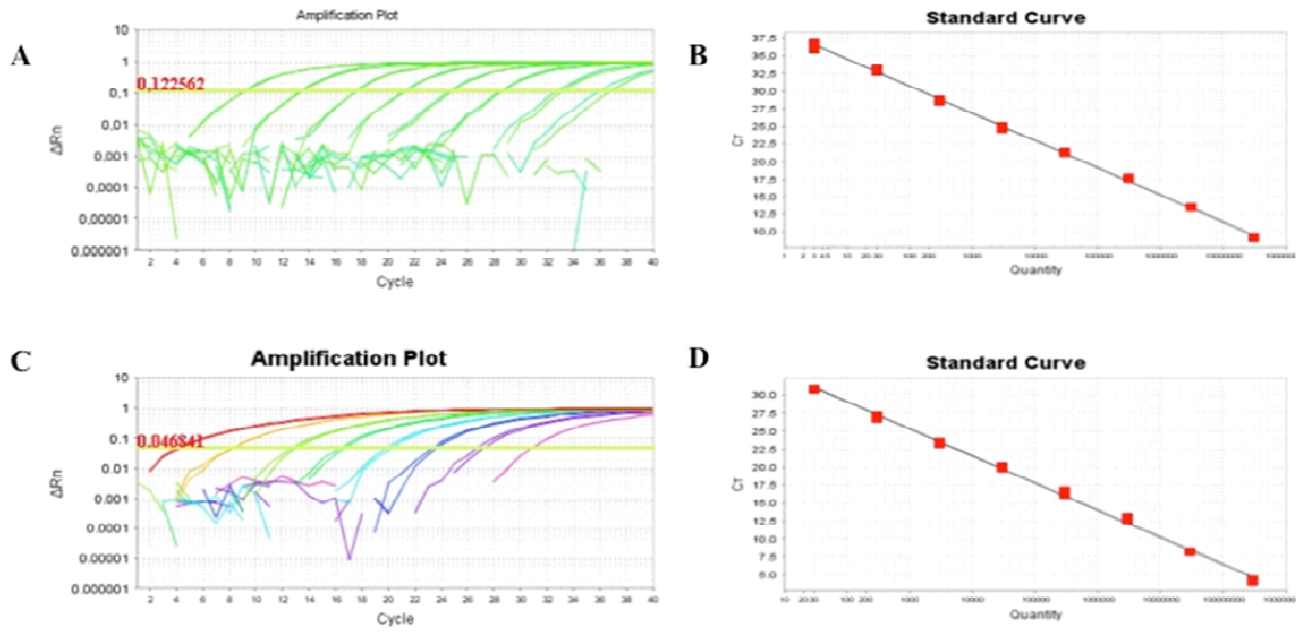

Fig. 2. Amplification plot and standard curve of *U. urealyticum* (A and B, respectively), *M. hominis* (C and D, respectively) using standard plasmids. Dilution series of the standard plasmids ranged from 3.0 x 10^{10} to 3.0 x 10^{0} copies/ µL. The results were expressed in copies/µL. The performance amplification was generated by the 7500 Fast Software v. 2.1 (Applied Biosystems, Foster CA, USA)

Limits of detection

To determine the detection limit for each test, monoplex reactions were carried out with each infectious agent separately (Fig. 1 and 2). According to the Ct values and the largest concentration of each point of the standard curve, the following detection limits, based on plasmid DNA, were determined: 29.7 copies/µL for *N. gonorrhoeae*, 30.1 copies/µL for *U. urealyticum*, 29.9 copies/µL for *C. trachomatis*, 29.7 copies/µL for *M. hominis* and 30.4 copies/□L for *M. genitalium*.

Multiplex real time PCR

The optimized real-time PCR assay consisted of two M-qPCRs: MI: for simultaneous detection of *N. gonorrhoeae*, *C. trachomatis*, and *U. urealyticum*; and MII: for simultaneous detection of *M. hominis, M. genitalium,* and *U. urealyticum*. Except for the specific primers and probes, all PCR components and thermocycling parameters were identical for both reactions, which enhanced the efficiency of reaction assembly.

All positive controls for each multiplex test amplified with Ct value less than 40, including the internal control (*β-actin* gene), which demonstrated that the extracted genetic material was of good quality. From a total of 136 samples analyzed, 66 (48.5%) were positive for one or more microorganisms with Ct < 40, and 70 (51.5%) were negative (Ct > 40). Among the positive samples, 36 (54.5%) samples were positive for *M. genitalium*, 36 (54.5%) for *C. trachomatis*, 6 (9%) for *U. urealyticum*, 6 (9%) for *N. gonorrhoeae,* and 1 (1.5%) for *M. hominis*. In the co-infections *M. genitalium/C. trachomatis* were the most frequent (12%), and 2 (3%) samples were coinfected with three or four microorganisms such as *M. gentitalium/C. trachomatis/N. gonorrhoeae* and *M. gentitalium/U. urealyticum/C. trachomatis/ N. gonorrhoeae* (Table 2).

DISCUSSION

Sexually transmitted infections (STIs) are a broad, but relatively well-defined, group of infections, usually characterized by an acute presentation that may progress to a chronic medical condition. Both men and women are affected, and according to the Center for Disease Control and Prevention (CDC/USA), an estimated 19 million new cases occur each year. Almost half of these new cases affect individuals between the ages of 15 and 24 years. The susceptibility to sexually transmitted infections, including the human immunodeficiency virus (HIV), and the high cost of treatment have led to the need for fast and reliable laboratory techniques for the identification of pathogens. Such methods can potentially improve diagnosis and therefore, reduce the complications and spread of the STIs (Samra *et al*., 2011). Procedures for nucleic acid amplification to detect sexually transmitted pathogens have been developed (Martin *et al*., 2000; Mallard *et al*., 2005; Wrobliwski *et al*., 2006). However, because STIs were polymicrobial infections, a number of studies have focused on new methods to identify multiple pathogens in a single sample simultaneously (Elnifro *et al*., 2000; Vancutsem, *et al*., 2010). Likewise, we describe here, the development of two M-qPCRs for the simultaneous detection of infection agents commonly associated with STIs based on the amplification of specific genes.

The selection of primers for multiplex PCR is a critical step in optimizing the reaction and depends a number of features, including homology to the target sequences, performance in *in silico* reactions, GC content, and amplicon size. Ideally, all primer pairs in multiplex PCRs will amplify only their respective targets. This can be achieved by using primers with similar annealing temperatures and that do not form secondary structures or dimer primers (Elnifro *et al*., 2000). In our evaluation, all primers and probes were considered ideal for multiplex tests, amplifying the specific regions of each agent simultaneously, using an initial volume of 200 µL clinical

sample. In this study, a plasmid containing the target sequence of each infectious agent was constructed and used as a positive control. To determine the limits of detection for each multiplex assay, 10-fold serial dilutions of the positive control plasmid were used in the reactions, ending with a total of 8 points in the standard curve. The results were reliable, significant, and consistent. A total of 136 clinical samples were analyzed for validation of each multiplex test. The results indicated that, in a single clinical sample, the multiplex tests could efficiently detect each of the five pathogenic agents used in this study, particularly in co-infections. Over the past three decades, diagnostics for STIs have depended on traditional methods, including culture, enzyme immunoassay and fluorescent antibody staining. However, in recent years several molecular methods have thought to become available. These newer methods have advantages because they are more sensitive, enabling detection in symptomatic and asymptomatic patients, including cases of viral infections (Souza *et al*., 2013). The multiplex tests developed in this study have the advantage of simultaneously diagnosing multiple diseases with a single diagnostic test, reducing costs and time. Moreover, the assays allowed the detection of pathogenic agents that are difficult to identify using other methods, simplifying the workflow and enhancing performance of these assays in routine diagnostic laboratories. In conclusion, these multiplex PCRs using TaqMan 5´nuclease real-time PCR provided a novel, qualitative method for the rapid detection of diagnosis of STIs associated with *N. gonorrhoeae*, *C. trachomatis*, *U. urealyticum, M. hominis,* and *M. genitalium*. Although additional studies with a greater number of clinical samples are needed, the results were notably encouraging, and we believe that these methods indicate an advance in clinical laboratory medicine. Therefore, these methods can be a valuable tool for routine laboratory diagnosis of infectious diseases.

Acknowledgement

This study was supported by grants from the Conselho Nacional do Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG).

Declaration of Conflicting interests

The authors declare that they have no conflict of interest.

REFERENCES

Elnifro, E. M., Ashshi, A.M., Cooper, R.J. *et al*. 2000. Multiplex PCR: optimization and application in diagnostic virology. *Clin Microbiol Rev*., 13, 559-570.

Fredlund, H., Falk, L., Jurstrand M. 2004. Molecular genetic methods for diagnosis and characterization of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*: impact on epidemiological surveillance and interventions. *Apmis*. 112, 771-784.

Frias, M.C.A., Pereira, C.F.A., Pinheiro, V.M.S. 2001. Frequência de *Chlamydia trachomatis*, *Ureaplasma urealyticum* e *Mycoplasma hominis* na endocévice de mulheres no menacme. *J Bras Doenças Sex Transm*. 13, 5-22.

Gdoura, R., Chaari, C., Znazen, L. 2007. *Ureaplasma urealyticum*, *Ureaplasma parvum*, *Mycoplasma hominis* and *Mycoplasma genitalium* infections and semen quality of infertile men. *BMC Infect Dis*., 8, 1-9.

Greer, L., Wendel, G.D. 2008. Rapid diagnostic methods in sexually transmitted infections. *Infect Dis Clin N Am*., 22, 601-617.

Imudia, A.N., Detti, L., Puscheck, E.E. 2008. The prevalence of *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections, and the rubella status of patients undergoing an initial infertility evaluation. *J Assist Genet*., 25, 43-46.

Lee, S.R., Chung, J.M., Kim, G. 2007. Rapid one step detection of pathogenic bacteria in urine with sexually transmitted disease (STD) and prostatitis patient by multiplex PCR assay (mPCR). *J Microbiol*. 45, 453-459.

Mallard, K., Schopfer, K., Bodmer, T. 2005. Development of real-time PCR for the differential detection and quantification of *Ureaplasma urealyticum* and *Ureaplasma parvum*. *J Microbiol Methods*. 60, 13-19.

Markoulatos, P., Siafakas, N., Moncany, M. 2002. Multiplex polymerase chain reaction: a practical approach. *J Clin Lab Anal*. 16, 47-51.

Martin, D., Cammarata, C., Van Der Pol, B. 2000. Multicenter evaluation of AMPLICOR and automated COBAS AMPLICOR CT/NG tests for *Neisseria gonorrhoeae*. *J Clin Microbiol.* 38, 3544-3549.

McKechnie, M.L., Hillman, R., Couldwell, D. 2009. Simultaneous identification of 14 genital microorganisms in urine by use of a multiplex PCR based reverse line blot assay. *J Clin Microbiol*. 47,1871-1877.

Nassar, F.A., Abu-Elamreen, F., Shubair, M. 2008. Detection of *Chlamydia trachomatis* and *Mycoplasma hominis*, *genitalium* and *Ureaplasma urealyticum* by polymerase chain reaction in patients with sterile pyuria. *Adv Med Sci*. 53, 80-86.

Olshen, E., Shrier, L. A. 2005 Diagnostic tests for Chlamydial and Gonorrheal infections. Semin Pediatr Infect Dis. 16, 192-198.

Sambrook, J., Fritisch, E.F., Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory. New York: Cold Spring Harbor.

Samra, Z., Rosenberg, S., Madar-Shapiro, L. 2011. Direct simultaneous detection of 6 sexually transmitted pathogens from clinical specimens by multiplex polymerase chain reaction and auto-capillary electrophoresis. *Diagn Microbiol Infect Dis*., 1, 17-21.

Souza, R. P., de Abreu, L. P., Ferreira, E. C. 2013. Simultaneous Detection of Seven Sexually Transmitted Agents in Human Immunodeficiency Virus–Infected Brazilian Women by Multiplex Polymerase Chain Reaction. *Am J Trop Med Hyg*. 89, 1199-1202.

Tucker, J.D., Bien, C.H., Peeling, R.W. 2013. Point-of-care testing for sexually transmitted infections: recent advances and implications for disease control. *Curr Opin Infect Dis*. 26, 73-79.

Vancutsem, E., Soetens, O., Breugelmans, M. 2010. Modified real-time PCR for detecting, differentiating, and quantifying *Ureaplasma urealyticum* and *Ureaplasma parvum*. *J Mol Diagn.* 13, 206-212.

Wrobliwski, J. K. H., Manhart, L. E., Dickey, K. A. 2006. Comparison of transcription mediated amplification and PCR assay results for various genital specimens types for detection of *Mycoplasma genitalium*. *J Clin Microbiol*. 13, 3306-3312.

****