Papers

Evaluation of an in-house polymerase chain reaction for detection of *Neisseria gonorrhoeae* in urogenital samples

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Abstract

***Aim*—To develop and evaluate a one day in-house polymerase chain reaction (PCR) assay for the detection of *Neisseria gonorrhoeae* DNA in urogenital samples.**

***Methods*—429 urogenital specimens were tested for the presence of *N gonorrhoeae* by in-house PCR and by Gen-Probe. The PCR assay amplifies target sequences within the *N gonorrhoeae* cppB gene on the 4.2 kb cryptic plasmid, after which amplicons are detected by a streptavidin-biotin based enzyme immunoassay using an internal probe. Discordant specimens were further evaluated by repeating the PCR and the Gen-Probe assay, and by an additional PCR using another set of 16S primers followed by radioactive detection of amplicons on a Southern blot.**

***Results*—Of the 429 samples tested, 15 were found positive by in-house PCR, eight of which were confirmed by Gen-Probe. Of the seven discrepant samples, five were confirmed by 16S PCR and are also considered true positive. The remaining two samples were positive in the in-house PCR only, and are considered false positive. After resolution of discrepant samples, the sensitivities of the *N gonorrhoeae* assays were 100% and 61.5% for the in-house PCR and Gen-Probe, respectively, while specificities were comparable at 99.5% and 100%.**

***Conclusions*—The in-house PCR for the detection of *N gonorrhoeae* DNA is at least comparable to Gen-Probe in performance. An extended evaluation period should elucidate if the additional five GO-PCR positive specimens, confirmed by 16S PCR, are caused by persistence of DNA or whether the method is indeed more sensitive.**

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Signs and symptoms of gonococcal infections in women are non-specific, and a substantial proportion of women with proven infection are asymptomatic.[1] Nonetheless, complications and sequelae of gonococcal infections may be severe, including pelvic inflammatory disease, infertility, and ectopic pregnancy.[2] Early treatment may prevent complications; therefore rapid and reliable diagnostic tests are essential.

The diagnosis of gonorrhoea by culture using a selective medium is believed to reach a sensitivity of 80% to 95%, with false negative results attributed to poor specimen storage, transport problems, and inhibition of growth by components of selective media.[3] Various non-culture diagnostic test alternatives such as direct immunofluorescence assay,[4] enzyme immunoassay,[5] nucleic acid hybridisation,[6-8] and amplification[9] have been developed and these tests have been reported to reach a sensitivity of 72% to 100% as compared with culture.

For the detection of *C trachomatis* we routinely perform an in-house polymerase chain reaction test (CT-PCR).[10] Since *C trachomatis* is the most prevalent organism found in sexually transmitted disease[11] and can be isolated from the cervix of up to 60% of women with gonorrhoea,[12] we developed a PCR for the detection of *N gonorrhoeae* (GO-PCR) which can be performed in parallel with the CT-PCR. The GO-PCR was compared with the Gen-Probe PACE 2 system (Gen-Probe, San Diego, USA), which is a nucleic acid hybridisation test. Currently, this assay is routinely performed in our laboratory for the detection of *N gonorrhoeae*. Discordant specimens were evaluated by an additional PCR using a target in the 16S region of the *N gonorrhoeae* genome with detection of amplicons on a Southern blot using a ^{32}P labelled internal probe.

Methods

STUDY POPULATION

The 429 specimens tested were obtained from 358 female and 71 male patients visiting obstetrics/gynaecology and dermatology clinics of five hospitals and the Rutgers foundation, all located in the neighbourhood of Eindhoven, The Netherlands. Patients were seen between September 1995 and March 1996.

SPECIMEN COLLECTION

From each patient a cervical or urethral sample was collected using a Dacron swab which was

placed in Gen-Probe transport medium. All hospitals were visited three times a day by our transport division to collect patient samples. On arrival in the laboratory, two drops of Fast Express were added (to decrease viscosity). Samples were incubated at 56°C for 10 minutes, split in equal portions, and stored at 4°C until analysis. Both assays were performed using aliquots from the same portion and treated as described.

IN-HOUSE PCR

A 100 µl aliquot of the sample was used for DNA extraction as described by Boom *et al.*[13] All primers used were HPLC purified and obtained from Isogen. Primers HO1 (GCTACGCATACCCGCGTTGC) and HO2 (biotin-CGAAGACCTTCGAGCAG-ACA) were derived from the cppB gene on the 4.2 kb cryptic plasmid.[3] The internal probe Roy1 (digoxigenin-AATACTGCCT-TGCTCATGCGTAACTGCCGG) was designed to contain the same number of G/C nucleotides as the internal probe used in the CT-PCR.[14] The PCR mix contained 10 mM Tris-HCl, pH 9.0; 50 mM KCl; 2.5 mM $MgCl_2$; 0.01% gelatine; 0.1% Triton X-100 (all from Merck); 0.2 mM each of dATP, dCTP, dGTP, and dUTP (Boehringer Mannheim); 0.25 µg of each primer per reaction mixture; and DNA polymerase (HT-Biotechnology), final concentration 0.25 units per reaction mixture. Ninety microlitres of the reaction mixture were pipetted into an Eppendorf tube and 10 µl of the purified sample DNA were added, followed by two drops of glycerol (Merck) to prevent evaporation. Amplification was performed in a PCR processor (Biomed) under the following conditions: delay-step 2 minutes at 94°C followed by 35 cycles of one minute at 94°C, one minute at 55°C, and one minute at 74°C.

PCR products were analysed by an enzyme immunoassay, as described before.[10] Briefly, streptavidin coated wells were incubated with 0.2 ml diluted PCR product, washed, and incubated with 0.1 M NaOH, washed again, and incubated with 5'-digoxigenin labelled probe. After incubation with conjugate (anti-digoxigenin peroxidase) and substrate (2,2 azino-di-[3-ethylbenzhiazoline sulphonate]) optical density (OD) was read at 405 nm. To prevent false positive results sterile procedures and guidelines for avoiding contamination were followed throughout.

Before actually starting our study, a panel of known positive and negative samples was used to optimise the PCR and EIA system. Optimal difference in OD values between positive and negative samples were found after 35 cycles with an annealing temperature of 55°C and a probe incubation of one hour at 37°C at a concentration of 10 pmol/ml. Incubation time and concentration of conjugate and substrate were as recommended by the manufacturer.

The specificity of the assay was evaluated by using a panel of 63 organisms (table 1) that can be found in the urogenital samples or that are closely related to *N gonorrhoeae*.

Table 1 DNA used to examine specificity of polymerase chain reaction test for N gonorrhoeae

Organism	*Number*	*Mean OD*
N gonorrhoeae	17	2.700
N meningitidis	1	0.011
N subflava	2	0.013
N flavescens	2	0.013
N sicca	2	0.008
N mucosa	1	0.013
N cinera	1	0.016
N elongata	1	0.049
N lactamica	2	0.010
N perflava	2	0.011
N denitificans	1	0.011
N subflava var flava	1	0.009
C trachomatis	2	0.012
Streptococcus mitis	1	0.005
Streptococcus agalactiae	1	0.005
Haemophilus influenzae	1	0.004
Haemophilus parainfluenzae	1	0.009
Haemophilus ducrei	1	0.009
Moraxella catarrhalis	1	0.009
Lactobacillus jensenii	1	0.022
Acinetobacter calcoaceticus	1	0.011
Acinetobacter lwoffi	1	0.011
Mobuluncus mulieres	1	0.012
Gardnerella vaginalis	1	0.007
Enterobacter cloacae	1	0.005
Klebsiella oxytoca	1	0.006
Escherichia coli	1	0.011
Staphylococcus aureus	1	0.008
Staphylococcus epidermidis	1	0.012
Corynebacterium species	1	0.005
Candida albicans	1	0.009
Candida tropicalis	1	0.009
Candida glabrata	1	0.008
Herpes simplex virus type 1	3	0.019
Herpes simplex virus type 2	2	0.018
Cytomegalovirus	1	0.020
Adenovirus	1	0.004
Human papilloma virus type 6	1	0.005
Human papilloma virus type 11	1	0.007
Human papilloma virus type 16	1	0.007

OD, optical density.

GEN-PROBE

The Gen-Probe PACE 2 system uses a single stranded acridinium ester labelled DNA probe complementary to rRNA of the target organism. Specimens were placed in transport medium in which rRNA is released from the organism. One hundred microlitres of the transport medium were subsequently added to the *N gonorrhoeae* specific DNA probe to form DNA–RNA hybrids. After incubation, 1 ml of separation solution containing metallic microbead and hydrolysis reagent was added. Metallic microbeads bind hybridised probe, while hydrolysis reagent destroys chemiluminescence label on unhybridised probe. Next, tubes were placed on a magnetic separator and supernatants were decanted while the tube and the base of the magnetic separator unit were held together. Finally, tubes were washed, and labelled DNA-RNA hybrids were measured in a chemoluminometer (Leader 1; Gen Probe). Results were reported in relative light units (RLU).[6–8] Samples with 300 RLU or more, after subtracting the mean RLU rate of the negative controls, were considered positive.

16s PCR

Primers Ngo1 (GACGGCAGCACAGGGAA-GCTTGCTTCTCGGG) and Ngo2 (CGCT-ACCAAGCAATCAAGTTGCCC) were used to amplify a target in the 16S region of the *N gonorrhoeae* genome. Thirty microlitre aliquots of the PCR product were analysed by electrophoresis on a 2% agarose gel.[15] For

Table 2 Results of GO-PCR, Gen-Probe, and 16S PCR, and clinical data on seven patients who were GO-PCR positive and Gen-Probe negative

Pt	Optical density: PCR 1	Optical density: PCR 2	Gen-Probe	16S PCR	Clinical data
1	0.372	0.353	Neg	Pos	Vaginal discharge
2	2.700	3.500	Neg	Pos	Vaginal discharge; frequent screening for syphilis and HIV
3	0.251	0.202	Neg	Pos	Urethral discharge
4	1.953	1.960	Neg	Pos	Vaginal discharge; abdominal complaints
5	0.478	0.832	Neg	Pos	Abdominal complaints; sex worker
6	0.385	0.576	Neg	Neg	Sex worker; drug addict
7	0.372	0.636	Neg	Neg	Urethral discomfort; no response to ciprofloxacin

PCR, polymerase chain reaction; Pt, patient.

Southern blot hybridisation, the amplified products were transferred from the gel to a nylon membrane (Hybond N+; Amersham) by electroblotting (Transblot SD; BioRad) and subsequent denaturation of the transferred DNA with 0.4 M NaOH. Prehybridisation was performed at 42°C in a solution containing 5× SSC (75 mM sodium citrate, 750 mM NaCl), 5× Denhardt solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% sodium dodecyl sulphate, 75 mM EDTA, and 0.1% mg of denatured sonicated herring sperm DNA per ml. Hybridisation was performed in the same solution at 42°C overnight with ^{32}P labelled oligonucleotide probe Ngo3 (AAGGCTGTTGCCA-ATATCGGCGGCCGA) directed against an internal sequence of the amplified product. Blots were washed three times in 2× SSC–0.1% sodium dodecyl sulphate at 42°C. Autoradiography was performed at −80°C for 3.5 hours and 24 hours on Kodak X-OMAT AR film by using intensifying screens.

BACTERIAL, YEAST, AND VIRAL DNA

Microorganisms included in this study are summarized in table 1. DNA was isolated according Boom *et al.*[13] Bacterial *Neisseria* species closely related to *N gonorrhoeae* were kindly provided by Dr A van der Embde, Academic Medical Centre, Amsterdam, The Netherlands.

Results

To evaluate the specificity of the in-house PCR test, we tested a wide range of organisms that can be found in urogenital samples. In addition, 17 different *N gonorrhoeae* strains and 16 closely related *Neisseria* species were analysed (table 1). The PCR was positive for all 17 *N gonorrhoeae* strains tested, while none of the 16 non-gonococcal *Neisseria* species, 17 bacterial genital commensal strains, three yeast strains, and 10 viral strains was positive. The mean (SD) OD was 2.7 (0.04) for the 17 *N gonorrhoeae* strains, and 0.011 (0.01) for all others.

Next we evaluated the performance of the GO-PCR on clinical samples, using Gen-Probe as a reference method. Of 429 samples tested, four male and four female samples were positive with both methods, while 414 were negative. Seven cervical samples gave discrepant results—all were positive by PCR and negative by Gen-Probe. The same results were obtained when PCR and Gen-Probe were repeated. In addition, a 16S PCR was performed, followed by detection of the amplicons using a ^{32}P labelled probe. Five of the seven were positive in the 16S PCR while the other two were negative (table 2). After resolution of discrepant samples, the sensitivities of the *N gonorrhoeae* assays were 100% and 61.5% for the in-house PCR and Gen-Probe, respectively, while specificities were comparable at 99.5% and 100%.

The in-house PCR revealed a mean extinction of 0.020 (0.02) OD for the 414 negative samples. The extinction of all eight Gen-Probe positive samples was higher than 3.500 OD. The five Gen-Probe negative but 16S PCR positive samples showed a mean OD of 1.260 (range 0.202 to 3.500) in the in-house PCR (table 2), while the OD values of the two samples negative in both Gen-Probe and 16S PCR were 0.576 and 0.636.

Discussion

We developed a PCR for detection of *N gonorrhoeae* in clinical samples in which amplicons are detected by hybridisation with an internal probe in an enzyme immunoassay (EIA) format. In an earlier study we compared EIA based hybridisation with conventional hybridisation on a Southern blot for the detection of *Chlamydia trachomatis*.[10] In this study both methods revealed identical specificity and sensitivity; however, EIA based detection of amplicons reduced the whole procedure with approximately 15 hours.[10]

The specificity of the assay was evaluated by using a panel of microorganisms that can be found in urogenital samples. This panel contained bacteria that can cause urogenital discomfort like *Gardnerella vaginalis, Mobuluncus mulieres*, and *Chlamydia trachomatis*, but also commensals that can be found in this region like lactobacilli. Moreover, we included yeasts, which can be found both as commensals and as a cause of vaginitis, and viruses, like herpes and papilloma virus, which can be found in this region and sometimes cause discomfort or even overt pathology. As an extra control, we also included some closely related *Neisseria* species, and 17 different strains of *N gonorrhoeae*. Of this panel of 65 organisms, only the 17 stains of *N gonorrhoeae* gave positive results, while none of the other microorganisms had an OD value exceeding 0.049, showing that the combination of primers and probe used here produces very accurate results. Specificity of the primers has been demonstrated before by Ho *et al* using *MspI* cleavage of the 390 bp amplicon into 250 and 140 bp fragments.[9]

The validation of this PCR for the detection of *N gonorrhoeae* in clinical samples is hampered by the low prevalence of gonorrhoea in

The Netherlands. In 1995 and 1996, 1425 and 1192 cases of gonorrhoea were reported, respectively[16] in a population of 15 million. We obtained 429 urogenital swabs from patients with complaints for which the physician included infection in the differential diagnosis. Of this population, only 13 (3%) actually proved to be infected with *N gonorrhoeae*, four men (4/71; 5.6%) and nine women (9/358; 2.5%), while 30 were infected by *C trachomatis*. In this study eight samples were found positive both by Gen-Probe PACE 2 and PCR. Seven samples were found negative by Gen-Probe and positive by PCR, all obtained from female patients. These samples were evaluated by repeating Gen-Probe and PCR. Similar results were obtained as in the first tests. When samples arrived in the laboratory they were split in equal portions. One was stored at 4°C and the other was used to perform the two assays. For retesting of samples, the second portion was used for Gen-Probe and for DNA isolation and GO-PCR. Part of the second portion was send to another laboratory, where a 16S PCR was performed. This procedure greatly reduces the risk of contamination. Moreover, the 16S PCR is not influenced by contamination with amplicons from the 4.2 kb cryptic plasmid, which are produced in our GO-PCR. Five of the seven discrepant samples were found positive by 16S PCR.

When the results of the seven discrepant samples were analysed in more detail, two were clearly positive (OD 3.500 and 1.960), and the other five all had extinctions between OD 0.202 and 0.832 (table 2). The three positive and two negative samples could not be distinguished from the results of the in-house PCR alone. None of the patients was known in our laboratory to be a patient with an earlier *N gonorrhoeae* infection. A possible explanation why Gen-Probe missed five of the 13 PCR positive samples might be that DNA is much more stable than RNA. Indeed, chlamydia DNA could be detected up to three weeks after successful treatment, while RNA could not be detected (data not shown).

The observed lower sensitivity of the Gen-Probe as compared with the in-house PCR might of course also be due to different techniques used. Gen-Probe relies on direct hybridisation of RNA released from the micro-organisms in the sample, while the target DNA in the PCR assay is first amplified before detection. The high copy ribosomal RNA is a good choice as a target for a direct hybridisation assay; however, this might not compensate for the extreme sensitivity of PCR resulting from amplification.

Another difference between the two assays is the preparation of the sample before aliquots are taken for the actual assay. Routinely we use the DNA extraction method described by Boom *et al* for PCR assays,[13] which provides high quality nucleic acids (almost) free of inhibitory substances, but which is rather labour intensive. Gen-Probe, on the other hand, allows direct transfer of the sample, because lysis of the microorganism takes place in transport medium. It might be that inhibition is responsible for some of the discrepant results. However, neither Gen-Probe nor GO-PCR provides an inhibition control.

From our data we can conclude that an OD below 0.150 can be considered negative, while an OD above 1.000 is positive. However, at present an OD between 0.150 and 1.000 has to be considered inconclusive. Therefore, we have decided to introduce an extended clinical evaluation period, during which a second sample and clinical information are requested for all specimens in which the test is repeatedly inconclusive.

CONCLUSION

The in-house PCR is a sensitive assay for the detection of *N gonorrhoeae* DNA in urogenital samples, at least comparable to Gen-Probe, and provides results within eight hours. The reason why GO-PCR detected five positive specimens which were confirmed by 16S PCR but missed by Gen-Probe has yet to be determined.

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