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Detection of Urogenital Mycoplasmas Using Culture and PCR: A Descriptive Pilot Study

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Abstract: Mycoplasmas as human urogenital tract pathogens are associated with infections, reproductive failures and adverse pregnancy outcomes and thus very important to diagnose. Several methods have been used to detect genitourinary Mycoplasmas, each having their own limitations, advantages and disadvantages. In the present study, researchers used microbial culture and PCR to detect Mycoplasmas in urogenital specimens with the aim of comparing detection rate, sensitivity and specificity of the two methods. It was a descriptive cross sectional pilot study. About 30 urogenital samples including 17 vaginal swabs, 7 male urine and 6 female urine samples were collected from patients referring to hospitals regardless of their disease and studied for Mycoplasmas by culture (using two media: PPLO agar and PPLO broth) and PCR. Of total 30 specimens, Mycoplasmas were detected in 14, 11 and 11 of them using PPLO broth culture medium, PPLO agar culture medium and PCR, respectively. Accordingly, the specificity and specificity of the PCR Method was determined 100 and 95%, respectively while culture was found to have a sensitivity of 77% and specificity of 66%. Researchers found PCR based on 16S rRNA sequences to be highly sensitive and specific offering a rapid, easy and cost benefit method for detection of Mycoplasmas in comparison to Microbial Culture Method.

Key words: Urogenital, mycoplasmas, culture, PCR, urine, Iran

INTRODUCTION

Mycoplasmas as the smallest free-living organisms are widely spread in the nature. Human Mycoplasma species are mainly colonized in genitourinary tract and although, some of them are normal commensals, others are proven pathogens of urogenital tracts (Taylor-Robinson, 2002). According to many studies they are known to have a role in infections of genitourinary tract (Keane *et al*., 2000; Nassar *et al*., 2008; Patel and Nyirjesy, 2010; Moi *et al*., 2009), cervicitis (Manhart *et al*., 2003; McIver *et al*., 2009; Schlicht *et al*., 2004) endometritis (Cohen *et al*., 2002), tubal infertility (Clausen *et al*., 2001) and Pelvic Inflammatory Disease (PID) (Taylor-Robinson, 2002). They can also cause adverse pregnancy outcomes such as abortion, preterm delivery and preterm birth and may lead to neonatal mortality and morbidity (Taylor-Robinson, 2007; Larsen and Hwang, 2010; Cassell *et al*., 1991; Govender *et al*., 2009). In addition, it is well established that they are associated with non-gonococcal non-chlamydial urethritis in men (Hooton *et al*., 1988; Horner *et al*., 1993; Taylor-Robinson *et al*., 1993; Takahashi *et al*., 2006). Accumulating evidence exists considering genitourinary Mycoplasmas as sexually transmitted infections (Ross and Jensen, 2002; Uuskula and Kohl, 2002; Manhart *et al*., 2007; Manhart and Kay, 2010).

Several methods have been used to detect genitourinary Mycoplasmas, each having its own limitations, advantages and disadvantages. The common method of microbial culture is not routine now since it requires special media that are complex and expensive and also the process is very time consuming as it takes at least 2-5 days to culture *U. urealyticum* and almost 8 weeks to culture *M. genitalium* (Razin *et al*., 1998; Stellrecht *et al*., 2004; Amirmozafari *et al*., 2009). Serological methods are not also widely used due to cross-reactivity with other species (Stellrecht *et al*., 2004; Amirmozafari *et al*., 2009; Agbakoba *et al*., 2006). With the revolutionary development of PCR Methods in the early 1990s, detection of such organisms that are difficult to cultivate became more feasible. These methods have been widely used to diagnose Mycoplasmas in the studies (Stellrecht *et al*., 2004; Amirmozafari *et al*., 2009; Agbakoba *et al*., 2006; Jensen *et al*., 1991; Timenetsky *et al*., 2006; Svenstrup *et al*., 2005; Dhawan *et al*., 2006; Stirling, 2003). In the present study, researchers examined 30 urogenital samples (from both

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male and female patients) for Mycoplasmas using culture (with two media: PPLO broth and PPLO agar) and PCR. The main purpose of the study was to determine and compare the sensitivity and specificity of these two methods for detection of urogenital Mycoplasmas.

MATERIALS AND METHODS

This descriptive cross sectional pilot study was performed during 2011-2012. Urogenital specimens were collected from patients referring to four major hospitals in Karaj with various chief complaints who needed urinary or vaginal tests according to their physicians' diagnosis. As a result, a total of 30 specimens including 13 urine samples (7 from male and 6 from female patients) and 17 vaginal swabs were gathered and analyzed. All patients gave informed consent agreements.

Specimens: Clean catch urine samples were collected in sterile plastic containers. Vaginal swabs were placed in test tubes containing Stuart Transport Medium (Merck, Germany) consisting of Pleuropneumoniae-Like Organism (PPLO) broth, yeast extraction, bovine serum, penicillin or streptomycin. Both swabs and urine samples were stored at 4°C and immediately transported to the lab. Vaginal swabs for PCR were frozen at -70°C until PCR.

Culture for urogenital mycoplasmas: All specimens were cultured within 12 h of collection. Filtration Method was used to separate Mycoplasmas from the specimens. Specimens were cultured in two culture media: PPLO broth (liquid culture medium) and PPLO agar (semi-solid culture medium) (DIFCO, USA). Specimens were filtered by syringe filter 0.45 μm in order to reduce bacterial and fungal contaminations. Then, 100 μL of each sample was inoculated into PPLO agar plates and 300 μL of each specimen was poured into tubes containing 2.7 μL of PPLO broth. All cultured tubes and plates were then incubated at 35°C with 80% humidity and 5% CO_2 for 14 days and examined daily for color change and fried-egg colonies characteristic of Mycoplasmas.

At days 10 and 14,200 μL of the PPLO broth in the cultured tubes was inoculated into separate plates containing PPLO agar medium and incubated at 35°C, 80% humidity and 5% CO_2. All plates were examined by microscope ×10 at days of 3, 7, 10 and 14.

PCR for urogenital Mycoplasmas: First, sample vials containing urine and vaginal specimens were filtered by 0.45 μm syringe filters. After vortexing for 5-10 sec, 100 μL of each sample vial was transferred to microtubes and centrifuged at 12000 rpm for 5 min at room temperature.

After centrifugation, the supernatant was discarded and the pellet was suspended in 1 mL of DBPS or PBS buffer. All microtubes were centrifuged twice more at 12000 rpm for 2 min with the pellet washed using 100 mL of the buffers. Then, they were heated for 10 min at 95-100°C bain-marie and again centrifuged at 12000 rpm for 5 min. The supernatants were transferred to additional sterile 0.5 mL microtubes and kept at 4°C until PCR.

PCR was performed using two specific primers (270 bp) including GPO3 (5' GGGAGCAAACACGAT AGATACCCT 3') and MGSO (5' TGCACCATCTGT CACTCTGTTAACCTC 3') in order to detect *Mycoplasma* genus in the specimens (Sinagene, Iran). Final PCR buffer included: 10 μL of the extracted DNA, 0.2 μM of each primer, 1.5 μM $MgCl_2$, 0.2 mM dNTP5, 5000 units of Taq polymerase and 5 μL 10×Amplitaq buffer. PCR reaction included an initial 5 min denaturation at 94°C followed by 35 cycles of 30-denaturation at 95°C, a 45 sec annealing at 56°C and a 60 sec extension at 72°C. A final extension was performed for 10 min at 72°C. Electrophoresis was performed on 15 μL of the reaction in a 3% agarose gel stained with Ethidium Bromide Solution (10 mg mL^{-1}). DNA bands were detected at 245-312 nm using UV transilluminator (Uvitec, UK).

Statistical analysis: Data was analyzed using SPSS Ver. 17. Statistical significance was determined using Chi-square and Exact Fisher tests. The $p<0.05$ was set.

RESULTS

A total of 30 urogenital specimens including 13 urinary samples and 17 vaginal swabs were gathered from patients (7 male, 23 female; mean age: 31.7±7.2) and tested for Mycoplasmas using culture (PPLO broth and agar) and PCR. Most patients were married (83.3%) and referred to the hospitals with chief complaints suggestive of urogenital infection (63.3%). Studying PPLO broth cultures showed that 14 cases (46%) were positive for Mycoplasmas including 6 vaginal samples and 8 urine samples (color change was considered positive). PPLO agar culture medium found 11 (30%) positive samples including 5 vaginal swabs and 6 urine samples (colony morphology was considered sufficient to identify Mycoplasmas). Using PCR Detection Method, Mycoplasmas DNA was found in 11 samples (30%) including 5 vaginal swabs and 6 urine samples. Table 1 shows the number of positive samples using different detection methods.

PPLO broth medium culture showed highest detection rate (46%) with a high false positive rate. Using

Table 1: Positive samples for Mycoplasmas using different detection methods

	No. of specimens			
Specimen type	Tested	PPLO broth positive (%)	PPLO agar positive (%)	PCR positive (%)
Vaginal swabs	17	6 (35)	5 (29)	5 (29.0)
Urine (female)	6	3 (50)	2 (33)	3 (50.0)
Urine (male)	7	5 (71)	4 (57)	3 (42.0)
Total	30	14 (46)	11 (30)	11 (33.3)

PPLO agar medium culture, Mycoplasmas were found in 33% of the samples, giving the method a sensitivity of 77% and specificity of 66%. In comparison to culture, PCR Detection Method with 33.3% detection rate the sensitivity and specificity were determined to be 100 and 95%, respectively improving test sensitivity and specificity by 23 and 29%, respectively in comparison to PPLO agar culture.

DISCUSSION

In this study, researchers used two culture media (PPLO broth and PPLO agar) and PCR to detect Mycoplasmas in 30 urogenital specimens and compared the detection rates and sensitivity and specificity of the tests. In general, the results finding Mycoplasmas in 11 of 30 urogenital specimens by PCR indicates a relatively higher infection rate compared with reports from other cities of Iran. However, the infection rate with Mycoplasmas in Iran is generally lower than other countries probably due to social and cultural factors.

Several other methods have been suggested to detect Mycoplasmas; however, using PCR along with microbial culture seems to be the most acceptable method in order to reduce false results (Amirmozafari *et al*., 2009). With considering the limitations of a pilot study, the results were generally in consistence with previous studies comparing PCR and culture for detection of Mycoplasmas (Stellrecht *et al*., 2004; Amirmozafari *et al*., 2009). In the study, researchers found 30% Mycoplasma positive specimens using culture (PPLO agar and PPLO broth) while PCR detected Mycoplasmas in 3.3% of the samples. Although, it has been reported that using both PPLO broth and agar media together might enhance the ability to isolate Mycoplasmas (Stellrecht *et al*., 2004), adding broth medium in the study didn't increase the ability to isolate Mycoplasmas and mostly increased the false positive results.

A previous study in Iran by Amirmozafari *et al*. (2009) compared PCR and culture for detection of Mycoplasmas in cervical specimens of 312 women and found detection rates of 16 and 2.8% for PCR and culture, respectively. Another study by Kathleen found detection rates of 25 and 33% for culture and PCR for diagnosing genital Mycoplasmas (Stellrecht *et al*., 2004). In several studies it has been shown that culture is not only time consuming and costly but also it has high false negative rates in comparison to PCR Methods and also there are some strains of Mycoplasmas which can't be detected by culture and besides PCR has the advantage of detecting nonviable organisms (Stellrecht *et al*., 2004; Amirmozafari *et al*., 2009; Stirling, 2003; Young *et al*., 2010). Of course PCR Methods might have some limitations and despite all the surveillance and precise environment settings, there is still a chance of DNA contamination in molecular detection systems leading to false positive results. This chance of contamination can be reduced by assigning appropriate environment settings and facilities as well as using internal controls in different phases of PCR (Potts, 2008). Previous studies have shown that diagnosing the strains of Mycoplasmas by PCR is superior to classic methods, proving it to be of higher sensitivity, specificity and reliability (Timenetsky *et al*., 2006; Stirling, 2003; Young *et al*., 2010).

CONCLUSION

The comparison between PCR and microbial culture showed a sensitivity and specificity of 100 and 95% for PCR and 77 and 66% for culture, respectively. This improvement of sensitivity and specificity by PCR methods for detection of urogenital Mycoplasmas is consistence with the literature (Stellrecht *et al*., 2004; Amirmozafari *et al*., 2009; Dhawan *et al*., 2006). In this study, researchers found PCR based on 16S rRNA sequences to be highly sensitive and specific offering a rapid, easy and cost benefit method for detection of Mycoplasmas in comparison to microbial culture method. Further studies with larger sample sizes are needed to confirm the results and they might focus on more issues such as using PCR for distinction of Mycoplasma strains in larger scales and comparing it with more detection methods other than culture.

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