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Molecular Detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*

JENS K. MØLLER, BJÖRN HERRMANN,
JØRGEN SKOV JENSEN, AND HENRIK WESTH

A nucleic acid amplification test (NAAT) may enable the laboratory to detect *Chlamydia trachomatis* and *Neisseria gonorrhoeae* with high sensitivity and specificity in traditional urogenital swabs and in different types of samples obtained noninvasively by patients at home or in other settings. NAATs can significantly improve diagnosis and treatment of sexually transmitted infections (STIs) by *C. trachomatis* and *N. gonorrhoeae*, but they may also more effectively reduce the spread of these two species of microorganisms by supporting systematic screening programs for testing of persons with no symptoms of urogenital infection.

The purpose of this chapter is to provide guidance in selecting the most appropriate NAATs for *C. trachomatis* and *N. gonorrhoeae* among available commercial and in-house assays. Furthermore, the aim is to describe good laboratory practice and issues that the laboratory needs to consider before and after implementation of NAATs for *C. trachomatis* and *N. gonorrhoeae*.

Table 1 summarizes major issues about implementation and use of a NAAT in the laboratory.

CULTURE OR NAAT?

Detection of *C. trachomatis* and *N. gonorrhoeae* can be accomplished by a variety of different principles and methods including culture and antigen and nucleic acid detection (3, 89). Historically, culture of *C. trachomatis* and *N. gonorrhoeae* has been the gold standard for detection of both of these two microorganisms. Relative limits of detection

of different technologies used to diagnose *C. trachomatis* are shown in Fig. 1. To culture or not is no longer the question for detection of *C. trachomatis*. Nucleic acid-based methods have in general replaced culture and antigen detection assays except in resource-poor settings. In contrast, culture still represents the gold standard for detection of *N. gonorrhoeae*, and it is the primary routine assay in most laboratories. The yield of culture is highly dependent on the transport conditions and adequately performed cell culture and plating of the respective bacteria. If optimal transport and culture methods are used, culture may be similar in sensitivity to NAATs for both organisms (48). The main advantages of culture are that it provides the basis for general antibiotic susceptibility testing, which is especially important for *N. gonorrhoeae* in situations where major drug resistance problems exist (9), and that the specificity is 100% per definition. This eliminates the risk of false-positive results, which is of major concern when low-prevalence populations are tested. The main disadvantage of culture is the potentially suboptimal sensitivity in areas where prolonged transportation of samples or the adequacy of culture techniques/facilities is of concern.

The decision on which assays the laboratory should use, thus, is a complex process, involving not only the laboratory but also the physicians who depend on the test results (9). We recommend the use of NAATs for all types of urogenital samples except in cases with medicolegal implications such as rape or sexual abuse, where culture should be a supplementary test.

CHOICE OF TARGET AND ASSAY

Ideally, the laboratory should use a method with a sensitivity and specificity close to 100%. However, even the perfect test would yield sensitivity and specificity values lower than 100% simply due to the inadequacy of the gold standard, be it culture or a combination of tests. In the choice of a new assay, it is important to consider the population to which the test is going to be applied. If the

Jens K. Møller, Department of Clinical Microbiology, Aarhus University Hospital, Skejby, Aarhus N, DK-8200, Denmark. Björn Herrmann, Department of Clinical Microbiology, University Hospital, Uppsala, S-751 85, Sweden. Jørgen Skov Jensen, Department of Bacteriology, Mycology and Parasitology, Statens Serum Institut, Copenhagen, DK-2300, Denmark. Henrik Westh, Department of Clinical Microbiology, 445 Hvidovre Hospital, Hvidovre, DK-2650, Denmark.

prevalence of the microorganism to be detected is high, a slightly lower specificity may be accepted, because the positive predictive value (PPV) of the test may still be high. With a decreasing prevalence, even specificity figures of >99% may create problems with an increasing proportion of false-positive tests (Fig. 2). In general, the choice of target influences the specificity and the choice of test method influences the sensitivity of the assay.

Choice of Target

The selection of targets for detection of *C. trachomatis* and *N. gonorrhoeae* is a major point in determining which assay to use for routine diagnostics. Sequence variation in the target region may lead to false-negative results, whereas the presence of the target gene in other species may lead to false-positive results.

In general, it is less problematic to select a target for detection of *C. trachomatis* than for *N. gonorrhoeae*. As *C. trachomatis* is an intracellular pathogen, it has fewer opportunities for exchange of genetic material with other species. In contrast, many of the NAATs for detection of *N. gonorrhoeae* have specificity problems due to the frequent genetic exchange occurring between *Neisseria* species leading to the acquisition of *N. gonorrhoeae* NAAT target sequences by commensal *Neisseria* species (33).

C. trachomatis

Three main targets have been used for detection of *C. trachomatis*: the cryptic plasmid, genomic sequences, or rRNA. Most commonly, sequences present on the cryptic plasmid are used. This is the case for three of the four major commercially available platforms, i.e., Abbott m2000, Becton Dickinson (BD) ProbeTec, and Roche Amplicor. The advantage of using target sequences present on the plasmid is the intrinsic amplification caused by its presence in multiple (7 to 10) copies. Although *C. trachomatis* isolates without the plasmid have been reported occasionally, this has not been found to be a major problem (43, 65). The plasmid gene sequence is generally believed to be relatively stable, but recently, a new *C. trachomatis* variant with a 377-bp deletion in the target region for the Abbott and Roche tests has been detected in Sweden (50, 69). This has led to modifications of the two assays with the introduction of dual targets. For the Abbott test, two targets on the cryptic plasmid were chosen, whereas the Roche test combines a plasmid target and *omp1*, the gene for the major outer membrane protein (MOMP). Obviously, the dual-

TABLE 1 Issues that need to be addressed before and after implementation of a NAAT for *C. trachomatis* and *N. gonorrhoeae*

Target population
Males versus females
Prevalence
Diagnosis versus screening
Test of cure
Specimen types
Urogenital swabs
Urine
Other samples (nonurogenital specimens)
Specimen collection and transport
Selection of assay
Commercial versus in-house
Non-FDA-cleared assays or application of assays
Potential legal cases
False-positive results
Confirmation of all specimens
Confirmation of certain ranges of results and patient populations
Control of inhibition
Control of contamination
Monitoring of the assay
Proportion of positive tests per run or day
Contamination monitoring
Monitoring for prevalence of inhibition or equivocal results
Reporting
Results versus interpretations

target approach may become the future standard, bearing in mind that diagnostic selective pressure led to a situation where more than one-half of the *C. trachomatis*-infected patients were missed by methods using a single plasmid target.

rRNA is used as target in the Gen-Probe Aptima Combo 2 assay (23S rRNA), the Gen-Probe single analyte APTIMA assay for individual supplemental testing for *C. trachomatis* (16S rRNA), and in-house nucleic acid sequence-based amplification tests (44). This target has the advantages of being present in high numbers, leading to an intrinsic amplification, and being indispensable for the organism; thus, the likelihood for genetic changes leading to false-negative results should be minimal. Genes encoding 16S rRNA have also been applied in in-house PCRs (42,

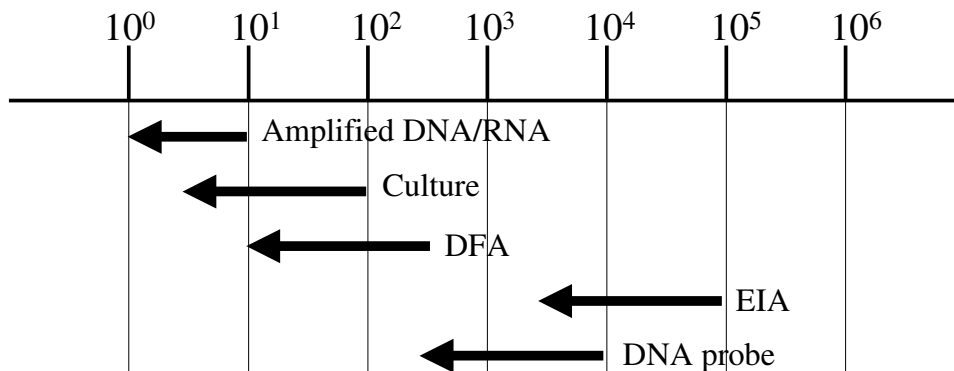


FIGURE 1 Relative limits of detection (log number of chlamydial elementary bodies per sample) of different technologies used to diagnose *C. trachomatis*. Modified from Carolyn Black, 1997 (3).

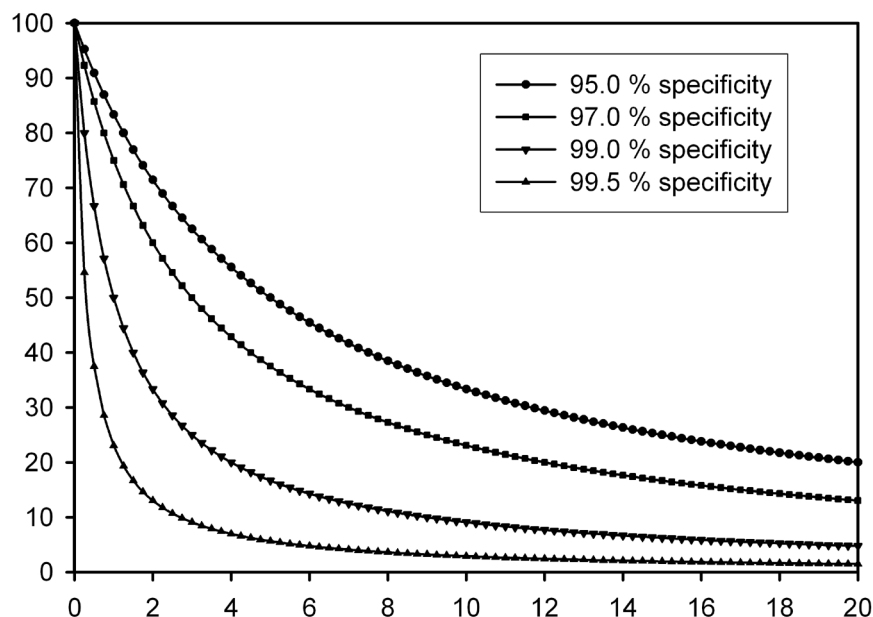


FIGURE 2 False-positive rate ($1 - \text{PPV}$) of an assay (y axis) with a given sensitivity of 100% expressed as a function of prevalence (x axis) and specificity.

84), but as only two copies of the rRNA operon are present in *C. trachomatis*, no intrinsic amplification is obtained. The same applies for single-copy genes such as the MOMP genes (29).

N. gonorrhoeae

The four major commercially available NAATs employ four different targets for detection of *N. gonorrhoeae*. Two of the targets are also found in other *Neisseria* species. Thus, the cytosine DNA methyltransferase gene used in the Roche Amplicor assays has been detected in *N. cinerea*, *N. flavescens*, *N. lactamica*, *N. subflava*, and *N. sicca*; except for *N. sicca*, representatives from the same species have been found to cross-react in the BD ProbeTec assay targeting the multicopy pilin gene-inverting protein homologue (89). Consequently, it is strongly recommended that positive results generated by these two assays be confirmed by an assay amplifying an independent target. Cross-reactions with other *Neisseria* species have not been reported for the Gen-Probe Aptima assay targeting the 16S rRNA or for the Abbott m2000 assay targeting the multicopy cell surface opacity protein (*opa*) genes.

Because of the specificity problems, a wide range of confirmatory assays have been published. Roche originally had a confirmatory assay amplifying the 16S rRNA gene, but this is no longer available. In-house 16S rRNA gene assays have performed well in a number of studies (5, 11), but a hybridization step has usually been necessary to obtain sufficient specificity. Some years ago, PCRs based on the *cppB* gene located on the gonococcal cryptic plasmid were widely used, but it was found that some *N. gonorrhoeae* strains lack this particular sequence (39, 85), leading to an unacceptably low sensitivity in some settings. NAATs based on the multicopy *opa* gene have proven to be very useful (79). Due to its multicopy nature, the sensitivity of the assay is enhanced, and the risk of false-negative reactions due to strain variability or deletions is reduced. Assays targeting the *porA* pseudogene also appear promising (25,

86). The gene is present in all *N. gonorrhoeae* strains studied and seems to be highly conserved (80). However, the possibility that this pseudogene could be deleted from the *N. gonorrhoeae* genome without serious consequences for the bacterium led to a larger validation study of 240 *N. gonorrhoeae* strains collected from geographically diverse sources. The study documented that all strains carried the pseudogene (85).

Dual-target NAATs for detection of *N. gonorrhoeae* may be a future way to avoid false-positive and false-negative results, but such assays have not yet been validated for diagnostic use.

Choice of Commercial Assays

Major Commercial Tests

The diagnostic market for NAATs detecting *C. trachomatis* and *N. gonorrhoeae* is dominated by four companies; Abbott, B-D, Gen-Probe, and Roche (Tables 2 and 3). Their choices of different NAAT principles, targets, and hardware solutions make the assays different, but the end results are assays that all perform well for *C. trachomatis* detection and with some limitations for *N. gonorrhoeae*. The choice of assay will depend on the needs of the end users as well as of the laboratory. The needs include, among others, (i) suitable performance of the assay, i.e., PPV, validated specimen types, availability of confirmatory tests, bar-coding of samples and reagents, bidirectional exchange of data between the laboratory information system and the assay instruments, contamination risk, maintenance (daily, weekly, and service), system-up time, and the shortest hands-on time possible to improve ergonomics; and (ii) availability of specialized laboratory space (the physical area taken up by the equipment and the need for dedicated rooms) and reliability of the local vendor. Finding room for storage of reagents and reagent shelf life can be [problems](#). All systems and their computers should be connected to an uninterruptible power supply to minimize the consequences of

TABLE 2 Comparison of methods for detection of *C. trachomatis* and *N. gonorrhoeae*^a

Method	Urine CT/GC	Rectal swabs CT/GC	Eye swabs CT/GC	Throat swabs CT/GC	Liquid cytology CT/GC	Automation available for batch test	Comments
Culture	N/N	N/A	A/A	N/A	N/N	No	Maintaining viable organisms a problem; gold standard for GC
DFA	N/N	N/N	A/Y	N/N	N/N	No	Experienced reader needed
EIA	N/N	N/N	R/N	N/N	N/N	Yes	Rarely used for GC; obsolete for laboratory testing
Probe	N/N	Y/Y	Y/Y	Y/Y	N/N	Yes	Combination of CT and GC assays possible
NAAT	A/A	Y/Y	Y/Y	Y/Y	Y/Y	Yes	Combination of CT and GC assays possible; de facto CT gold standard

^aAll commercial NAATs are FDA approved for urogenital swabs. Abbreviations: CT, *C. trachomatis*; GC, *N. gonorrhoeae*; A, approved; Y, to be used with confirmatory test only and to include the notification "Assay not approved for sample material" on the final report; N, not recommended; R, ●●●; DFA, direct immunofluorescence assay; EIA, enzyme immunoassay.

main power failures. It is advisable never to choose a new system without having visited laboratories experienced with the system. Further issues that should be taken into consideration may be found in Table 1. Note that national legislation or guidelines may necessitate absolute requirements. Before choosing among assay platforms, their respective performances can be compared in large quality control programs, which are supplied by the College of American Pathologists or European organizations such as

United Kingdom National External Quality Assessment Service (UK NEQAS) and Quality Control for Molecular Diagnostics (QCMD). QCMD is a "not for profit" organization dedicated to advancing the quality of molecular diagnostics through external quality assessment (EQA). In surveys or reports from these EQA programs, the rate of false-positive and false-negative results according to product manufacturer can be estimated. False-positive results are usually due to sample carryover or amplification prod-

TABLE 3 Commercially available molecular methods for detection of *C. trachomatis* and *N. gonorrhoeae*

Organism and company	Assay	Gene target(s)	Molecular principle	Target purification before amplification/hybridization	Amplification control
<i>C. trachomatis</i>					
Abbott	m2000 v2	Plasmid (2 targets)	NAAT-real-time PCR	Silica-based magnetic particles	Yes
BD	ProbeTec	Plasmid	NAAT-SDA	Pouch ^a	Yes
Digene	Hybrid Capture II	Plasmid and genome	Hybridization	No	No
Gen-Probe	PACE 2 CT	23S rRNA	Hybridization	No	No
Gen-Probe	APTIMA Combo 2	23S rRNA	NAAT-TMA	Specific target capture	No
Gen-Probe	APTIMA CT	16S rRNA	NAAT-TMA	Specific target capture	No
Roche	TaqMan48 v2	Plasmid and <i>omp1</i>	NAAT-PCR	Wash only	Yes
<i>N. gonorrhoeae</i>					
Abbott	m2000 v2	Opacity protein gene	NAAT-real-time PCR	Silica-based magnetic particles	Yes
BD	ProbeTec	Pilin gene-inverting protein homologue	NAAT-SDA	Pouch ^a	Yes
Digene	Hybrid Capture II	Plasmid and genome	Hybridization	No	No
Gen-Probe	PACE 2 GC	16S rRNA	Hybridization	No	No
Gen-Probe	APTIMA Combo 2	16S rRNA ^b	NAAT-TMA	Specific target capture	No
Gen-Probe	APTIMA GC	16S rRNA ^b	NAAT-TMA	Specific target capture	No
Roche	TaqMan48 v2	Cytosine DNA methyltransferase gene	NAAT-PCR	Wash only	Yes

^aA magnetic particle-based purification system was recently developed for the BD Viper platform.

^bDifferent targets are used.

TABLE 4 Sensitivity and specificity of commercial NAATs for detection of *C. trachomatis*^a

Cohort	NAAT	No. of studies included	Sample type	% Sensitivity (95% CI)	% Specificity (95% CI)
Women	PCR	14	Cervix	85.5 (80.3–90.6)	99.6 (99.4–99.8)
			Urine	83.3 (77.7–88.9)	99.5 (99.3–99.8)
	SDA	2	Cervix	93.6 (91.2–96.1)	97.9 (97.3–98.5)
			Urine	79.9 (73.3–86.4)	99.1 (97.7–100)
	TMA	4	Cervix	96.7 (93.0–100)	99.1 (98.2–100)
			Urine	92.5 (88.0–97.0)	98.6 (97.7–99.6)
Men	PCR	12	Urethra	87.5 (82.4–92.5)	99.2 (98.8–99.6)
			Urine	84.0 (78.5–89.4)	99.3 (98.9–99.7)
	SDA	1	Urethra	92.4 (86.8–96.2)	96.3 (94.3–97.8)
			Urine	93.1 (87.7–96.7)	93.8 (90.7–95.1)
	TMA	2	Urethra	95.9 (91.3–100)	99.4 (98.7–100)
			Urine	87.7 (80.1–95.2)	99.4 (98.7–100)

^aCI, confidence interval. Data from Cook et al., 2005 (12).

uct contamination and are problems that have to be continuously addressed. More information on the subject may be gathered from the CLSI manual, *Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline* (55). The guideline addresses among other things selection and qualification of nucleic acid sequences, establishment and evaluation of test performance characteristics, controlling false-positive reactions, quality assurance, and regulatory issues.

Sensitivity and Specificity of Commercial NAATs

A systematic review assessing the sensitivity and specificity of commercial NAATs for detection of *C. trachomatis* and *N. gonorrhoeae* in urine and cervical (women) and urethral (men) swab samples, respectively, was published by Cook et al. in 2005 (12). The combined study sensitivities and specificities are shown in Tables 4 and 5. However, few published studies at the time of data selection presented data on the transcription-mediated amplification (TMA) and the strand displacement amplification (SDA) assays. For *C. trachomatis* the sensitivities were between 83.3 and 96.7% with specificities in the range of 93.8 to 99.6%. For *N. gonorrhoeae* testing, the sensitivities were between 55.6 and 96.7% with specificities in the range of 98.7 to 99.7%.

TMA and SDA found significantly more *C. trachomatis* than PCR in cervix specimens (Table 4). In female urine, TMA found significantly more *C. trachomatis* than SDA. For male samples (urethra and urine), no significant differ-

ence in sensitivity was observed for PCR, SDA, and TMA. Results of TMA and PCR were nearly identical for urine and cervix specimens, whereas the sensitivity of SDA for *C. trachomatis* in cervix and urine specimens was significantly different (Table 4).

For NAAT detection of *N. gonorrhoeae*, similar results were obtained for PCR, SDA, and TMA on cervix specimens (Table 5). For female urine specimens, the PCR assay had a significantly lower sensitivity than SDA and TMA. For male samples, PCR had a significantly higher sensitivity for urethra specimens than for urine specimens (Table 5). Three studies reported data separately for symptomatic and asymptomatic women and found identical results in the two groups (12). Similar results were also seen for the groups of symptomatic and asymptomatic males.

Discrepancy Analysis

In general, assays are evaluated by comparing their results to a gold standard and subsequently calculating the sensitivity and specificity. Unfortunately, there is no uniform method that the companies have to adhere to, no consensus on an appropriate reference standard, and no well-defined clinical state that allows differentiation between infected and uninfected persons (22). Many scientific publications have used discrepant analysis with a third assay to determine the status of the specimen. Using this approach, discrepant results for the test under evaluation are reevaluated with one or more different NAATs. The sensitivity and specificity for these NAATs can greatly af-

TABLE 5 Sensitivity and specificity of commercial NAATs for detection of *N. gonorrhoeae*^a

Cohort	Test	No. of studies included	Sample type	Sensitivity% (95% CI)	Specificity% (95% CI)
Women	PCR	4	Cervix	94.2 (90.5–98.0)	99.2 (98.4–100)
			Urine	55.6 (36.3–74.9)	98.7 (97.5–99.9)
	SDA	1	Cervix	96.5 (90.1–99.3)	99.5 (99.0–99.8)
			Urine	84.9 (75.6–91.7)	99.4 (98.9–99.8)
	TMA	1	Cervix	99.2 (95.7–100)	98.7 (98.0–99.3)
			Urine	91.3 (85.0–95.6)	99.3 (98.6–99.6)
Men	PCR	4	Urethra	96.1 (94.4–97.7)	99.0 (98.2–99.8)
			Urine	90.4 (87.9–92.9)	99.7 (99.4–100)

^aFrom Cook et al., 2005 (12).

fect the performance estimates of the test under evaluation. Some have argued that discrepant analysis is a statistically inappropriate practice (21, 23), while others believe that while the discrepancy analysis biases towards an improved sensitivity, the bias may be relatively negligible (20, 72).

Other Commercial Assays

Other commercial companies have CE-marked assays (Artus and Digene), and a growing number of companies have tests that currently have a "Research only" label. Siemens HealthCare diagnostics have very recently developed a kinetic amplification-based assay for their VERSANT™ kPCR Molecular System, which detects both *C. trachomatis* and *N. gonorrhoeae*. Several companies are developing multiplex sexually transmitted disease (STD) panels, for example, AutoGenomics (Infiniti™ STD-6) and Seegene (Seeplex™ STD 7-plex test and 9-plex test). Spartan Bioscience has developed a platform (Spartan Dx) for very low volume testing (four wells). Two DNA hybridization tests are on the market, the Gen-Probe Pace 2® test and the Digene Hybrid Capture II test. Both tests can be used for detection of *C. trachomatis* and *N. gonorrhoeae* alone or in combination. The methods are still commonly used but are being phased out by NAATs. The Digene Hybrid Capture II test has been cleared for use on an automated instrument and compares well with NAATs, although the sensitivity is somewhat lower.

Platforms for High-Throughput STD Testing

Laboratories needing high-throughput automated platforms for detection of both *C. trachomatis* and *N. gonorrhoeae* currently have two options; BD and Gen-Probe both offer a solution capable of analyzing more than 350 samples for both *C. trachomatis* and *N. gonorrhoeae* per shift. BD provides a platform called Viper, and Gen-Probe offers a platform called Tigris. The BD Viper can test 460 patient samples per shift in the present nonextracted mode and 368 samples for *C. trachomatis* and *N. gonorrhoeae* in a newly launched extracted mode adding an automated nucleic acid purification step before amplification of the specimen. In both modes, an initial 30-min prewarming step of the specimens outside the Viper is needed. The Gen-Probe Tigris can test 492 samples for *C. trachomatis* and *N. gonorrhoeae* in an 8-hour shift, and pretreatment of samples is not required before processing the specimens in the Tigris. Compared to manual platforms these robots have a huge advantage, as they significantly reduce manual labor and repetitive movements for the staff. These two platforms were recently evaluated and compared with the more recent Abbott RealTime PCR system m2000 (36), which has a lower throughput (186 specimens per 8-hour shift). The study comprised only 500 first-void urines including a number of known positive specimens in order to increase the ability of the study to detect differences in performance of the three assay systems. Specimens that gave discrepant results were retested with in-house real-time PCR assays. In all, 98 true positive *C. trachomatis* and 24 true *N. gonorrhoeae* specimens were detected. It was concluded that all three assays were suitable for the detection of the two microorganisms (36).

Sensitivities for *C. trachomatis* were between 96.9 and 99.0%, and specificities were 99.5 to 100%. Sensitivities for *N. gonorrhoeae* were between 95.8 and 100%, and specificities were 100%. More studies are needed to assess the true performance of the m2000 system and compare the results with the data shown in Tables 4 and 5.

The ideal robot has a nucleic acid extraction module for improving sensitivity and specificity. Penetrable caps for samples and reagents are essential. Reagent storage for the assays but also for swab and urine sample kits must be taken into consideration as well as response time for backup deliveries. A laboratory with only one robot must have a contingency plan for breakdowns.

In-House Detection Assays

In-House *C. trachomatis* PCR Assays

Routine diagnostics of *C. trachomatis* infections is predominantly performed with commercial NAAT high-volume test systems, but there are still applications where in-house-developed methods are useful. The first PCR methods used ethidium bromide detection in agarose gels or isotope-labeled hybridization (14, 16) and were later followed by colorimetric detection that is still used in some commercial assays. Nowadays, several amplification techniques are available and detection assays are mostly in a real-time format, which facilitates the detection, reduces the contamination risk from amplicons, and enables quantification.

In-house assays can be used for confirmatory testing of commercial tests as a second target. See also "Choice of Target and Assay" above. Combinations of assays may also be used for detection of *C. trachomatis* in investigations of sexual abuse or rape (9) or for identification of plasmid-free strains (43). There are also several applications for in-house methods where subspecies identification or typing is used on specimens that are found to be positive by the commercial tests. In recent years the incidence of lymphogranuloma venereum, caused by infection by *C. trachomatis* strains, especially genotype L2b, has drastically increased among men having sex with men in Europe, North America, and Australia. This has required specific tests for diagnosis of lymphogranuloma venereum, and different real-time PCRs have been developed (52). Another example is the appearance of a new variant of *C. trachomatis* that was discovered in Sweden in 2006. A deletion in the cryptic plasmid affected the target region of two commonly used commercial tests and caused false-negative test results (24). Specific PCRs were used for identification of this new variant (8, 69). For more specific research purposes, there is a continuous development of new assays.

In-House *N. gonorrhoeae* PCR Assays

Several in-house *N. gonorrhoeae* PCR assays have been published based upon the same targets as used by the commercial assays described above but also based on *cppB* and the *porA* pseudogene (89). Many screening assays for *N. gonorrhoeae* exhibit low PPVs, particularly in low-prevalence populations. The main purpose of an in-house assay is to provide a confirmatory test for one of the four major commercial assays. Today only Gen-Probe offers an alternative commercial *N. gonorrhoeae* NAAT. Their APTIMA GC (AGC) assay may be used for the confirmation of *N. gonorrhoeae*-positive specimens with the APTIMA Combo 2 test (AC2). The Aptima GC assay targets a nucleic acid sequence in the gonococcal 16S rRNA molecule that is different from the one targeted by the AC2 assay and is thus suitable for confirming AC2-positive results (6). To embark on developing your own in-house assay requires not only the choice of a target sequence in *N. gonorrhoeae* and a NAAT principle but also, more importantly, a prospective evaluation of the in-house test on sample material and groups of individuals from your

routine setting before practical use of the *N. gonorrhoeae* assay. Based on that evaluation, the level of false-positive and false-negative results can be determined.

Diagnosing *N. gonorrhoeae* by use of NAATs might increase the sensitivity, compared to cultivation. However, using NAATs can also be problematic due to the close genetic relationships between different *Neisseria* species, resulting in false-positive diagnoses. Thus, there is a need for a confirmatory test identifying the non-*N. gonorrhoeae* species responsible for false-positive results from a commercial screening test as shown for the COBAS Amplicor CT/NG assay (11).

Performance of Different Sample Types

It is a prerequisite for a well-performed NAAT that the sample material investigated be properly collected and contain an adequate number of epithelial cells infected with *C. trachomatis*. However, the direct immunofluorescence assay is the only type of method that allows a concomitant assessment of the quality of the specimen collection by microscopy. Urine is effective as a specimen for detection of *C. trachomatis* and *N. gonorrhoeae* only if it contains an adequate number of cells, and hence, a “first-void” urine sample should be collected at a time when the last urination was no less than 1 hour earlier. The laboratory should provide guidelines for proper sampling and educate health care personnel to ensure correct use of the assays and interpretation of results (Table 2).

The NAATs are not FDA approved for use on samples from extragenital sites, such as eye, pharynx, and rectum. Specimens from extragenital sites should only be tested if a thorough in-house validation has been performed. Preferably, an independent NAAT should be performed for all positive results. When reporting the test results, the laboratory should emphasize that the result was produced on a specimen not approved for the assay and that the result should be interpreted with caution (Table 2), especially if an independent NAAT has not been used for confirmation.

Influence of Anatomical Site on Bacterial Load

The advent of real-time PCR allows the quantification of bacterial load. Michel et al. (46) used a quantitative PCR to evaluate the *C. trachomatis* load in patient samples from matched anatomical sites. Briefly, the bacterial load was lowest in female urine (47 elementary bodies [EBs]/100 μ l) and increased by a factor of 3.4 in female urethral swabs, by a further factor of 4.8 in vaginal swabs, and by a further factor of 2.9 (2,230 EBs/100 μ l) in cervical swabs. For men, bacterial load was highest in urine (1,200 EBs/100 μ l) and significantly lower for urethral swabs (821 EBs/100 μ l). For females, a markedly lower sensitivity of NAATs for urine compared to endocervical samples has been observed during parts of the menstrual cycle (49). This suggests that menstrual cycle factors (estrogen?) are inhibitory in the urine but not present in the vaginal secretions. This may preclude the use of urine sampling for systematic screening of *C. trachomatis* in asymptomatic females.

Pharyngeal and Rectal Swabs

Currently no commercial companies have chosen to validate their assays for pharyngeal and rectal swabs. This is surprising, as the market for these tests could be quite large. Use of NAATs for samples from these sites has two major advantages over culture: it has fewer problems with specimen storage and transport, and the sensitivity is higher due to less contaminating flora in the culture systems. As was

discussed previously (see “Choice of Target” above), several *N. gonorrhoeae* assays have specificity problems that are enhanced when the swab is applied to areas with other *Neisseria* species. For Roche Cobas Amplicor PCR the problematic species are *N. flavescens*, *N. lactamica*, *N. sicca*, and *N. subflava*, and for ProbeTec SDA they are *N. subflava* and *N. cinerea*. Cross-reactivity has not been reported for the Abbott m2000 assay or for the Gen-Probe Aptima Combo 2 assay. Furthermore, a single-analyte confirmatory test (Aptima GC) is available for the latter (6, 71).

Many laboratories have been forced to perform verification of their commercial assays on rectal and throat swabs with an in-house PCR for *C. trachomatis* and *N. gonorrhoeae* or by culture, which has a low inherent sensitivity. In larger laboratories, another commercial NAAT is occasionally used, but for smaller laboratories, the cost is too high. Confirmation is further complicated by cross-reactivity reported also for some in-house PCRs targeting other *N. gonorrhoeae* sequences (17, 60). Valuable advice for gonococcal NAAT use can be obtained from Whiley et al. (87).

Sample Preparation Methods

Inhibition of the NAAT may constitute a serious problem for many assays using urine or other specimen types containing a high level of potential inhibitors. Sample extraction methods, therefore, play an important role in the overall performance of an assay. Two principles exist: the extraction can be generic, i.e., a general extraction of all nucleic acids in the sample, or it can be target specific, i.e., extraction of the nucleic acid containing the target of the assay. Most automated extraction methods apply magnetic particles to which the nucleic acids are bound by various principles. The particles are kept in the sample tube by a magnet throughout the washing procedures during which potential inhibitors and other substances are removed. The Gen-Probe Aptima test uses a target capture technique whereby capture probes attached to magnetic beads specifically bind the rRNA molecules containing the 16S rRNA or 23S rRNA targets used in their APTIMA assays. Use of target capture increases the specificity of the assay, whereas the generic methods may provide purified material for other commercial or in-house assays.

Combination Testing

NAATs make it easier to test for both *C. trachomatis* and *N. gonorrhoeae* in the same sample. Dual detection of the two microorganisms in the same urogenital sample (“combo-test”) has been FDA approved for all the major commercially available NAATs. It is important to realize that the advantage of combo-testing may be outweighed by its inappropriate use in testing of populations with a low prevalence of, for example, gonorrhea. Even with an acceptable specificity of the combo-test, the false-positive rate may be dramatically increasing for a prevalence below 2%, as shown in Fig. 2.

From a management point of view, workflow in many laboratories could benefit from a single method allowing the combined testing for *C. trachomatis* and *N. gonorrhoeae* in a patient sample easily obtained and transported to the laboratory without significant loss of the target material. However, if a proper transport and plating of the specimens for culture of *N. gonorrhoeae* can be secured, the NAATs do not provide higher sensitivity than culture for urogenital specimens. A universal transport medium that allows both culture and NAAT for *C. trachomatis* and *N. gonorrhoeae*

would potentially be of great benefit, enabling a NAAT screening followed by culture of positive samples only. This would allow antimicrobial susceptibility testing and monitoring of gonococcal resistance.

Decisions on NAAT combo-testing in both resource-rich and resource-poor settings should be influenced by clinical and laboratory considerations rather than commercial pressures.

Pooling

Pooling of samples is a strategy that takes advantage of the high analytical sensitivity of NAATs in order to reduce laboratory costs and may allow the use of NAATs in resource-poor countries (74). Pooling may be considered if the prevalence of *C. trachomatis* or *N. gonorrhoeae* is low in a given population or category of samples. The number of samples in a pool depends on the prevalence, and it is calculated from the number of samples, which needs to be tested individually from positive pools. Samples from a negative pool should be reported as negative. The laboratory should carefully consider the economical benefits reaped by reducing the number of tests to be performed against the risk of mixing up samples, creating sample-to-sample contamination, lowering the sensitivity, delaying positive sample reports, and spending more time managing samples and results. In St. Petersburg, Russia, an in-house PCR was used on 1,500 endocervical samples tested both individually and pooled in groups of 5 and 10 samples (73). The sensitivity and specificity of the PCR were not affected by either pooling strategy. The pooling strategy resulted in cost savings of 53.3% (5 samples per pool) and 44.0% (10 samples per pool). In another study pooled PCR samples (compared to individual PCR) had a sensitivity of 100% and a specificity of 98.9%. For pooled SDA tests (compared to individual SDA), a sensitivity of 86.5% and a specificity of 98.9% were found (1). The lower sensitivity of SDA on pooled samples could have been caused by the use of 2-sucrose phosphate buffer for sample collection, the dilution of samples in a pool, and/or by the slightly lower analytical sensitivity of SDA compared to PCR.

QUALITY CONTROL

A range of factors, some of which can be controlled by good laboratory practices and thorough quality control, can affect the performance of NAATs negatively. Factors concerning sequence-related problems have been addressed in the section about choice of target.

The general considerations for design of the molecular diagnostic laboratory have been described elsewhere (see chapters 55 through 58 in this volume).

For the diagnosis of STIs, optimal sensitivity of the test is crucial; however, it is important also to consider the consequences of false-positive results in the context of diseases that are considered psychologically and socially stigmatizing, not to mention the medical and legal aspects in some situations. Therefore, good care should be taken when results are reported.

Even when a commercially available approved test is implemented, an internal validation should be performed. This could be a comparison with the existing methods for diagnosis, but if a less sensitive assay is being replaced with a more sensitive test, confirmatory testing may often be needed. Exchanging specimens for NAAT validation between laboratories using different NAAT methodologies is usually a rewarding exercise. For validation of in-house as-

says, a more thorough validation is needed, even if the method is adapted from a published validated method. The technical sensitivity and specificity should be estimated by determining the limit of detection (LOD) with known amounts of purified DNA as well as with quantified cultured bacteria spiked into the relevant specimen matrix subjected to the chosen sample extraction procedure. Specificity should be checked with isolates covering a broad geographical and temporal spectrum and preferably isolated from different patient populations. Lack of amplification with phylogenetically closely related species should be demonstrated to control for cross-reactions, but testing other urogenital tract pathogens seems less important considering the fact that less than 10% of the urogenital bacterial flora is cultivable. It is by far more important to test the clinical specificity on a large panel of specimens collected from patients with the relevant disease manifestations but without evidence of infection with the microbe in question.

It is important to include internal amplification controls (IACs), i.e., a small amount of target added to the reaction in order to demonstrate lack of inhibition. The IAC can be designed in a variety of ways (26), and it should preferably be added before the nucleic acid extraction procedure in order to control for inadvertent loss of the specimen. By keeping the concentration of the IAC as close to the LOD as possible, the presence of minor inhibition can be detected, and the IAC is less prone to compete for the amplification reagents with a subsequent increase of the LOD and hence a false-negative test. Apart from detecting inhibition of the NAAT, amplification of the IAC will also document reagent or instrument failure, and since instrument failure may be partial and affect only a subset of the specimens, an IAC is important for high-quality diagnostics.

Confirmation of positive results is a controversial issue. The U.S. Centers for Disease Control and Prevention recommend confirmatory testing if the PPV is <90% (26). Even with very specific testing methods, a PPV of <90% may be reached whenever the prevalence of the pathogen is <2% (Fig. 2). This would be the case for most *N. gonorrhoeae* testing performed in Northern Europe, whereas the prevalence of *C. trachomatis* in samples most often would be above that value. Obviously, repeating the test with the same assay would most often only reveal cases where the false-positive results were caused by mislabeling or mix-up of samples. Using an alternative target, preferably after a renewed nucleic acid extraction of the original sample specimen, and at the same time checking of the identity labeling on the original specimen tube would significantly increase the specificity and consequently the PPV. Therefore, confirmatory testing of positive results is highly recommended by some, although the procedure has been criticized for being unnecessary (51).

Environmental monitoring of the laboratory is a good way of controlling the efficiency of decontamination procedures. Surfaces are swabbed using sample collection kits appropriate for the detection system. If positive results are encountered, more rigorous decontamination procedures should be implemented, and the cleaning staff should be instructed in using an appropriate workflow moving from clean areas to less clean areas.

Environmental contamination of sites for sample collection has been reported as a risk for false-positive results when using RNA as target (45). Whether this is a problem in daily practice remains to be determined.

Internal quality assessment can easily be arranged by selecting a panel of specimens with known results and then relabeling them before repeat testing as for ordinary clinical specimens. This could be done monthly or even more often. Monitoring the positive rate on a weekly basis is generally recommended for surveillance, but seasonal fluctuations make it a rather insensitive tool, although it was the basis for detecting the new variant *C. trachomatis* strain (68). Constant monitoring of positive controls is advised. It is recommended that a positive control close to the LOD be included in every setup and that the percentage of amplification failures be monitored on a weekly basis. For real-time PCR assays, or other tests where semiquantitative results are registered, positive controls should be monitored for drift.

The laboratory should also participate in organized, interlaboratory comparisons such as EQA schemes. The EQA schemes should reflect the full spectrum of sample types, and the samples should be tested according to the standard method used in the laboratory including all pre- and post-examination procedures.

Commercially available EQA schemes for *C. trachomatis* and *N. gonorrhoeae* NAATs are available from several sources, e.g., QCMD, as mentioned in "Major Commercial Tests" above. Use of these schemes is easy for the laboratory and has the advantage of providing a larger statistical basis for comparison of the results. Usually specimens are sent out twice a year.

APPLICATIONS OF MOLECULAR DETECTION METHODS

Screening Programs

After the introduction of *Chlamydia* testing of the population in general, an initial decline in the prevalence of

Chlamydia-positive individuals occurred, but a marked rise in the prevalence has been noted again in recent years in several countries (Fig. 3) (18, 38). The uniform rise in these countries seems to reflect a true increase in the rate of urogenital *Chlamydia* infections and not only the increased use of the more sensitive NAATs and testing of larger groups of individuals. NAATs offer a reliable method for the use of noninvasive samples such as urine or self-taken vulvovaginal or penile swabs, which makes larger screening programs possible. Such programs are targeting healthy individuals at the community level, including home screening and school- or other institution-based screening with the purpose of detecting asymptomatic persons infected with *C. trachomatis* or *N. gonorrhoeae*, and should take into account the strong age-dependent distribution of *Chlamydia* infections (57, 67).

Previous studies have demonstrated the cost-effectiveness of annual NAAT-based *Chlamydia* screening for women (27, 59). The majority of *Chlamydia* cases are seen in women ages 15 to 24. Young men are less often tested than females, but they can be reached by the increased use of urine samples. The study of Blake et al. (4) shows that the use of NAATs to screen both men and women for *Chlamydia* upon entry to a National Job Training Program may be cost-effective and cost-saving and provide an opportunity to substantially reduce chlamydial infections among young people at risk for STDs.

Home Screening

Self-collected specimens offer both genders an acceptable and sensitive alternative method to testing for STIs (58). Different specimen types have been used for self-collected specimens, e.g., vaginal pipette, first-void urine, vulvovaginal swab, tampon, and penile swab (35). Considering that one-half of the individuals infected with *C. trachomatis*

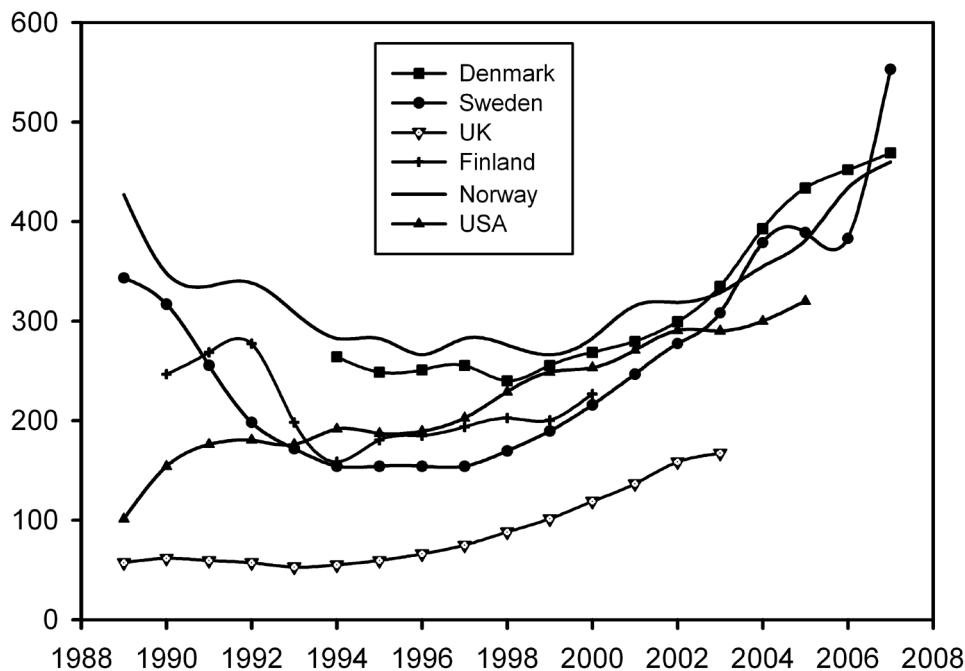


FIGURE 3 Rates (cases per 100,000 population) of reported *Chlamydia* infection in selected countries from 1989 to 2007. Compiled from various sources including references 18 and 38.

have no recognizable signs or symptoms of urogenital infection, it is important to reach out to these groups of persons, as they may not turn up in health care facilities. Home-based screening for *C. trachomatis* or other STIs by mailed samples may effectively increase the number of persons tested (57), thereby reducing the epidemic spread, and hopefully prevent pelvic inflammatory disease, ectopic pregnancies, and tubal factor infertility.

Test of Cure

Test of cure for *C. trachomatis* infections is not routinely indicated if the prescribed treatment has been taken and possible symptoms disappear. NAAT is not ideal for test of cure because it cannot distinguish between dead and live bacteria. Test of cure using NAATs may lead to false-positive results in a cured patient within 4 weeks after completion of treatment. If tested within that period, a culture or other nonamplification assay should be used, bearing in mind that these assays often are less sensitive than NAATs and consequently may miss a persistent infection. In medicolegal cases, cultures and NAATs should always be requested. For urogenital infections with *N. gonorrhoeae*, culture should always be preferred, and test of cure should be performed 1 week after completion of antibiotic treatment. It is important that the sexual partner(s) be treated and reinfection avoided after treatment. Repeat testing is recommended for *C. trachomatis*-positive individuals within 3 to 6 months posttreatment because of the high risk of reinfection/relapse (15, 32). A repeat test is always indicated in individuals with persistent symptoms or in cases where nonstandard treatment regimens have been used. Persistent symptoms after treatment may indicate double infection with another STD pathogen, and additional testing and treatment should be considered.

MOLECULAR EPIDEMIOLOGY (GENOTYPING)

C. trachomatis

There are several reasons and applications for typing *C. trachomatis*: to examine association between types and clinical manifestations and pathogenicity; in investigations of sexual assaults; for analysis of transmission patterns in sexual networks; and for examination of persons with repeatedly positive *Chlamydia* tests to examine for persistence versus reinfection.

Typing of *C. trachomatis* has mainly been based on discrimination of the 15 different serovars of the MOMP encoded by the *ompA* (*omp1*) gene. Serovars A to C infect the eye and lead to trachoma with blindness as final end point (subtype Ba is also sexually transmitted). Serovars D through K are sexually transmitted and may lead to sequelae including ectopic pregnancy and infertility in women and epididymitis in men. Among heterosexuals serotype E is predominating and comprises about 40% of all *Chlamydia* cases in most countries. In contrast, among men having sex with men certain subtypes of serovars D, G, and J may predominate (53). The serovars L1 through L3 are also sexually transmitted but are more invasive and have previously been confined to developing countries mainly. However, in recent years they have spread among men having sex with men in Europe and North America (81). Phylogenetic characterization of strains based on *ompA* results in serovar groupings that are inconsistent with the patho-

biology of *C. trachomatis* (7). Some studies have reported associations between clinical symptoms and certain serovars of the D through K group, but several large studies have not found any such correlation (40, 47, 64).

Molecular genotyping of *ompA* can be performed by using restriction fragment length polymorphism on PCR products from culture isolates (54, 56), but also directly from clinical specimens (19, 70). A higher discrimination of *ompA* variants is achieved by sequence determination. In some studies with small study populations and/or recruitment from high-risk groups, a high sequence variation has been seen (13, 62, 78). However, in studies with large and unselected populations the discriminating capacity has been low (30, 31, 40). Similar limitations have also been found for *ompA* typing of trachoma infections (41). Increased possibilities for genotyping have been achieved by multiplex real-time PCR (29) and hybridization arrays (66, 90). All these methods can discriminate mixed infections and provide sensitive detection and typing of *C. trachomatis* directly from clinical specimens.

The nine polymorphic membrane protein genes, *pmfA* to *pmfI*, have been hypothesized to be useful for typing, but the discriminating capacity is limited (77).

The highly conserved genome of *C. trachomatis* has given difficulties in developing discriminating typing methods based on single genes. However, improved resolution has been achieved using multilocus target systems. Analysis of variable numbers of tandem repeats in three loci combined with *ompA* sequencing has been shown to reach a significantly higher diversity index than by using *ompA* alone (63). The combination of *ompA* sequencing and variable-number tandem repeat genotyping was readily performed with samples submitted in the transport media for the routine assay for *C. trachomatis*. An alternative system is based on sequencing of five target regions and in analysis of 47 clinical isolates of representative serotypes resulted in 32 genetic variants among 12 *ompA* variants (34). It was also applied in differentiating serotype E strains with identical *ompA* from sexual networks. A genotyping system has also been developed for the entire *Chlamydiaceae* family (61). It is based on seven housekeeping genes and aims to analyze evolutionary changes rather than be a tool for partner notification.

N. gonorrhoeae

The highly variable genome of *Neisseria* species is caused by frequent mutations and constant genetic transformation as well as frequent recombination between strains both within and between species. Thus, genotyping of *N. gonorrhoeae* achieves a much higher discrimination than what can be obtained for *C. trachomatis*. Genotyping can be used for short-term epidemiological characterization including partner notification, identification of core groups or clusters of gonorrhea cases, and performance of precise epidemiological surveillance. It can also be a tool to examine the effects of public health interventions on the size and nature of sexual networks. Another important application is monitoring the spread of antibiotic resistance. Since some of the targets used are surface-exposed antigens, genotyping can also be applied for analysis of immunity and pathogenicity.

Auxotyping and serotyping were used before for discrimination of *N. gonorrhoeae* strains, but the discriminatory power is considerably lower than that of genotyping methods such as pulsed-field gel electrophoresis and *opa* typing

(82). The *opa* typing is based on 11 chromosomal genes and includes a PCR amplification combined with restriction enzyme digestion and in its original form also isotope labeling. It provides the highest discrimination achieved by currently used methods and has been applied for transmission investigations (83). However, it is laborious and results of the obtained band patterns may be inconvenient to share between laboratories.

Sequence-based methods produce more unambiguous data, and the entire *por* gene provides high discrimination. A simplified system (*N. gonorrhoeae* multiantigen sequence typing) is based on sequencing of the internal fragments of *por* and *tbpB* and enables a high-throughput typing procedure (10).

The capacity of *N. gonorrhoeae* for natural mutation and recombination has led to abundant resistance mechanisms, and genotyping is an important tool to characterize the stepwise acquisition of resistance and to perform epidemiological surveillance of antibiotic resistance (37).

Neisseria was the first bacterial genus that was explored for multilocus sequence typing, and systems are typically based on seven housekeeping genes. This enables strain characterization that addresses the population and evolutionary biology of the species. Such a system has also been developed for *N. gonorrhoeae* (2).

Although there are limitations to the use of multilocus target systems, they can be applied to clinical specimens from routine NAAT diagnostics and enable improved partner notification as well as research studies. Detection in array format for multilocus systems will further facilitate genotyping of *C. trachomatis* and *N. gonorrhoeae*.

The advent of new multitarget typing systems provides more information and highlights the need for standardized nomenclature systems. User-friendly typing systems require easy interpretation and communication of data. This is an urgent challenge to be solved.

FUTURE PERSPECTIVES

NAAT-Based Detection of Susceptibility to Antibiotics

Penicillin-resistant *N. gonorrhoeae* strains have for a long time demanded alternative treatments for gonococcal infections. Increasingly prevalent infections by ciprofloxacin-resistant *N. gonorrhoeae* now necessitate treatment with the more expensive injectable ceftriaxone. Detection of gonococcal drug resistance at present requires culture, but non-culture tests for *N. gonorrhoeae* are rapidly replacing culture. Therefore, there is a growing need for NAAT-based detection of drug-resistant *N. gonorrhoeae* strains directly in patient samples. A real-time PCR assay for detection of mutations in the Ser91 codon of the *gyrA* gene of *N. gonorrhoeae* in urine specimens has recently been described by Siedner et al. (75). Their determination of a susceptibility genotype in 72% of a collection of female urine specimens correlated well with the susceptibility pattern for ciprofloxacin. With treatment options for gonococcal infections diminishing, the recent isolation of *N. gonorrhoeae* strains in Australia with reduced susceptibilities to the newer cephalosporins represents a worrisome trend that also requires monitoring and further investigation (88).

Drug-resistant *C. trachomatis* strains have been described (76), but they seem not to constitute a widespread clinical problem. However, the rare use of culture today for

detection of chlamydial infection and the lack of nonculture assays for drug susceptibility tests applied directly to the specimens examined for *C. trachomatis* in combination with asymptomatic infections may obscure inadequate antimicrobial treatment. The rapid spread of a mutant variant of *C. trachomatis* in Sweden escaping detection by some NAATs (69) has been a warning that vigilance for drug-resistant mutants should be enforced.

Thus, one cannot safely rely on a given antibiotic for treatment of gonococcal or chlamydial infections in the future without some knowledge of the prevalence of drug resistance in these microorganisms. However, it is important to stress the added complexity in resolving antimicrobial susceptibility by molecular techniques compared to the present detection of the microorganisms by NAAT. Genotypes do not necessarily represent the phenotype or clinical sensitivity of a given microorganism towards an antibiotic. Furthermore, when using direct molecular detection of drug resistance genes in the patient sample, we have to be sure the genes come from the particular microorganism under investigation. If the drug resistance gene is not restricted to the particular microorganism, we need to develop tests whereby the drug resistance gene and another sequence unique for the microorganism are detected at the same time, as was devised, for example, for the detection of methicillin-resistant *Staphylococcus aureus* strains directly from specimens containing a mixture of staphylococci (28).

Array Technology

The molecular array technology, with its potentially high capacity embedded in a single detection chip, may revolutionize the detection of STD pathogens in the future by creating multiplex PCR detection systems that not only detect all relevant microorganisms but also determine their drug resistance pattern and genotype, providing concomitant information for treatment and prevention of STIs.

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Author Queries

<QU1>Au/Some references renumbered per ASM style (CLSI manual moved to References as no. 55). Please check references and in-text citations throughout.

<QU2>Au/Please define CE (used here only).

<QU3>Au/Sentence beginning with "In recent years...." has been edited to reflect the fact that lymphogranuloma venereum is the name of the disease, not the causative agent.

<QU4>Au/Please define "R" in Table 2 footnote.