Multiplex AMPLICOR PCR Screening for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in Women Attending Non-Sexually Transmitted Disease Clinics

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A new PCR kit (AMPLICOR CT/NG; Roche Diagnostic Systems, Inc., Branchburg, N.J.) was used as a screening tool for the detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in first-void urine (FVU) specimens from 3,340 asymptomatic women attending European health care units for contraceptive advice or pregnancy termination. All samples were kept frozen (−20°C) prior to testing. Chlamydia-positive samples were retested once by the plasmid-based PCR kit and also by a major outer membrane protein (MOMP) primer-based PCR. Discrepancies were resolved by using the direct immunofluorescence test (DIF) with the centrifuged sediment of the FVU specimens. Samples positive for *N. gonorrhoeae* were retested by chromosomal primer-based PCR and verified by a 16S RNA PCR. Of the samples tested, 1.8% were considered inhibitory by using the internal amplification control. Of 81 samples positive for *C. trachomatis*, 74 samples were positive by both plasmid- and MOMP-based PCRs, 6 samples were positive by plasmid-based PCR and DIF, and one sample was positive by both MOMP-based PCR and DIF. Nine samples (0.3%) were positive for *N. gonorrhoeae* by the chromosomal primer-based PCR; however, none of the results could be confirmed. The test offers the unique ability to identify inhibition of amplification with the optional internal control.

The frequent lack of obvious symptoms in persons affected by *Chlamydia trachomatis* genital infections is one major reason for introducing screening programs for their detection. The most significant consequences of genital chlamydial infections in females include the risk of tubal infertility, ectopic pregnancy, and pelvic inflammatory disease (19). The same holds true for infections caused by *Neisseria gonorrhoeae* (11).

Over the past few decades a number of techniques have been used to detect genital infections caused by *C. trachomatis*, i.e., isolation in McCoy cells (26) in combination with staining the cell cultures with fluorescein (fluorescein isothiocyanate)-labelled monoclonal antibodies. This was until recently considered the "gold standard" in the diagnosis of such infections. Later, antigen detection tests, such as the direct immunofluorescence test (DIF) (34) and enzyme immunoassay (EIA) (13), have been applied for their detection. Many have found that nucleic acid detection assays, such as nonamplification (14) and, lately, amplification assays, e.g., PCR (5, 12, 25) and ligase chain reaction (LCR) (1, 7), possess higher sensitivities than the detection methods used earlier.

Thus, PCR and LCR have proved useful for the detection of *C. trachomatis* in cervical and urethral samples both in symptomatic and in nonsymptomatic women (7, 10, 17, 21). Recently, tests with first-void urine (FVU) specimens by these techniques have shown that the amplification tests are as sensitive as tests with endocervical swab cultures (1, 10, 22, 25, 31).

Traditionally, urethral and cervical samples (and in certain countries, rectal samples as well) have been routinely used to detect *N. gonorrhoeae*. In male patients with urethritis, the diagnosis can be made by direct microscopy of Gram- or methylene blue-stained smears of urethral discharge. However, for the optimal microbiological diagnosis of gonorrhea in asymptomatic male and female patients and for tests for cure of gonorrhea, culture should be the diagnostic method of choice (18). The sensitivity of gonococcal cultures for patients with acute gonorrhea is believed to be 80 to 95%, but it is not higher than approximately 50% for females with long-standing infections. False-negative *N. gonorrhoeae* culture results have been attributed to poor sampling technique, toxic sampling equipment, inhibition of growth by components of body secretions as well as by inappropriate antibiotics in selective culture media, and finally, self-treatment. Improper specimen storage and transport also contribute to the number of false-negative culture results. A variety of nonculture test alternatives for the diagnosis of gonorrhea have been developed, such as DIF, EIA and nonamplified DNA hybridization assays (6, 28). DNA amplification techniques (32) hold promise for the elimination of many of the specimen collection, transport, and laboratory diagnostic problems.

The present study was aimed at screening women attending European health care units for contraceptive advice or pregnancy termination for *C. trachomatis* and *N. gonorrhoeae*.

MATERIALS AND METHODS

Study population. A total of 3,340 women (age range, 15 to 44 years; median age, 26 years) were asked to participate in the study. The women had attended 24 European family planning centers or gynecological clinics in 14 European countries for contraceptive advice (*n* = 2,987; age range 15 to 45 years; median

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age, 26 years) or pregnancy termination (*n* = 353; age range 16 to 43 years; median age, 26 years).

Sampling. The women were instructed not to wash or otherwise clean their genitalia before voiding or to spread the labia during urine collection (as should be recommended when collecting samples for bacterial urine cultures). The FVU specimen was collected after bladder incubation (women were asked not to urinate) for at least 1 h. Of the 15 to 30 ml of urine collected (in a 50-ml plastic beaker), 4 ml was transferred to a polypropylene tube and was frozen at −20°C (some were frozen after overnight incubation at 2 to 8°C) and kept frozen for at most 2 months. Urine specimens which had been left at room temperature for more than 24 h were excluded from the study.

Specimen transport. After the agreed upon numbers of urine samples were collected by each center, they were shipped to the laboratory in Uppsala, Sweden. Frozen samples were sent on dry ice by 24-h delivery. In all cases the samples arrived before they had thawed.

PCR testing. The PCR tests were performed with the Multiplex AMPLICOR CT/NG kit (Roche Diagnostic Systems, Inc., Branchburg, N.J.) as instructed by manufacturer.

(i) Contamination prevention. The amplified material created by PCR contains uracil as opposed to thymine. To prevent carryover contamination from previously amplified material, the kit contains an enzyme which, at the end of the PCR, is used to destroy the uracil-containing amplicon. Foreign DNA, if present, remains intact. To further reduce any additional risk of contamination, the tests were performed in three separate areas of our laboratory, i.e., one area for reagent and specimen preparation, another area for amplification, and still another area for detection. In order to avoid contamination, plugged micropipettes with an aerosol barrier (Labsystems, Helsinki, Finland), disposable gloves, and separate laboratory coats were used for each of three steps.

(ii) Sample preparation. Five hundred microliters of the each thawed sample was mixed with 500 μl of urine wash buffer. The mixture was incubated for 15 min at 37°C and centrifuged at 14,000 × *g* in a microcentrifuge (Eppendorf; Netheler-Hinz GmbH, Hamburg, Germany), after which the supernatant was decanted and discarded. The pellet was dissolved in 250 μl of lysis buffer in the CT/NG kit, and the mixture was incubated at room temperature for 15 min. Two hundred fifty microliters of specimen diluent in the CT/NG kit was added to each test tube, and the tube was centrifuged at 14,000 × *g* for 10 min. Positive and negative controls for *C. trachomatis* and *N. gonorrhoeae* (included in the Multiplex AMPLICOR CT/NG kit) were always used in each test run. Fifty microliters of the processed urine specimens and 50 μl of the controls were transferred to reaction tubes (MicroAmp; Perkin-Elmer, Norwalk, Conn.), which contained the master mixture from the CT/NG kit and an internal control for each individual sample.

Plasmid DNA of *C. trachomatis* or an transcript RNA of *N. gonorrhoeae* with primer binding region sequences identical to the test-specific target sequence served as internal controls in the AMPLICOR CT/NG kit. It also contains a unique probe binding region that differentiates internal control and the test-specific target sequences.

(iii) Evaluation of test results. Samples were considered positive when they gave an optical density (OD) of ≥0.8 at 450 nm and the internal control had an OD of ≥0.25. Negative samples were those with ODs of <0.2 and an internal amplification control OD of ≥0.25. Samples in the grey zone were those with ODs between 0.2 and 0.8 when the internal control OD was ≥0.25.

Samples were considered inhibitory if the OD was <0.2 for both *C. trachomatis* and *N. gonorrhoeae* and the internal amplification control OD was <0.25.

The total time required for the PCR analysis and detection was approximately 5 h, while the pure hands-on time was approximately 3 to 3.5 h. With very few exceptions, the tests had acceptable controls.

Confirmatory testing of positive samples and analysis of discrepant results. All samples positive for *C. trachomatis* were coded and retested by the same plasmid-based Multiplex AMPLICOR CT/NG PCR assay and thereafter were retested by a major outer membrane protein (MOMP)-based PCR assay (RDS; Roche Laboratories, RDS, Kaizeraugst, Switzerland). Samples giving discrepant results between the two plasmid-based PCR tests were tested a third time by the plasmid-based PCR test. The results for samples giving discrepant results between the plasmid- and MOMP-based PCRs were resolved by DI(MicroTrak *Chlamydia trachomatis* Direct Specimen Test; Syva, San Jose, Calif.). Confirmatory testing was done in a blinded manner.

Plasmid PCR-positive samples were those giving positive results in two consecutive plasmid PCR runs. Samples that were twice negative by this test were regarded as plasmid PCR negative.

C. trachomatis-positive samples were those giving positive results in the plasmid- and MOMP-based PCR assays or those giving positive results in any of the two PCRs and a DIF.

Samples which were positive for *N. gonorrhoeae* by the Multiplex AMPLICOR CT/NG PCR test, which targets the putative methyltransferase gene, were retested once more by the Multiplex PCR assay. The positive samples were also tested by a 16S RNA-based PCR performed at Roche Diagnostic Systems Laboratories.

DIFs. For DIFs, 500 μl of urine was centrifuged at 14,000 × *g* and the supernatant was discarded. The pellet was dissolved and mixed for 5 s in 100 μl of urine wash buffer. Twenty microliters of the suspension was spread onto a glass slide. The slides were air dried, fixed with methanol, stained with fluores-

TABLE 1. Test results for 3,340 FVU samples from women[a]

No. of samples	PCR result		DIF result	No. of samples regarded as positive after resolution
	Plasmid-based PCR[b]	MOMP-based PCR		
3,258	−	NT[c]	NT	0
74	+,+	+	NT	74
6	+,+	−	+	6
1	+,−,−	+	+	1
1	+,−,−	+	−	0

[a] The women were attending clinics for pregnancy termination or contraceptive advice and were tested for *C. trachomatis* by a plasmid-based PCR (Multiplex AMPLICOR CT/NG kit), and the results were resolved by an MOMP-based PCR and DIF.
[b] Multiple results are the results of the consecutive runs.
[c] NT, not tested.

cein-labelled monoclonal antibodies, and tested with a fluorescence microscope (screened at ×400 magnification and confirmed at ×1,000 magnification; Diaphot-TMD; Nikon, Tokyo, Japan). A sample was considered positive when at least five elementary bodies (EBs) were found.

RESULTS

Of the 3,340 FVU samples tested, 80 were positive for *C. trachomatis* in both plasmid-based PCR runs (Table 1). Among those samples, the results between the plasmid- and MOMP-based PCRs disagreed for six samples; i.e., the result could not be confirmed by the latter test. However, DIF revealed the presence of chlamydial EBs in the sediment of the FVU specimen.

For two additional samples there was disagreement between the two Multiplex PCR runs for each sample; i.e., the samples were positive in the first run and were negative in the following runs, therefore being considered to have a negative result by the plasmid-based PCR. Those two samples were, however, positive by the MOMP-based PCR (Table 1), while only one of them remained DIF positive.

Of the 81 samples confirmed to be positive for *C. trachomatis*, 48 (59.2%) had ODs of >3.0, while those with ODs falling between 2.5 and 3.0, 2.0 and 2.5, and 1.5 and 2.0 made up 16.2, 12.3, and 7.4% of the samples, respectively. Only 3.7% of the samples gave ODs between 1.0 and 1.5 and 1.2% of the samples gave ODs between 0.5 and 1.0 (Fig. 1).

Of the 3,340 samples tested, 61 (1.8%) were considered inhibitory and remained so when they were retested by the plasmid-based PCR.

Of the 3,340 samples tested, 9 (0.3%) were positive for *N. gonorrhoeae* with the Multiplex AMPLICOR PCR kit; the results for all of these samples were confirmed by repeat testing by this assay. When those nine samples were tested for *N. gonorrhoeae* by the 16S RNA-based PCR, all nine proved to be negative.

DISCUSSION

During recent years, tests based on the amplification of nucleic acids have been developed as alternatives to conventional methods for the diagnosis of sexually transmitted diseases, such as culture, DIF, and EIA. So far PCR and LCR are the two most frequently used DNA-based methods (1, 5, 7, 10, 12, 17, 21, 22).

Testing of female FVU specimens by nucleic acid amplification assays has been shown to be a highly sensitive approach to the diagnosis of genital *C. trachomatis* infections (10, 25, 31). The excellent performance of PCR with male FVU specimens
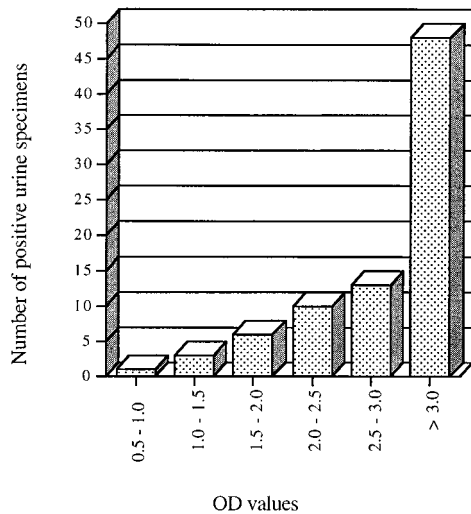
FIG. 1. Distribution of ODs for 81 chlamydia-positive FVU samples obtained by PCRs with the Multiplex AMPLICOR CT/NG kit.

(13) and swabs containing female endocervical and urethral specimens (3, 5) has been documented. The sensitivities of the LCR test with FVU specimens was shown to vary from 87.5 to 100% compared to the results of testing urethral and cervical samples (1, 33).

We used the Multiplex AMPLICOR CT/NG PCR assay for the simultaneous detection of infections caused by *C. trachomatis* and *N. gonorrhoeae*. *C. trachomatis* was detected in 2.4% of women who had considered themselves gynecologically healthy.

Previous studies with female FVU specimens used to detect *C. trachomatis* by EIA demonstrated a sensitivity of approximately 40% (8, 29). DNA amplification tests performed with urine from asymptomatic women have shown higher sensitivities than those of culture with cervical specimens and enzyme-linked immunosorbent assays with urine (1).

Studies of *C. trachomatis* infection by testing cervical and urethral samples by culture have shown that approximately half of all infected women are positive at both sites, with 30% of the women being positive at the cervix only and 5 to 30% being positive at the urethra only (4, 23). FVU specimens have proved to be suitable for testing, provided that DNA amplification is used. Urine can be contaminated with chlamydiae from the urethra, and it also most likely will contain chlamydiae shed from the vaginal introitus and the vulva because urine comes into contact with these parts of the genital tract when females urinate.

The concern has also been raised that PCR tests may provide positive results for patients who are cured. PCR assays may remain positive for *C. trachomatis* for some weeks after the completion of a course of antibiotic therapy (9). Patients' antibiotic intake may thus explain differences between the culture and DNA-based test results (2), because culture requires the presence of live organisms, while the DNA-based assay may detect the nucleic acids of dead bacteria that have not yet been shed. It is notable that 11% of the women in our study had taken an antibiotic between 6 and 2 weeks before sampling. The level of antibiotic consumption might be representative of that among females in age groups in the general European population attending clinics for contraceptive advice and pregnancy termination. Because culture was not per-

formed in our study, this particular diagnostic dilemma could not be evaluated.

Several factors may influence the performance of amplification tests with urine, i.e., the volume of urine available for testing, use of FVU versus midstream samples (30), time of sampling since the last micturation, and the possible presence of inhibitors in FVU samples.

In our study, we required at least 1 h of bladder incubation before voiding. This was supposed to ensure that microorganisms were not washed out from the urethra or cervix just before the urine sample was obtained. If a longer incubation time was necessary, this could restrict the usefulness of this method for the detection of chlamydial and gonococcal infections in gynecological clinics, because many women prefer to empty their bladders just before they are subjected to pelvic examinations. It may cause practical problems to have patients remain at the clinic for some hours before they are able to provide an adequate urine sample. Because no comparison was made by testing multiple urine specimens collected after different times of bladder incubation from each patient, it was impossible to assess the impact of this factor on the performance of the test.

Inhibitors present in clinical samples may reduce the sensitivity of PCR (24) and might explain the discrepancies in the results obtained by tests with different types of samples (20). One major factor affecting the sensitivity of PCR tests seems to be the presence of *Taq* polymerase inhibitors that block the activity of this enzyme (16).

The AMPLICOR CT/NG PCR kit contains an optional internal control which enables the routine detection of the presence of inhibitors in test samples. Of the 3,340 urine samples that we tested, 1.8% were found to be inhibitory. By exploiting the opportunities given by the test, one can try to reduce the inhibitory capacities of such samples by diluting (8) or freezing-thawing the urine (20). Both of these methods have been claimed to increase the sensitivity of the test. On the other hand, inhibitors may not be equally distributed in the urine and might not be present in the new portion of urine. The use of an internal control offers the possibility of disclosing persons producing inhibitory samples. Such persons might be recalled for resampling or other tests may be performed with the same sample.

Most of the chlamydia-positive samples (i.e., 95%), had high OD values, which made them easy to identify as positive. An OD exceeding 3.0 was found for 59.2% of such samples. Another large group (i.e., 36% of positive samples) had ODs between 1.5 and 3.0. Only 5% of the positive samples had ODs of 0.5 to 1.5. However, one needs to stress that the OD value does not reflect the severity of infection. No correlation between the OD in the plasmid-based PCR and positivity by the MOMP-based PCR was observed.

Chlamydiae may not be equally distributed in urine samples. This may explain discrepancies between the consecutive PCR runs for an individual sample.

A possible explanation for discrepancies in the results between the plasmid- and MOMP-based PCRs may be a difference in the target used for the amplification. The PCR assay that we used to detect *C. trachomatis* is based on a cryptic plasmid primer, which has been proved to be a more sensitive approach than that used for the MOMP primer-based PCR (15, 27), because plasmids are present in EBs in multiple copies. This may explain why six samples were positive only by the former test but not by the latter test. MOMP-based PCR assays have almost exclusively been used for confirmatory testing (12).

The identification of *N. gonorrhoeae* in clinical specimens is essential for the effective control of gonorrhoea. Diagnosis of

gonorrhoea by culture involves several factors that may present obstacles, such as the availability of nontoxic sampling swabs and appropriate fresh, nonoxidized, nontoxic transport media, the requirement for a short transport time, the availability of high-quality culture media (preferably both nonselective and selective), and the availability of a temperature- and a CO_2-regulated incubator and appropriate species identification methods. The use of a PCR method may overcome several of these potential diagnostic pitfalls.

In the present study nine (0.3%) of all samples were positive for *N. gonorrhoeae* in the primary tests by the chromosome-based PCR test. The samples positive by this test, however, all turned out to be negative by the 16S rRNA backup test. Because these samples were positive only by the chromosome-based PCR, they were resolved to be false-positive specimens. One may speculate that the initially positive results may have been true positive and that the 16S RNA-based PCR tests were false negative. It is also possible that these specimens were falsely positive due to cross-reactivity with nonpathogenic *Neisseria subflava* or *Neisseria cinerea* strains, which can be amplified by the chromosomal DNA-based PCR test but not by the 16S RNA-based system. The negative results for 99.7% of the specimens provide a very high negative predictive value (>99%), which is particularly important for the value of tests to be used for populations in which the prevalence of gonorrhoea is low, which is likely to be the case, for example, in family planning units in the catchment region of our study, i.e., Europe. The true value of the PCR test could, however, not be established for *N. gonorrhoeae* by our study design because tests for gonorrhea were not available in many local laboratories. On the other hand, samples could not be sent on a regular basis to our laboratory in Uppsala for testing.

We have demonstrated that chlamydial infections do occur in women consulting European health care units for contraceptives and pregnancy termination. Therefore, screening for *C. trachomatis* in conjunction with regular gynecological examinations, which are highly recommended, should be considered. On the basis of the results of our study, it is not possible at present to recommend routine screening of asymptomatic women at such centers for *N. gonorrhoeae*. Evaluations based on cost-effectiveness of establishing universal or target screening programs for asymptomatic women attending different health care units are warranted.

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