Clinical Validation of a Real-Time Polymerase Chain Reaction Detection of Neisseria gonorrhoeae porA Pseudogene Versus Culture Techniques

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Background: Diagnosing Neisseria gonorrhoeae using nucleic acid amplification tests (NAATs) might increase the sensitivity, compared to cultivation. However, using NAATs has also been problematic mainly due to the close genetic relationships between different Neisseria species, resulting in false positive diagnoses. This study was conducted to clinically validate a previously published real-time polymerase chain reaction (PCR) method targeting the porA pseudogene in N. gonorrhoeae in comparison to culture techniques.

Methods: In total, 360 samples, urethra (n = 109), rectum (n = 84), pharynx (n = 119), and cervix (n = 48) from 185 males and 57 females, were analyzed using porA pseudogene PCR and cultivation. Sequencing of the entire porA pseudogene and the 16S rRNA gene were used to resolve discrepant results.

Results: Of the 360 samples, 37 were positive by both culture and PCR, however, the PCR identified 15 additional confirmed positive samples. The PCR method showed a sensitivity, specificity, positive predictive value, and negative predictive value of 100% in a preselected population. The preselected population had a true gonorrhoea prevalence of 17.4%.

Conclusions: The present porA pseudogene real-time PCR comprises a valuable supplement to the traditional culture techniques for diagnosis of N. gonorrhoeae, especially for samples from extragenital sites such as pharynx and rectum.

NEISSERIA GONORRHOEAE IS ONE OF TWO pathogenic neisseria species and the causative agent of gonorrhoea, which is a major sexually transmitted infection (STI) in many countries.1–5 The gold standard for N. gonorrhoeae diagnostics remains culture, despite the low viability of the bacteria in vitro,6–8 mainly due to the opportunity to perform antimicrobial susceptibility testing.

Autolysis is a major reason why culture has reduced sensitivity, compared to nucleic acid amplification tests (NAATs), even under more optimized conditions.9–14 Accordingly, detection technologies such as NAATs that are independent of bacterial viability have a potential to limit false negative samples and in some geographic areas, NAATs are rapidly replacing culture for diagnosis of gonorrhoea. However, the Neisseria genus comprises many closely related species,15,16 which have been causing significant problems mainly in regards of suboptimal specificity of most N. gonorrhoeae NAATs.13,17–24 When using NAATs for diagnosis of N. gonorrhoeae, a very high specificity is essential, especially for samples from extragenital sites25 where commensal Neisseria species are exceedingly prevalent. Besides increased sensitivity, other advantages of NAATs are the opportunities of using noninvasive samples (e.g., urine), rapidness, high automation, and simultaneous detection of several etiological agents of STIs such as Chlamydia trachomatis and N. gonorrhoeae.

Extensive sequencing of the porA pseudogene26–27 and previous work on porA targeting polymerase chain reactions (PCRs)28–30 have supported this pseudogene as a highly suitable chromosomal target. The porA gene/pseudogene is absent in commensal Neisseria species26,29,31 and the porA gene of the human pathogen N. meningitidis is sufficiently divergent to be distinguished from the N. gonorrhoeae porA pseudogene by PCR.27,30

In the current study, we evaluated our previously published real-time porA pseudogene PCR method for detection of N. gonorrhoeae against conventional culture. The main priority was to establish a clinically validated, highly sensitive, and species specific real-time PCR suitable for detection of N. gonorrhoeae in genital as well as extra genital samples.
Materials and Methods

Study Population and Data Collection

All consecutive patients attending a venereological polyclinic (Olafiatlinikken) in Oslo, Norway, from January 2006 through May 2006 with suspected gonorrhea were enrolled in the study. Reasons for suspecting gonorrhea were: purulent discharge from urethra or cervix, typical diplococci in stained urethral smear, partner with gonorrhea, unprotected sex in a country high endemic for gonorrhea, and receptive anal or pharyngeal sex in males who have sex with males.

Of the eligible patients, 269 patients were recruited. However, 42 samples from 27 patients were excluded as they where taken for test of cure after treatment (n = 21), Herpes simplex virus testing (n = 7), or due to technical failure resulting in unavailable samples (n = 14). Consequently, 360 samples from 242 patients were finally included into the current study, i.e., 284 samples from 185 men and 76 samples from 57 women. The samples were taken from urethra (n = 109), rectum (n = 84), pharynx (n = 119), and cervix (n = 48). The study was approved by the regional ethics committee.

Clinical Samples

Samples were collected using either a urethral flocked swab (Copan) or an endocervical flocked brush (Copan). The urethral swab was used for sampling in the urethra. The endocervical brush was used to take samples from the cervix, rectum, and pharynx. All sampling sites were based on the patient’s sexual anamnesis. Each sample for PCR analysis was collected with individual swabs that were placed and transported in 3 mL UTM-RT transport media (Copan) for PCR analysis. A separate set of swabs were collected from the same sites, but placed and transported in M40 transport medium (Copan) for culture diagnostics. The samples for culture were consistently taken before the samples for PCR to avoid reducing the quality of the routine diagnostics. In addition, a urethral smear was taken with a metal curette and in females also a cervical smear for direct microscopy. Samples were transported 3 km daily from Olafiatlinikken to the Ullevål University Hospital, Oslo, Norway by courier for cultivation and to the Department of Microbiology and Infection Control, Tromsø, Norway by mail (1640 km) for real-time PCR testing. All samples for culture, inoculated in the transport media, were stored a maximum of 3 hours at room temperature before being sent the microbiology laboratory and inoculated on culture media upon arrival. The samples for PCR were kept at 4°C before shipping by regular mail. After arrival at the laboratory the next day, the PCR samples were stored at 4°C before analysis, usually performed twice a week.

Microscopy of Stained Genital Smears

All smears were methylene blue or Gram-stained and examined for typical intracellular, i.e., in polymorphonuclear leukocytes, diplococci in high-power field microscopy using oil immersion.

Culture

The culture diagnostics were performed at Ullevål University Hospital as part of their routine diagnostic services by identification of characteristic colonies on chocolate agar medium supplemented with inhibitory antimicrobials, i.e., 5 µg/mL trimethoprimlactate, 100 IE/mL colistin, 0.5 µg/mL lincomycin, and 1 µg/mL Amphotericin B. For thorough species confirmation, oxidase test, identification of Gram-negative diplococci, sugar oxidation tests, and Phadebact GC Monoclonal test (Bactus AB) were used.

DNA Preparation

All the UTM-RT samples were shaken for 10 seconds and the brush removed. Then 700 µL of the UTM-RT sample was put in a Tecan Miniprep-75 (Tecan) modified by Genpoint to perform DNA preparation with the BUGS’S BEADS version U kit (Genpoint). The Tecan Miniprep-75 automatically sets up the PCR reaction after DNA isolation.

Real-Time PCR

Our previously developed real-time TaqMan FAST PCR assay, which targets the porA pseudogene of N. gonorrhoeae, was utilized as previously described with 1 modification. The internal amplification control probe was modified to be as follows: Yakima yellow-CCATAGTTGCGCTACTCCCGTCG-Eclipse DarkQuencher.

Discrepancy Resolution Using porA Pseudogene and 16S rRNA Gene-Sequencing

All samples that were positive in both culturing and real-time PCR were considered as true positives. In cases of discrepant results, the samples were sent to the National Reference Laboratory for Pathogenic Neisseria, Department of Clinical Microbiology, Örebro University Hospital, Örebro, Sweden, for discrepant analysis using sequencing of the 16S rRNA gene (primers used for PCR: 5′-TGATCCARCCCGGASSTTC-3′, 5′-AGATTTGTAT-CYTGGYTYAG-3′; primers used for sequencing: the two PCR primers, and 5′-GTGCCCAAGCCGCGGTAA-3′, 5′-TTAC-CGCCGGCTGCTGCCCAC-3′, 5′-GCAAGACGCCACCCC-3′, 5′-AGGGTTCGCTCCTGTTG-3′) and the entire porA pseudogene as previously described.

Reporting Results

Results were reported continuously to Olafiatlinikken. The two microbiologic laboratories were not informed of the results of the other laboratory before the study ended.

Results

Of the 360 examined samples, 52 samples were positive for N. gonorrhoeae using the porA pseudogene real-time PCR method, and 37 were positive using culture techniques (Table 1). All 15 discrepant samples were, after the disclosure of the results, confirmed as true positive for N. gonorrhoeae by sequencing of the 16S rRNA gene and the entire porA pseudogene. The discrepant samples were from urethra (n = 1), rectum (n = 6), and pharynx (n = 8) and collected from 14 different, symptomatic patients.

Consequently, based on the results of the cultivation, the real-time PCR, and the subsequent confirmatory sequencing, 308 true negatives and 52 true positive samples from 39 men and 3 women, were identified. The sensitivity was 100% for the PCR method and 71% for culture, whereas the specificity was 100% for both methods. The positive predictive value and negative predictive value were both 100% for the PCR method, but for culture the results where 100% and 93%, respectively.

Inhibition of the PCR reactions was observed in 35 samples from urethra (n = 13), rectum (n = 6), pharynx (n = 12), and cervix (n = 4). DNA was consequently extracted once more and the samples are analyzed both undiluted and after twofold dilution. By using this procedure, all the samples displayed negative results for N. gonorrhoeae and positive results for the internal amplification control.
and consequently can act as reservoirs of infection. Kent et al.34 found that nearly 85% of rectal N. gonorrhoeae infections among men who have sex with men were found only at extragenital sites and countries in which an effective culturing is not possible seems very promising and can be highly valuable in geographic and temporal analyses in these locations can often be asymptomatic, harder to treat, and consequently can act as reservoirs of infection. Kent et al.34 found that nearly 85% of rectal N. gonorrhoeae infections among men who have sex with men were found only at extragenital sites. In addition, the inoculum in urethra tends to be greater than in the rectum and pharynx, making the latter samples easier to culture. Of course, “bed-side” culturing may have been able to improve the sensitivity of culturing. However, in the current study, we wanted to comprehensively compare our PCR method to the present routine diagnostics.

The use of extragenital sites, such as pharynx and rectum, for diagnosing N. gonorrhoeae is crucial, particularly because infections in these locations can often be asymptomatic, harder to treat and consequently can act as reservoirs of infection. Kent et al.34 found that nearly 85% of rectal N. gonorrhoeae and C. trachomatis infections were asymptomatic and that 64% of gonococcal infections among men who have sex with men were found only at nonurethral sites. In addition, it has been suggested that rectal gonorrhea is an independent risk factor for human immunodeficiency virus transmission.35

All the discrepant results between PCR and culture were resolved by sequencing. Since the porA pseudogene was the target in our PCR, we used a second genetic target for sequencing, i.e., the 16S rRNA gene as recommended for Australian public health laboratories.36 However, based on this and earlier studies,26–29,37 verification of PCR positive samples may also be done with the reported method. Of course, the DNA extraction from the primary clinical sample has to be repeated and this material used as template in the verification. Using the same assay for verification, however, requires utilization of effective internal control systems and participation in at least one, preferably several comprehensive, external quality programs. The samples included in such programs should reflect not only currently transmitted strains, but also temporally, geographically and genetically diverse strains. In addition, as for all genetic diagnostic methods, a regular validation of the method besides participation in internal and external quality control program is exceedingly valuable. Accordingly, at our diagnostic laboratory it is mandatory that all our assays should be rescanned every year against all published sequences available in databases.

To determine the antimicrobial susceptibility of N. gonorrhoeae, culture remains essential for diagnosis. However, as shown in the current study, our porA pseudogene real-time PCR can help reduce false negative diagnoses, which are mainly due to the viability limitations for N. gonorrhoeae.38 This is especially important for rural areas with suboptimal transportation, including medium, conditions, and rapidness or culture. In the current study, the laboratory used for culture diagnostics was situated only 3 km away from the venereological polyclinic. However, in Norway as well as many other countries, most clinics or general practitioners have considerable longer transportation times.

The identified PCR inhibition of 35 samples was most likely due to residual lysis-buffer in the DNA eluate because of insufficient removal by the pipeting robot. This was a temporary problem that was immediately discovered due to the very sensitive internal amplification control. The problem was addressed by readjusting the Tecan Miniprep-75 and receiving new batch of reagents from the manufacturer. Effective inhibition controls are necessary in order to eliminate false negative results which is in full accordance with recommendations proposed.36

In conclusion, the previously performed thorough evaluation of the performance characteristics of the present fast real-time PCR assay28 in combination with the hereby reported clinical validation warrants the use of the present PCR assay as a supplement for diagnosing gonorrhea. Furthermore, this method makes it possible to also screen extragenital sites when indicated by sexual anamnesis. The results of the current study are also in full concordance with previous studies using analogous porA pseudogene PCR.30 However, introduction of real-time PCR detection of N. gonorrhoeae for diagnostic purposes also poses a question of when to most effectively sample for test of cure, which is presently under investigation.

**Discussion**

For the fastidious N. gonorrhoeae, optimal sampling, transport medium, transport time, and transport conditions are essential for performing culture diagnostics with high sensitivity.32 This study demonstrates that real-time PCR, targeting the porA pseudogene can be a fast, highly sensitive, specific, automated, and valuable supplement to culture, in order to reduce false negative N. gonorrhoeae results. However, the study was performed on patients attending only 1 clinic in Norway, and the number of examined patients were limited. Consequently, further evaluation and validation in different populations as well as over longer time periods is necessary and also in progress. However, the present method seems very promising and can be highly valuable in geographic areas and countries in which an effective culturing is not possible to perform, even for primary diagnostics of N. gonorrhoeae. For pharyngeal and rectal samples, in comparison with culture, the sensitivity of the PCR was significantly higher. The majority of the discrepant samples were from rectum or pharynx. Furthermore, 9 of the corresponding 14 patients displayed negative culture results for all specimens. Overgrowth of other microorganisms may partly explain the observed discrepancy.33 In addition, the inoculum in urethra tends to be greater than in the rectum and pharynx, making the latter samples easier to culture. Of course, “bed-side” culturing may have been able to improve the sensitivity of culturing. However, in the current study, we wanted to comprehensively compare our PCR method to the present routine diagnostics.

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**References**


