A Fast Real-Time Polymerase Chain Reaction Method for Sensitive and Specific Detection of the *Neisseria gonorrhoeae* porA Pseudogene

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Ever since the advent of molecular methods, the diagnostics of *Neisseria gonorrhoeae* has been troubled by false negative and false positive results compared with culture. Commensal *Neisseria* species and *Neisseria meningitidis* are closely related to *N. gonorrhoeae* and may cross-react when using molecular tests comprising too-low specificity. We have devised a real-time polymerase chain reaction (PCR), including an internal amplification control, that targets the *N. gonorrhoeae* porA pseudogene. DNA was automatically isolated on a BioRobot M48. Our subsequent PCR method amplified all of the different *N. gonorrhoeae* international reference strains (*n = 34*) and *N. gonorrhoeae* clinical isolates (*n = 176*) but not isolates of the 13 different nongonococcal *Neisseria* species (*n = 68*) that we tested. Furthermore, a panel of gram-negative bacterial (*n = 18*), gram-positive bacterial (*n = 23*), fungal (*n = 1*), and viral (*n = 4*) as well as human DNA did not amplify. The limit of detection was determined to be less than 7.5 genome equivalents/ PCR reaction. In conclusion, the *N. gonorrhoeae* porA pseudogene real-time PCR developed in the present study is highly sensitive, specific, robust, rapid and reproducible, making it suitable for diagnosis of *N. gonorrhoeae* infection. (*J Mol Diagn 2006, 8:574–581; DOI: 10.2353/jmoldx.2006.060024*)

Neisseria gonorrhoeae is a difficult organism to diagnose by culture, mainly due to its low viability in transportation media.[1] This means a significant risk of false negative samples. The sensitivity of culturing may be quite low even under optimal conditions.[2,3] *N. gonorrhoeae* detection based on techniques that are independent of bacterial viability would improve gonorrhea diagnostics radically.

N. gonorrhoeae belongs to the *Neisseria* genus of bacteria and is the causative agent of gonorrhea,[4] which is a major sexually transmitted infection in many countries.[5] The *Neisseria* genus comprises many closely related species,[4] of which only *N. meningitidis* and *N. gonorrhoeae* are primarily pathogenic to humans. Sites of infection for *N. gonorrhoeae* are mainly urogenital, anorectal, and oropharyngeal mucous membranes. Many of the other *Neisseria* species are commensal to humans and can commonly be found on mainly extra genital mucous membranes. *Neisseria* species are also found on several animal hosts.[4] *Neisseria* genomes are highly homologous,[6–8] and interspecies genetic exchange has been described. This could be facilitated by a system secreting chromosomal DNA found in *N. gonorrhoeae*[9] and specific uptake systems for extracellular DNA such as the one described for *N. meningitidis*.[10]

The gold standard for diagnosing *N. gonorrhoeae* remains culture; however, the very fastidious *N. gonorrhoeae* does not survive very long outside the host. This emphasizes the importance of optimized sample collection, transportation, and storage of the specimens as well as adequate culture methods. Consequently, an increasing number of nucleic acid amplification tests (NAATs) have been developed for diagnosis of *N. gonorrhoeae*. Commercially available NAATs have been important supplements to the laboratory diagnosis of *N. gonorrhoeae*, largely due to the increased sensitivity compared with culture techniques and the possibility for using noninvasive samples such as urine. However, due to the interspecies homology, concerns have frequently been raised about the specificity of many published NAATs, both commercial and in-house methods.[11–22] When using NAATs for diagnosis of *N. gonorrhoeae*, a very high specificity is essential, especially for extragenital specimens.[19] For example, the pharynx commonly harbors

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commensal *Neisseria* species and/or *Neisseria meningitidis* and hence is not suitable as a collection site unless a highly specific test is used.[19] Some commercial NAATs have even been withdrawn from the market due to cross-reactivity with commensal *Neisseria* spp. and/or an insufficient analytical sensitivity.[23] Due to the social impact of false positive test results, retesting of all positives with a second NAAT targeting another gene is recommended.[24] This is an unacceptable solution for most laboratories as it is laborious, expensive, and time-consuming. Therefore, a highly specific NAAT that can be used for both routine analysis as well as confirmative retesting of positive samples is highly needed.

The choice of target gene is the main key to a successful *N. gonorrhoeae* NAAT. Previous methods have used targets such as the cryptic plasmid (*cppB* gene),[25–27] *opa* genes,[11] *orf*1 gene,[28] cytosine DNA methyltransferase gene,[18] and *16S rRNA* gene.[29,30] However, the *cppB* gene is missing in many *N. gonorrhoeae* strains,[20,29] and most of the other targets have been shown to cross-react with the equivalent genes in commensal *Neisseria* species. A novel in-house PCR reported by Whiley et al[31,32] that targets the *porA* pseudogene of *N. gonorrhoeae* has so far proved to have satisfactory specificity. Extensive sequencing of the *porA* pseudogene[33,34] has supported this gene as a suitable target for *N. gonorrhoeae* PCR. The *porA* gene/pseudogene is absent in commensal *Neisseria* species,[33,35,36] and the *porA* gene of *N. meningitidis* is sufficiently divergent to be discriminatory between the two human pathogenic *Neisseria*.[34] Thus, this pseudogene is a nonexpressed equivalent of the *N. meningitidis porA* gene.[33,34] There are a few inactivating mutations that make the *N. gonorrhoeae porA* a pseudogene.[33,34] The pseudogene seems very stable, and it is not subjected to any positive selective pressure; consequently, the pseudogene has stayed virtually unchanged over time.[34]

Real-time PCR have made PCR more user-friendly and reduced hands-on time, time for receiving results, and level of contamination. There are several real-time PCR systems on the market that ramp temperature up to 50% faster than a conventional PCR machine. This allows the user to run up to 96 or 384 samples (40 cycles) in 35 or 55 minutes, respectively, providing a 20-μl reaction mix. These protocols are typically referred to as rapid cycle PCR or fast cycle PCR, but we call it fast real-time PCR in the present article.

In the present study, we designed and evaluated a real-time PCR for specific detection of *N. gonorrhoeae* that can supplement our culture method and/or be used as single diagnostic method in other geographic areas, in which adequate culturing is not possible to perform. The main priority was optimal specificity and sensitivity. We collected international *N. gonorrhoeae* reference strains and clinical isolates to verify the sensitivity of the method. As many isolates and international reference strains as possible of commensal *Neisseria* and *N. meningitidis*, along with other microorganisms often found in the genital area, were gathered to ensure maximum specificity. Another important criterion was that the method should be possible to automate, and an appropriate sample collection and an optimal transportation buffer should be found.

Materials and Methods

Bacterial Isolates, Clinical Samples, and Culture Conditions

International *Neisseria* reference strains were collected from American Type Culture Collection (ATCC), Culture Collection University of Gothenburg, National Collection of Type Cultures, World Health Organization (World Health Organization), Swedish Reference Laboratory for Pathogenic *Neisseria*, and Statens Serum Institut, Denmark. These reference strains included *N. gonorrhoeae* (*n* = 34), *N. meningitidis* (*n* = 4), *Neisseria sicca* (*n* = 2), *Neisseria subflava* (*n* = 1), *Neisseria flavescens* (*n* = 2), *Neisseria mucosa* (*n* = 2), *Neisseria lactamica* (*n* = 2), and *Neisseria cinerea* (*n* = 1). The *N. gonorrhoeae* reference strains originated from different geographic locals worldwide and have been isolated during the last four decades. Furthermore, in 29 of the 34 *N. gonorrhoeae* reference strains, the entire *porA* pseudogene has previously been sequenced.[34]

In addition to international reference strains, we examined 176 clinical *N. gonorrhoeae* isolates. These included 76 isolates cultured in Archangelsk, Russia, in 2004. These isolates were initially cultured on nonselective media (NPO Microgen, Stavropol, Russia), but species were subsequently confirmed as *N. gonorrhoeae* by colony characteristics on selective media,[37] rapid positive oxidase test, presence of gram-negative diplococci in microscopy, sugar oxidation test,[38] and specific monoclonal antibodies (Phadebact GC Monoclonal test; Boule Diagnostics AB, Huddinge, Sweden). Fourteen isolates were cultured at University Hospital of North Norway, 2003 to 2004, and nine isolates were received from Norwegian Organization for Surveillance of Antibiotic Resistant Microorganisms; all these were identified as *N. gonorrhoeae* by rapid positive oxidase test, the presence of gram-negative diplococci, sugar oxidation test,[38] and specific monoclonal antibodies (Phadebact GC Monoclonal test). Thirteen isolates from Statens Serum Institut in Denmark were species identified by culturing on selective medium and MINIBACT-N.[39] In addition, five confirmed *N. gonorrhoeae* isolates donated by Helen Palmer[19] and 51 genetically distinct, Swedish-confirmed *N. gonorrhoeae* isolates from 1998 to 2001 with known *porA* pseudogene sequence[34] were included.

Furthermore, clinical isolates (*n* = 54) of other *Neisseria* species were included. These comprised *N. gonorrhoeae* subspecies *kochii* (*n* = 4), *N. meningitidis* (*n* = 7), *N. sicca* (*n* = 7), *N. subflava* (*n* = 11), *N. flavescens* (*n* = 3), *N. mucosa* (*n* = 5), *N. lactamica* (*n* = 7), *N. cinerea* (*n* = 7), *Neisseria caviae* (*n* = 1), *Neisseria animalis* (*n* = 1), and *Neisseria polysaccharea* (*n* = 1). The commensal *Neisseria* were identified by sugar oxidation,[38] oxidase testing, and, if needed, API NH or RapID NH (bioMerieux, La Balme-les-Grottes, France).

To exclude cross-reaction with genomes from other bacterial species, ie, non-*Neisseria* species, we tested DNA from a panel of gram-negative bacterial (*n* = 18), gram-positive bacterial (*n* = 23), fungal (*n* = 1), and viral (*n* = 4) as well as human DNA.

All *Neisseria* spp. were cultured from frozen stocks ($-70°C$) for 24 to 48 hours at 37°C in 5% CO_2 on nonselective chocolate agar[40] containing GC-Agar Base Medium CM367B (Oxoid, Basingstoke, UK), distilled water, 5% defibrinated horse blood, 25% glucose solution, and Vitox solution (Oxoid SR090H and SR090A).

Isolation of Bacterial DNA

Genomic DNA from the bacteria was isolated with a BioRobot M48 from Qiagen (Hilden, Germany) using the MagAttract DNA tissue kit with the Infectious disease protocol and an elution volume set to 100 μl, according to the manufacturer's specifications. All bacteria and fungi were initially suspended in 200 μl of TE buffer, pH 8 (Ambion, Austin, TX), to approximately 1.5×10^8 colony-forming units (CFU)/ml before isolation of DNA. All viruses were suspended in a virus in-house transport medium made from minimum essential medium (Gibco, Carlsbad, CA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (Gibco), and Gentamicin (Gibco).

Primer Design and PCR Conditions

Primers and probes against the *N. gonorrhoeae porA* pseudogene were designed as a TaqMan assay by using Primer Xpress 2.0 (Applied Biosystems, Foster City, CA) according to manufacturer's guidelines. The forward and reverse primer sequences for the *N. gonorrhoeae porA* pseudogene were 5′-CCGGAACTGGTTTCATCTGATT-3′ and 5′-GTTTCAGCGGCAGCATTCA-3′, respectively. The sequence for the TaqMan probe for the *porA* pseudogene was 5′-FAM-CGTGAAAGTAGCAGGCGTATAGGCGGACTT-BHQ-1-3′. The primer sequences, probe sequence, and the amplicon were compared with the *porA* pseudogene sequences from a previous study[34] as well as all of the genetic sequences deposited in GenBank.

For the *N. gonorrhoeae*-specific PCR, we used TaqMan Fast Universal PCR Master Mix (×2), No AmpErase UNG on a 7900 HT Fast Real-Time PCR System (Applied Biosystems). The Fast-PCR was run with 20-second activation of the polymerase at 95°C, followed by 50 cycles of 95°C for 1 second, and 60°C for 20 seconds. We used a 11.5-μl template added to 13.5 μl of reaction solution for each PCR sample. We also tested the robustness of the method by varying the sample volume in the PCR between 9 and 15 μl while keeping the reaction solution constant at 13.5 μl. The primers amplify a 102-bp fragment of the *porA* pseudogene. The *porA* primers and probe were optimized as recommended by Applied Biosystems, and a final concentration of 900 nmol/L of each primer and 200 nmol/L probe was found to be optimal.

A serial dilution of commercially available quantitated DNA from Tebu-bio (Le Perray en Yvelines, France) was used to generate a standard curve for efficiency (*E*) calculation as well as deciding the limit of detection. Efficiency was calculated using the formula: $E = 10^{-1/slope} - 1$.[41] To estimate the efficacy also of the transportation buffer and DNA preparation method used in the present study, a suspension was made of *N. gonorrhoeae* reference strain ATCC 19424 in phosphate-buffered saline. This was then diluted 10^{-1} through 10^{-7}, and 100 μl of each dilution was plated on a modified Thayer-Martin medium[40] and incubated overnight at 37°C in the presence of 5% CO_2. Another 100 μl of each dilution 10^{-1} through 10^{-5} was transferred to 3 ml of UTM-RT sample transportation buffer (Copan, Brescia, Italy) and stored overnight at 4°C. The following day, 200 μl of each UTM-RT dilution was used to prepare DNA on the BioRobot M48, as previously described.
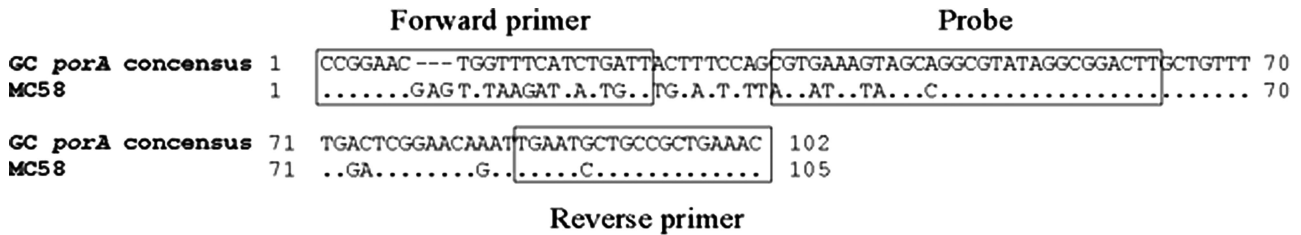
Internal Amplification Control

For inhibition control, an internal amplification control (IAC) was constructed by using composite primers that have *N. gonorrhoeae* specific 5′-end and a pGEM-luc plasmid (Promega, Madison, WI) specific 3′-end, which amplifies a 181-bp fragment of the β-lactamase coding region (positions 2988 to 3169). The forward and reverse primer sequences for making the IAC were 5′-GTTTCA-GCGGCAGCATTCATGGTCTGACAGTTACCAATGCTT-AA-3′ and 5′-CCGGAACTGGTTTCATCTGATTGGCTGG-CTGGTTTATTGCTG-3′, respectively. The sequence for the TaqMan probe for the *porA* pseudogene was 5′-FA-M-CGTGAAAGTAGCAGGCGTATAGGCGGACTT-BHQ-1-3′. This makes a DNA amplicon that is 255 bp long with ends that is recognized by the forward and reverse *porA* primers. Five μl of a 10^{-4} dilution of the pGEM-luc plasmid was used as a template in a total volume of 50-μl reaction solution with no UNG. Fifty nM of each of the composite primers were used. First, the reaction mixture was heated at 95°C for 10 minutes, then 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute followed.

Results

All *N. gonorrhoeae* international reference strains ($n = 34$) and clinical *N. gonorrhoeae* isolates ($n = 176$) examined in the present study were positive in our developed in-house *porA* pseudogene real-time PCR. In addition, all international reference strains and clinical isolates of other *Neisseria* species than *N. gonorrhoeae* were negative. Furthermore, neither of the 46 non-*Neisseria* bacterial species nor the nonbacterial agents tested positive. Every test we performed to optimize the method, determine specificity, etc, or analyze samples was tested twice in parallel. In addition, the PCRs of representative strains/isolates were repeated to analyze the reproducibility and all of the initial results were confirmed.

An alignment of the primers and probe on a partial consensus sequence based on recent sequencing[34] of *N. gonorrhoeae porA* pseudogenes and the corresponding region of the *N. meningitidis* MC58 gene[42] shows that the primer and probe regions of the *N. gonorrhoeae porA* pseudogene (Figure 1) is 100% conserved. Furthermore, the figure shows that the *N. gonorrhoeae porA* pseudogene is sufficiently different from the *N. meningitidis porA* gene to effectively discriminate between the two pathogenic *Neisseria*.

Forward primer   **Probe**

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GC porA concensus  1  CCGGAAC --- TGGTTTCATCTGATTACTTTCCAGCGTGAAAGTAGCAGGCGTATAGGCGGACTTGCTGTTT  70
MC58               1  .......GAGT.TAAGAT.A.TG..TG.A.T.TTA..AT..TA...C..................... 70

GC porA concensus 71  TGACTCGGAACAAATTGAATGCTGCCGCTGAAAC  102
MC58              71  ..GA........G..[.....C............] 105
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Reverse primer

Figure 1. Nucleotide sequence alignment of partial *N. gonorrhoeae* consensus *porA* pseudogene compiled from 87 sequences[34] and the corresponding *N. meningitidis* sequence. The boxed sequences on the flanks are the forward and reverse primer location, respectively. The boxed sequence in the middle is the location of the probe. The MC58 sequence is the partial *porA* sequence of the previously published whole-genome sequenced *N. meningitidis* strain MC58.[42]

As previously mentioned, optimal concentration of each primer was shown to be 900 nmol/L. The probe concentration was optimized to 200 nmol/L of the *porA* probe, and 100 nmol/L of the IAC probe. The IAC was optimized to give a cycle threshold (Ct) of 37 to 39 in known negative samples. With this master mix, we see a PCR efficiency of 99% as derived from the dilution series in Figure 2 by making a standard curve (Figure 3) and using the slope in the equation $E = 10^{-1/\text{slope}} - 1$. The DNA isolation procedure used in the present study can be performed in approximately 2.5 hours, whereas the present PCR takes about 40 minutes. Consequently, for 44 clinical samples (DNA isolation of two batches of samples) the total assay time is only 4 hours and 10 minutes (96 PCR reactions including controls).

The IAC was only amplified in very low concentrations of competing *N. gonorrhoeae* DNA or in the total absence of *N. gonorrhoeae* DNA (Figure 4). A twice-repeated dilution series of *N. gonorrhoeae* DNA using master mixes with and without IAC showed that there was no significant competitive inhibition of the target PCR (Figure 5). The

amplification efficacy was shown to be 99.3 and 94.9% for the master mix with and without IAC, respectively. The master mix with IAC had the greatest amplification efficacy. In Figure 5, the two amplification plots overlay each other with very little difference, yet there is an insignificant difference in efficacy of 4.4%.

One *N. gonorrhoeae* reference strain (ATCC 19424) was aliquoted and stored at −20°C and thawed for DNA purification and PCR. At the same time a commercially purified DNA sample (Tebu-bio) was aliquoted and stored at −20°C. These two samples were run 7 different days over a 2-week period with an average Ct value for the ATCC 19424 strain of 33.98 and a SD of 0.8. For the commercially purified DNA, the Ct was 36 on average with a SD of 0.4.

The limit of detection for the commercially isolated and quantified *N. gonorrhoeae* DNA was determined to 7.5 Geq/PCR reaction in 5:5 parallel reactions. In 3:5 parallels, we detected 1.5 Geq/PCR reaction. When evaluating a dilution series of *N. gonorrhoeae* spiked UTM-RT medium, we found the colony-forming units/ml
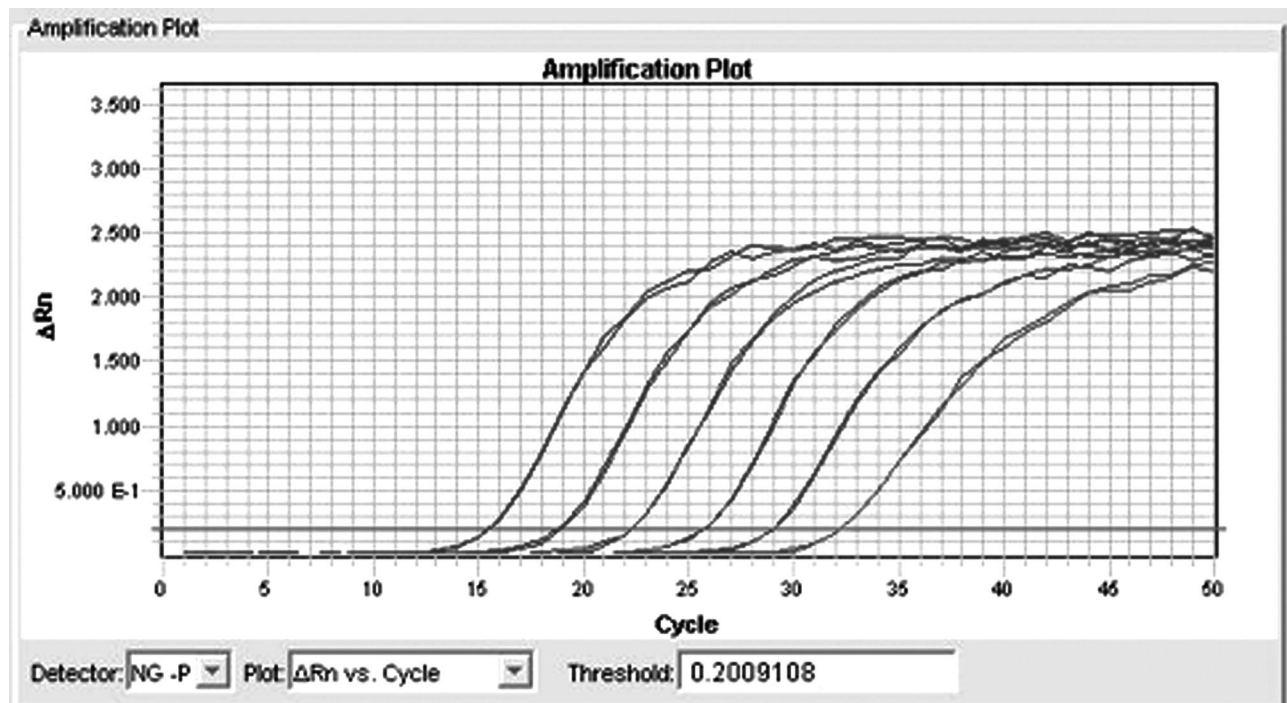

Figure 2. Dilution series of commercially purified *N. gonorrhoeae* DNA (Tebu-bio) and detection in the *N. gonorrhoeae porA* pseudogene real-time PCR developed in the present study. The six 10-fold dilutions showed good linearity.
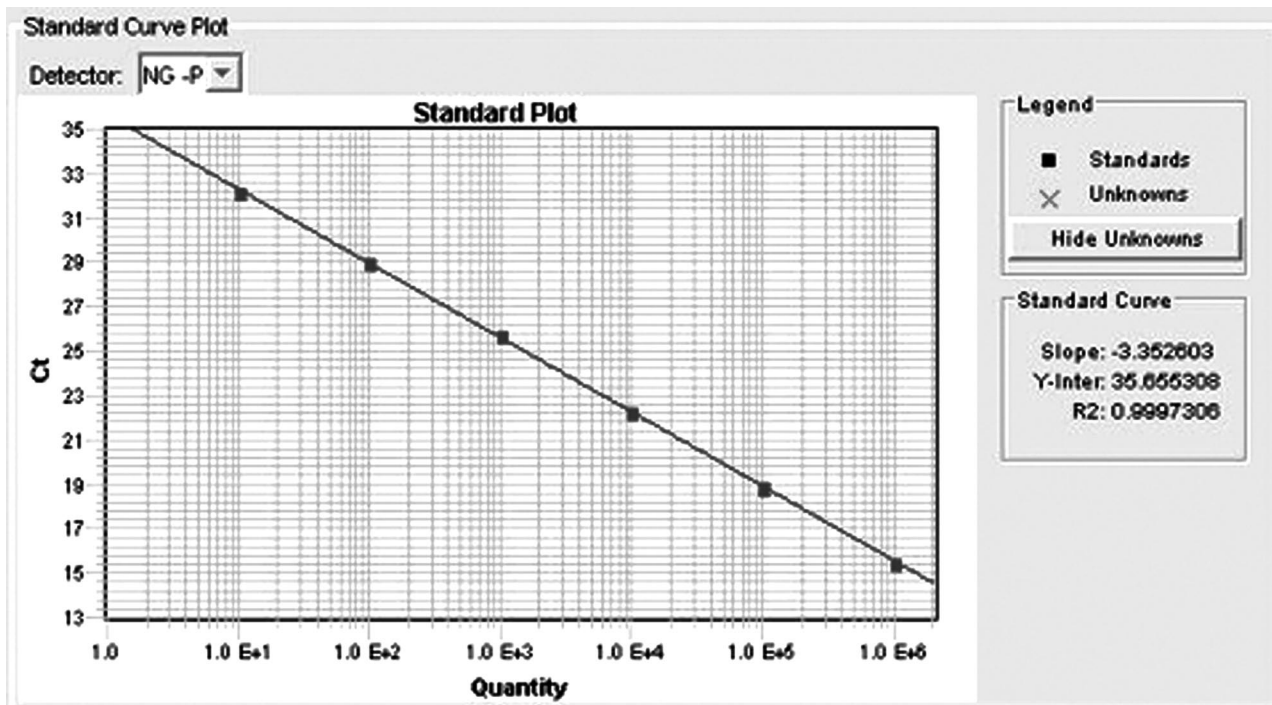
Figure 3. Standard curve based on the dilution series in Figure 2. The slope is the basis for the amplification efficacy (*E*) calculation. In a 100% efficient PCR, the DNA will double per cycle or use 3.333 cycles to increase DNA copies by 10-fold ($2^{3.33} \approx 10$).

UTM-RT to be 80 in one experiment and 53 the next experiment, which in average translates to 0.13 colony-forming units/μl DNA eluate, given a 100% recovery in the DNA isolation. This was based on one experiment where the dilutions were plated in duplicates, and the PCR was run in parallels.

Discussion

Our results show that the real-time *porA* pseudogene PCR, developed in the present study, was sensitive and specific enough to detect all of the 210 highly different *N. gonorrhoeae* international reference strains and clinical
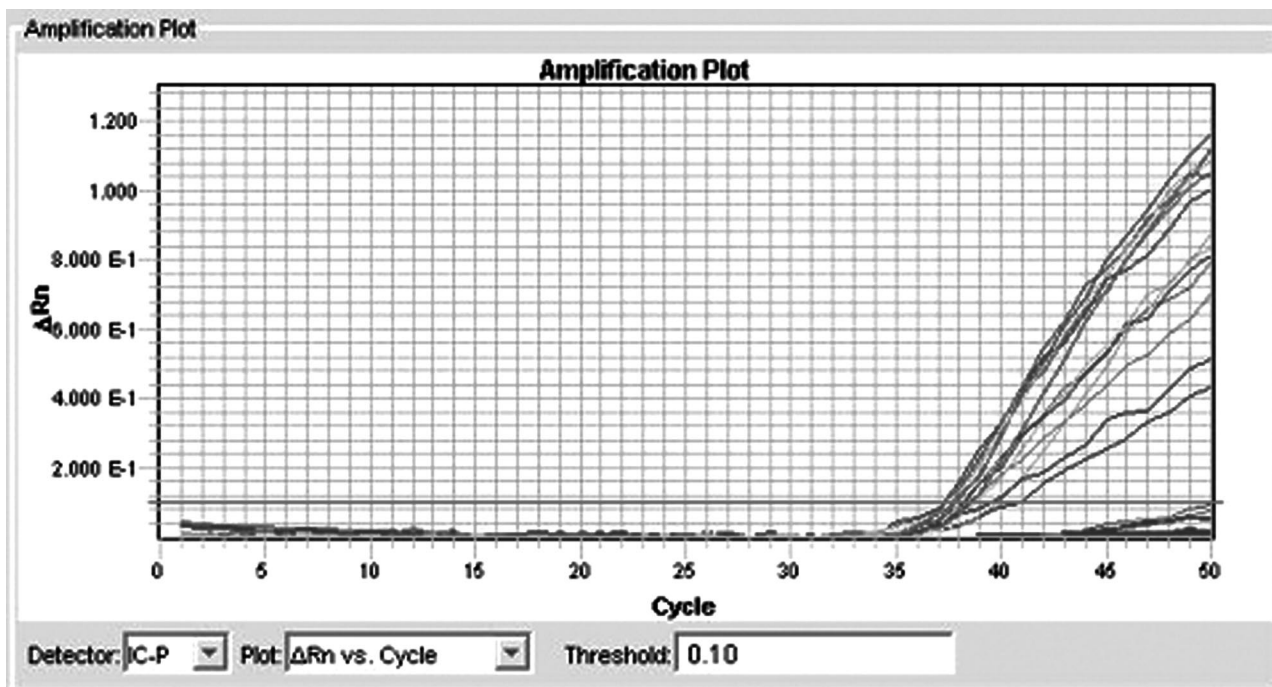

Figure 4. IAC amplification with low-level *N. gonorrhoeae*. The figure shows the internal amplification control amplified in dilutions of increasing concentrations of *N. gonorrhoeae* DNA. The amplification lines that do not cross the thick line are negative due to larger amounts of *N. gonorrhoeae* DNA.
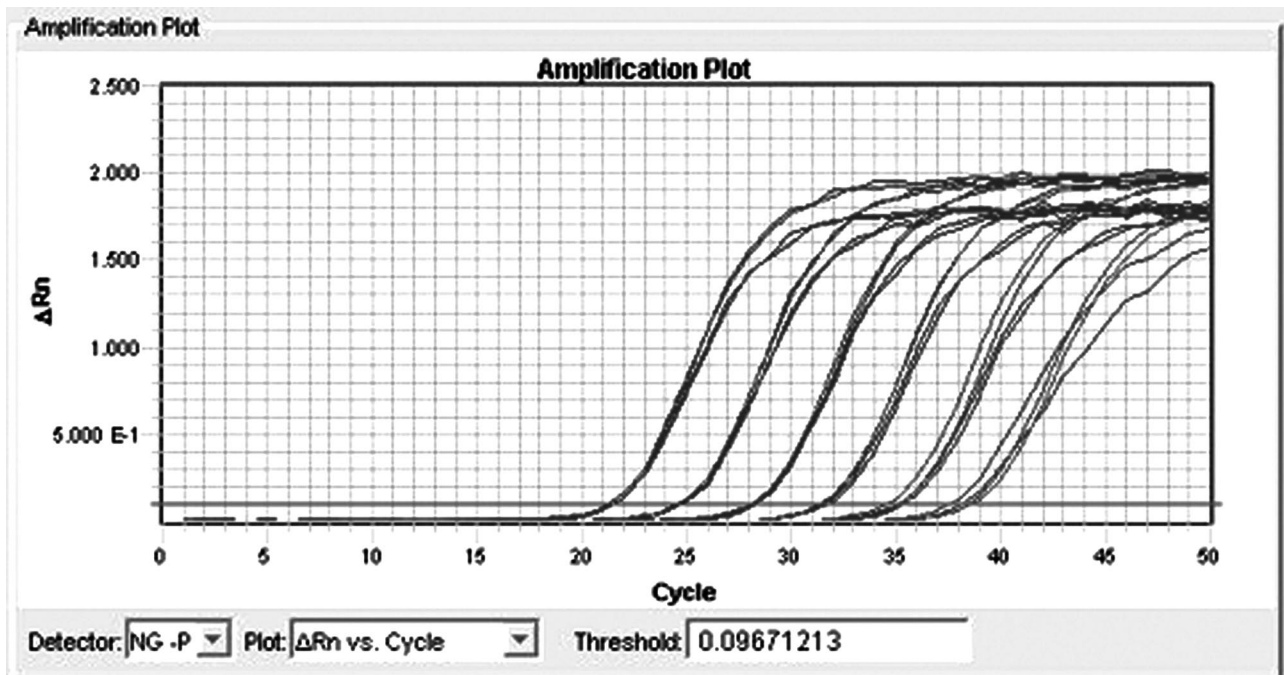
Figure 5. Dilution series of *N. gonorrhoeae* DNA using two parallels from master mixes with and without IAC. There was no significant difference in the amplification of *N. gonorrhoeae* DNA between the two master mixes as all four parallels are approximately equal. The mastermix without IAC shows a slightly higher ΔRn than the master mix with IAC.

isolates examined in the present study, ie, had a sensitivity of 100%. In addition, the detection limit was low (7.5 Geq/PCR reaction).

Regarding the specificity of the method, in Figure 1 a partial alignment of *N. gonorrhoeae porA* consensus pseudogene (composite of 87 previously sequenced *N. gonorrhoeae* isolates[34]) and the corresponding region of the *N. meningitidis porA* gene show that the primers and probe are located in a highly conserved area of the *porA* pseudogene and that the regions are sufficiently different from the *N. meningitidis porA* gene to discriminate the two bacteria. Furthermore, no international reference strain or clinical isolate of any other *Neisseria* species was amplified. In the heterogeneous panel of other infectious agents, we included all microorganisms that we routinely analyze with PCR. In addition, we included pathogens and commensal nonpathogens available in our collection of isolates. The choice of commensals aimed to cover a representative selection of microorganisms that can be derived from all main test sites used for gonorrhea sampling; ie, cervix, urethra, oropharynx, and anorectum. None of these non-*Neisseria* agents were amplified by the *porA* pseudogene PCR. Consequently, the assay comprised 100% specificity.

Several commercial suppliers have developed NAATs for *N. gonorrhoeae*, but many of these as well as the developed in-house methods suffer from too-low specificity.[19,36,43,44] This problem has been due to limited sequencing of equivalent genes in commensal *Neisseria* species and the overall great homology between most *Neisseria* species. Recent work on sequencing of the entire *porA* pseudogene in *N.* gonorrhoeae[34] has facilitated the discovery of this gene as a promising target for

highly sensitive and specific molecular diagnostics of *N. gonorrhoeae*.

The *porA* gene exists as a highly conserved, nonexpressed pseudogene in *N. gonorrhoeae* and is therefore not subjected to a high evolutionary selective pressure for changes as is the case for the expressed *N. meningitidis porA* gene.[34] This is an advantage, as it might limit the risk of false negatives because of mutations in the gene in the future. On the other hand, bacteria are not believed to retain unnecessary genes, and this may predict a loss of the *porA* pseudogene from the *N. gonorrhoeae* genome in an evolutionary time perspective.[45] However, the *porA* pseudogene seems to have been retained since *N. gonorrhoeae* and *N. meningitidis* diverged into two different species from their common ancestor.[33,46] In the present study, we tested isolates cultured during a time period spanning from the 1960s to the present date, which at least demonstrates a certain degree of stability of the *porA* pseudogene. There might even be an unidentified reason for keeping this gene.

Previously, we have tried numerous other genes as possible targets for a *N. gonorrhoeae*-specific PCR, such as *traG*,[47] *traH*,[47] *dcmG*,[48] carbonic anhydrase,[49] and pJD1.[26] However, all these PCRs have either amplified commensal *Neisseria* spp. genes or failed to identify some *N. gonorrhoeae* isolates. The results of the present study and the increasing data regarding the characteristics of the *porA* pseudogene,[33,34] strongly support that so far the *porA* pseudogene is the most suitable target for *N. gonorrhoeae* diagnostic NAATs.

One major advantage with the real-time diagnostic assay developed in the present study is that the TaqMan chemistry can be used on most existing real-time PCR

platforms, making it more easily accessible than FRET (free resonance energy transfer)-probe chemistry, which was used by Whiley et al.[27,31,32,36] Furthermore, because there is a significant difference (140 bp) in the size of the *N. gonorrhoeae porA* pseudogene amplicon and the IAC, the different amplicons are easily distinguished by melting-point analysis using, for example, SYBR Green I fluorescence in non-probe-based real-time PCR or on agarose gel electrophoresis after conventional PCR (data not shown). A PCR with our primers used without the probe was able to discriminate against *N. meningitidis* and commensal *Neisseria* (data not shown). Consequently, the PCR developed in the present study can readily be adapted and optimized to be used also in resource-poor settings, in which probe-based real-time PCR systems are not accessible or afforded.

In conclusion, the *N. gonorrhoeae porA* pseudogene real-time PCR developed in the present study is highly sensitive, specific, robust, rapid, and reproducible, making it suitable for diagnosis of *N. gonorrhoeae*. Comparison against culturing on samples taken from several different collection sites is necessary for further evaluation. We are currently doing this comparison in collaboration with external laboratories.

Acknowledgments

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