

SPECIAL REPORT

Guidelines for the Laboratory Diagnosis of *Mycoplasma genitalium* Infections in East European Countries

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The present guidelines aim to provide comprehensive information regarding laboratory diagnosis of *Mycoplasma genitalium* infections in East European countries. These guidelines are intended primarily for laboratory professionals testing specimens from patients at sexual health care clinics, but may also be useful for community-based screening programmes. Diagnosis of *M. genitalium* infection is performed exclusively using nucleic acid amplification tests (NAATs), owing to the poor and slow growth of the bacterium in culture. Because no internationally validated and approved commercial NAAT for *M. genitalium* detection is presently available, it is necessary that laboratories performing *M. genitalium* diagnostics not only carefully evaluate and validate their in-house PCRs before using them routinely, but also use comprehensive internal controls and take part in external quality assessment programmes. The guidelines were elaborated as a consensus document of the Eastern European Sexual and Reproductive Health Network, and comprise one element of a series of guidelines aimed at optimizing, standardizing, and providing guidance on quality laboratory testing for reproductive tract infections. Key words: *Mycoplasma genitalium*; laboratory diagnosis; guidelines; Eastern Europe.

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Mycoplasma genitalium is a relatively newly discovered microorganism, which was first isolated in 1980 from two of 13 men with non-gonococcal urethritis (1). *M. genitalium* is a very fastidious bacterium, and cultivation of this organism is exceedingly difficult and time-consuming; therefore data regarding its clinical relevance have become available only after introduction of PCR assays (2, 3). With the use of PCR tests, it has been shown that *M. genitalium* is a sexually transmitted pathogen and that the spectrum of diseases in both males and females

is similar to that caused by *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (4).

In Eastern Europe, the true incidence and prevalence of most sexually transmitted infections (STIs), including *M. genitalium* infection, remain unknown. This situation results primarily from suboptimal diagnostics, case reporting and surveillance systems (5–11). The present guidelines are the most recent in a series of guidelines (12–15) developed by the Eastern European Sexual and Reproductive Health (EE SRH) Network (11, 16, 17) aimed at establishing quality laboratory testing for STIs in East European countries. It is envisaged that different countries may need to make minor national adjustments to the guidelines presented here in order to meet local laws, health strategies and the availability of kits and reagents.

CLINICAL PRESENTATION OF *M. GENITALIUM* INFECTIONS

Considerable evidence has accumulated in recent years to suggest that *M. genitalium* has an aetiological role in urethritis in males (4). An association and/or suggestive evidence have been demonstrated, but a causal role of *M. genitalium* has not been proven for:

- cervicitis in females (18–21),
- pelvic inflammatory disease (22),
- prostatitis (23),
- epididymitis (24),
- tubal factor infertility (25, 26),
- sexually acquired reactive arthritis (27, 28).

Lack of a clear association with *M. genitalium* has been reported for:

- bacterial vaginosis (3, 19, 29),
- adverse pregnancy outcomes (30, 31).

LABORATORY DIAGNOSIS OF *M. GENITALIUM* INFECTIONS

Although culture techniques have improved in recent years, it takes several weeks or even months for each

isolate to grow (32, 34), which makes culture impossible to use for diagnostics in routine clinical practice.

M. genitalium shares several structural properties with another human pathogen, *M. pneumoniae*, and cross-reactivity between these two Mycoplasma species can result in lack of adequate specificity when using serology for diagnosis (35). Recently, several techniques have been developed and some have shown usefulness in epidemiological studies (25, 36); however, none of the available tests have been validated for use in the diagnosis of individual cases.

Since traditional diagnostic methods, such as culture and serology, are not suitable for routine diagnosis of *M. genitalium*, identification of infected individuals has been entirely dependent on nucleic acid amplification tests (NAATs). Table I presents *M. genitalium* NAATs used in published studies.

Most of the PCR assays are based on detection of sequences within the *MgPa* adhesin gene of *M. genitalium*. Some parts of the *MgPa* gene, however, are highly variable and primers targeting these regions will not perform well with clinical specimens. In Table I, the maximal number of mismatches found with a single *M. genitalium* strain in the target region is given for each *MgPa* gene primer aligned with 22 known sequences. These sequences ($n=22$) were obtained from the seven *M. genitalium* strains deposited in the ATCC with known high sequence homology to the G37 type strain, from an early passage of the M30 strain isolated by David Taylor-Robinson in 1980 (1), from three consecutive isolates from a French patient, and from 11 unrelated *M. genitalium* strains isolated from patients from Scandinavia and Japan.

The *16S rRNA* gene is also used as target in *M. genitalium* PCRs (46, 48, 52); however, owing to the homology between *M. genitalium* and *M. pneumoniae*, design of specific and sensitive primers and probes is relatively difficult (45). For some *16S rRNA* gene PCR assays, detection of *M. genitalium* is based on amplification with Mollicutes (*Mycoplasma* and *Ureaplasma*) universal primers and subsequent hybridization with species specific probes (50, 51). Although this approach allows detection of several Mycoplasma species from the same primary amplification reaction, significant competition, in particular with amplification of ureaplasma *16S rRNA* gene sequences, can result in poor sensitivity for detection of *M. genitalium* DNA (55).

Several real-time *M. genitalium* PCR tests have been developed since the first real-time assay was published in 2002 by Yoshida et al. (51). The combination of high sensitivity, specificity, robustness and reduced risk of contamination with amplicons suggests that real-time PCR should be the main method for *M. genitalium* diagnostics in the future. Quantification of *M. genitalium* DNA, which is feasible using real-time PCR, may provide important information regarding a number of research questions such as treatment efficacy (53) or

clinical relevance of *M. genitalium* DNA load in clinical specimens (44).

As an alternative to PCR, a transcription-mediated amplification (TMA) assay targeting the 16S rRNA, a molecule present in up to 100–1000 copies per bacterial cell, thereby increasing the sensitivity of detection compared with the PCR assays that target single-copy genes, has been offered recently (56). This NAAT was shown to be a sensitive, specific and high-throughput test for *M. genitalium* detection. In Russia, a nucleic acid sequence-based amplification (NASBA) assay (57) also targeting 16S rRNA is commercially available.

Although NAATs are the only effective tools available for detection of *M. genitalium* at present, and some are commercially available, no approved commercial NAAT is available. It is important to note, that for diagnostic use in the European Union, commercial tests need to be CE (Conformité Européenne) marked according to the In Vitro Diagnostic Medical Devices Directive 98/79/EC. However, in Article 1.5 of the Directive, devices “manufactured and used only within the same health institution and on the premises of their manufacture, or used on premises in the immediate vicinity, without having being transferred to another legal entity” are excluded. This allows in-house NAATs to be used for diagnostic purposes within the institution if this service is provided without a fee or as part of any commercial transaction, but even so, quality assurance is of utmost importance.

INDICATIONS FOR TESTING FOR *M. GENITALIUM*

The main indications for testing for *M. genitalium* are presented in Table II.

At present, population screening for *M. genitalium* is considered premature because prospective data on the natural history of disease, as well as clear evidence regarding *M. genitalium* as a cause of severe complications and sequelae is lacking (59).

CLINICAL SAMPLES FOR TESTING

General

In recent years, many NAATs have been used to detect *M. genitalium* in patient specimens (Table I), and some studies have also assessed the sensitivities of different specimen types for the detection of *M. genitalium* (52, 56, 60–62). In one large study, male first voided urine (FVU) was found to detect more *M. genitalium* infections (98%) than urethral swabs (82%). In females, however, FVU detected only 71% of the *M. genitalium* infections, while using both FVU and a cervical swab specimen increased the sensitivity to 96% (60).

In a study by Wroblewski et al. (56), it was concluded that in women, the most sensitive specimen type for the detection of *M. genitalium* was a vaginal swab (84% of

Table 1. Published diagnostic PCR tests for *M. genitalium*. Positions with sequence variation in bold

Target gene and PCR assay methodology	Forward primer			Reverse primer			Ref.
	Name	Sequence	Position	Name	Sequence	Position	
<i>MgPa</i>	MgPa-1	AGTTGATGAAACCTTAACCCCTTGG	180–204	MgPa-3	CCGTTGAGGGGTTTCCCATTTTGG	436–460	(2)
Conventional PCR		1 mismatch in 1 strain			0 mismatches		
<i>MgPa</i>	Mg1 (outer f)	TGCTATGACCAATGATGAC	3837–3856	Mg2 (outer r)	CTGCTTTGGTCAAAGACATCA	4191–4210	(3)
Conventional PCR (semi-nested)		1 mismatch in 3 strains			1 mismatch in 6 strains		
	Mg3 (inner f)	GTAATTAGTTACTCAGTAGA	3910–3929				
		0 mismatches					
<i>MgPa</i>	Mg1a (outer f)	GGTTAACTTACC*AGTGGCTTTGATC	3864–3889	Mg2	Same as (3)		(37)
Conventional PCR (semi-nested)		1 mismatch in 5 strains					
	Mg3	Same as (3)					
<i>MgPa</i>	G3A	GCTTTAAACCTGGTAAACCAGATTGACT	3755–3781	G3B	GAGCGTTAGAGATCCCTGTTCTGTGA	4236–4261	(38)
Conventional PCR		1 mismatch in 1 strain			0 mismatches		
<i>MgPa</i>	MgPaW1	AAGTGGAGCGATCACTACTAAC	–85– –63	MgPaWR1	CCGTTGTTAATCAIACCCTTCTGA	389–410	(39)
Conventional PCR		0 mismatches			0 mismatches		
<i>MgPa</i>	MGS-1	GAGCCTTTC ^a TAAACCGCTGC	38–56	MGS-4	GTTGTTATCATAACCTTCTGAT	388–408	(40)
Conventional PCR (semi-nested)		0 mismatches			0 mismatches		
	MgPa-1-mod	TGAAACCTTAAACCCCTTGG	186–204	MGS-2	GTGGGGTTGAAA GGATGATG	691–710	
<i>MgPa</i>		1 mismatch in 1 strain			Up to 10 mismatches in 11 strains		(41) from (2)
Conventional PCR		1 mismatch in 2 strains		MgPa-3-mod	AGGGGTTTCCCATTTTGG	436–454	
<i>MgPa</i>	MgPa-476	ATGGCAGCCTATCTTGAICCTTTTAA	476–503	MgPa-903	TTCACCTCCCACTACT CTT ATGC	903–929	(42)
Conventional PCR		1 mismatch in 2 strains			Up to 15 mismatches in 15 strains		
<i>MgPa</i>	MgPa-1-mod	Same as (41)		MgPa-1-mod	Same as (41)		(43)
Conventional PCR (microplate hybridization)							from (41)
<i>MgPa</i>	MgPa-355F	GAGAAATACCTT G ATG JCA GGCAA ^b	355–378	MgPa-432R	GTTAAATATATAATAAAGCTCTACCGTTGTTATC	400–432	(44)
Real-Time PCR (TaqMan)		1 mismatch in 1 strain			0 mismatches		
<i>I6S rRNA</i>	Mge 1	GAATGACTCTAGCAGGCAATGGCTG	451–475	Mge 2	AITTGCTCACTTTTACAAAGTTGGCT	1236–1260	(45)
Conventional PCR							
<i>I6S rRNA</i>	Mg16S-45F	TACATGCAAAGTCGATCGGAAAGTAGC	57–81	Mg16S-447R	AAACTCCAGCCAATTCCTGCTAG	459–481	(46)
Conventional PCR (microplate hybridization)							
<i>I6S rRNA</i>	16SFG2	CCTTATCGTTAGTTACATTTGTTTAA	1108–1132	16SRG	TGACATGCCGCTTCCAATAAA	1429–1448	(48)
Conventional PCR					1 mismatch with 16S rRNA SNP (47)		
<i>I6S rRNA</i>	My-ins	GTAATACATAGGTCGCAAGCGTTATC	532–557	MGSO	TGCACCAICTGTCACCTCTGTAAACCTC	1024–1050	(49)
Conventional PCR							
<i>I6S rRNA</i>	My-ins	Same as (49)		MGSO-2	CACCACTGTCACTCTGTAAACCTC	1026–1050	(50)
Conventional PCR (microplate hybridization)							
<i>I6S rRNA</i>	My-ins	Same as (49)		MGSO-2	Same as (50)		(51)
Real-time PCR (TaqMan)							
<i>I6S rRNA</i>	Mg16S-45F	Same as (46)		Mg16S-447R	Same as (46)		(52)
Real-time PCR (FRET probes)							adapted to real-time PCR from (46)
	p115-f	CCCAATCGTCAAGGTACAATGATGA	2817–2840	p115-r	GCATTTTCAAGTTCAACTGC AAAAGG	2892–2916	(53)
Real-Time PCR (FRET probes)							
gap (MG301)	Mg-gap-605f	GTGCTCGTGTGCAGCTGT	605–623	mg-gap-794r	GCTTGTATTACTTGTTC AACAGATGGAC	767–794	(54)
Real-time PCR (FRET probes)							

^aThe nucleotide is misprinted according to *M. genitalium* G37 sequence in the original publication. ^bUnderlined variation was reported in additional sequences, but this has been shown not to influence amplification efficiency (44).

Table II. Clinical indications for testing for *M. genitalium*

Patients	Indications
Males	Urethritis (especially in urethritis after empirical doxycycline therapy ^a) Epididymitis Prostatitis (with concurrent urethritis) Sexual partners of <i>M. genitalium</i> positive patients
Females	Cervicitis Urethritis Pelvic inflammatory disease (PID) Sexual partners of <i>M. genitalium</i> positive patients

^aA substantial proportion of *M. genitalium* infected patients with urethritis have persistent infection and often experience recurrent symptoms after doxycycline therapy (53, 58).

infections by TMA, 91% by PCR), followed by a cervical swab (60% by TMA, 53% by PCR) and urine (58% by TMA, 65% by PCR). Their findings that vaginal swab specimens are more sensitive than urine specimens for the detection of *M. genitalium* infection in women differ from those of Shipitsyna et al. (62), who showed that FVU was superior to both vaginal samples in females (100% vs. 57%) and to urethral swabs in males (83% vs. 75%) for the detection of infection.

Although the ideal genital specimen type for the detection of *M. genitalium* in men and women has not been thoroughly assessed, FVU seems to be the most sensitive specimen for the detection of *M. genitalium* infection in men, whereas in women, the use of more than one specimen may significantly improve the diagnostic sensitivity. In a recent study, endocervical swab specimens mixed and transported in FVU demonstrated a trend towards a higher sensitivity than FVU specimens alone as well as a significantly increased sensitivity compared with endocervical swab specimens transported in 2-SP medium for detection of *M. genitalium* DNA (61). It is important to note, however, that the ideal specimen type is highly dependent on the method applied for nucleic acid extraction and that the optimal specimen type may also differ between NAATs. Unfortunately, no systematic comparative studies on sample preparation before NAAT have been performed, but important issues to consider are: (i) sample collection without unnecessary volumes of transport medium, i.e. avoiding dilution of FVU specimen by mid-stream urine; (ii) concentration of the bacterial cells by centrifugation, and use of methods for purification that yield sufficiently clean nucleic acids without loss of target or excessive number of manipulations that would increase the risk of sample cross-contamination.

Sample collection, transportation and storage

The performance characteristics of the diagnostic tests are largely dependent upon the quality of the sampling. Even the best tests may give inadequate results owing to inappropriate sampling.

It is important to remember that:

- specific therapy should not have been initiated prior to sampling;
- if urethral and/or urine samples are collected, a patient should not have urinated for at least two hours prior to sampling.

Sampling devices:

- gynaecological speculum;
- cotton (gauze) swab to remove contaminating discharge;
- sterile cotton/Dacron swab;
- sterile container for urine;
- tubes with transport medium.

Specimens are collected in transport medium supplied/recommended by the manufacturer of the specific NAAT. Ideally, sampling and transport should be identical to that for *C. trachomatis* NAAT detection as the same specimen is usually tested for both agents. If no transport medium is specified in the manufacturer's instruction, samples may be collected in 2-SP medium (sucrose-phosphate buffer; 0.2 M sucrose, 0.02 M phosphate) according to the following procedures (Table III).

Sample transportation and storage:

- clinical materials should be transported to the laboratory as soon as possible.
- if storage conditions are not indicated in the manufacturer's instruction, samples should be kept at room temperature for up to 6 h or in a refrigerator for up to one week. Freezing should be avoided if possible as it decreases the sensitivity in most transport media (unpublished data¹).

PCR-BASED DIAGNOSIS OF *M. GENITALIUM* INFECTION

Analysis procedures

All analysis procedures should be performed in accordance with the instructions of the manufacturer of the specific PCR, using equipment, reagents and disposables supplied/recommended. Each run should include all necessary quality controls: positive (amplification) control, negative (contamination) control, and inhibition control.

PCR tests used in East European countries for diagnosis of *M. genitalium* infection

The information regarding performance characteristics of NAATs used in East European countries for the detection of *M. genitalium* is limited, but an evaluation of some commercially available Russian PCRs has recently been published (62).

¹Carlsen KH, Jensen JS. Does freezing of specimens affect sensitivity? Submitted.

Table III. Methods of collection of specimens for detection of *M. genitalium*

Patients	Collection methods
Males	Urethral swab In case of abundant discharge clean the external opening with a cotton (gauze) swab Introduce a sampling swab into the urethral meatus (0.5–2 cm), slightly rotate the swab in the urethra for few seconds, then take it out and place into a tube with transport medium
	First catch urine Ask the patient to collect the first 5–10 ml of the freely voided urine without previous washing
	Females
Females	Cervical swab Introduce warmed gynaecological speculum and clean the external cervical os to remove contaminating vaginal discharge using a large cotton (gauze) swab Introduce a sampling swab into the endocervical canal (1–2 cm), slightly rotate the swab inside the endocervical canal, then take it out and place into a tube with transport medium
	Urethral swab In case of abundant discharge clean the external opening with a cotton (gauze) swab Introduce a sampling swab into the urethral meatus (0.5–2 cm), slightly rotate the swab in the urethra for few seconds, then remove it and place into a tube with transport medium
	Vaginal swab Obtain material under direct vision using a gynaecological speculum from the posterior and lateral fornices of the vagina using a sterile swab, then place it into a tube with transport medium Self-obtained vaginal swabs can be used in certain situations. Women are instructed to insert the swab approximately 5 cm into the vagina and rotate three times. The procedure should not elicit any pain. After retraction, the swab is placed in an appropriate transport medium
	First catch urine Ask the patient to collect the first 5–10 ml of the first voided urine without any previous washing

QUALITY ASSURANCE

At present, NAATs are the only tools available for *M. genitalium* detection. A quality assurance scheme for nucleic acid-based diagnostics is presented in Fig. 1.

Prior to implementation in routine diagnostics, methods should be fully evaluated and validated. General guidelines and the minimum requirements for validation of a new or modified test have been published (63, 64). Based on these principles, for validation of a new test for *M. genitalium* infection, we recommend:

- Validation of the proposed test against an internationally validated and published test;
- A minimum of 50 positive clinical specimens and 100 negative specimens should be tested (as shown by the reference test);
- Specimens that are weakly positive should be included. Replicate dilutions of a strong positive specimen should also be included to assess the reproducibility of detection at low copy number;
- The sensitivity of the proposed test should not be more than 5% below that of the chosen reference test and the specificity should be >99%.

Recently, a series of studies has been conducted in Russia to evaluate NAATs used for diagnosis of STIs, including *M. genitalium* infection (62, 65, 66). The diagnostic performance of the *M. genitalium* PCRs displayed a high level of concordance with the reference assays, but the results of the study indicated that only one of the five PCRs evaluated had a reasonable clinical sensitivity, whereas the specificities of all the tests were high.

RECOMMENDATIONS

- A diagnosis of *M. genitalium* infection should be performed exclusively using NAATs, owing to the poor and slow growth of the bacterium in culture.
- Since no internationally validated and approved commercial NAATs for *M. genitalium* detection are presently available, it is necessary that laboratories performing *M. genitalium* diagnostics carefully evaluate and validate in-house PCRs before using them in routine clinical practice. Comprehensive internal controls should be used and laboratories should participate in external quality assessment programmes.

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Quality assurance				
Quality control			Quality assessment	
Evaluation and validation of tests	Internal quality control	Equipment evaluation and monitoring	Internal quality assessment	External quality assessment
An evidence-based assessment of how a test performs in the laboratory	<ul style="list-style-type: none"> • Sampling control • Control of nucleic acid isolation • Amplification control • Contamination control • Inhibition control 	Checking the performance of the equipment at regular basis and recording the data obtained	Periodical resubmitting and testing of randomly selected anonymised clinical samples	Testing of coded clinical or "spiked" samples distributed by national or international reference laboratories

Fig. 1. A quality assurance scheme in diagnostic microbiology using nucleic acid amplification tests.

G. Askarova, A. Utegenova (Kazakhstan), D. Yusupova, E. Al-Kilani (Kyrgyzstan), A. Rubins (Latvia), V. Kucinskiene (Lithuania), T. Krasnoselskikh, A. Guschin (Russia), A. Kasymov, O. Kasymov (Tajikistan), G. Mavrov, N. Kochetova, G. Bondarenko (Ukraine), S. Ibragimov, O. Izvekova, and T. Nabiev (Uzbekistan).

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