

# Polymerase chain reaction for the detection of *Neisseria gonorrhoeae* in clinical samples

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## Abstract

**Aims:** To evaluate the use of a *cppB* gene derived polymerase chain reaction (PCR) assay for direct detection of *Neisseria gonorrhoeae* in clinical samples.

**Methods:** A PCR assay was performed on 33 *N gonorrhoeae* strains and 12 other *Neisseria* species and other normal genital flora to evaluate the specificity of the chosen *cppB* primers. The assay was subsequently evaluated with 52 clinical swab samples collected from China.

**Results:** An amplified product of 390 base pairs (bp) was observed with all the *N gonorrhoeae* strains, each of these products on digestion with the restriction enzyme *MspI* produced two bands of 250 bp and 140 bp respectively. This set of primers did not produce any amplified product of the expected length with the other non-gonococcal strains tested. For the 52 clinical swabs, 34 were culture positive and PCR successfully detected all these positives. In addition the PCR was positive for two swabs which were culture negative but positive for *N gonorrhoeae* antigens when tested with the ELISA method (Gonozyne).

**Conclusions:** This PCR assay is a promising diagnostic tool for detection of gonococci directly from clinical swab samples. Further evaluation is necessary.

The use of hybridisation with DNA probes as a tool for detection of gonococci in urogenital specimens has been investigated extensively in recent years. Probes using nucleotide sequences of the pilin gene,<sup>1</sup> the IgA1 protease gene,<sup>2</sup> ribosomal RNA<sup>3,4</sup> and the cryptic plasmid<sup>5</sup> have been reported. The pilin genes of the gonococci have more than one copy, but they are highly homologous with those of the meningococci. All gonococci have a single copy of the IgA1 protease gene,<sup>2</sup> however detection is difficult when few organisms are present in the clinical sample. The rRNA-derived oligonucleotide probes to different regions of the 16 S ribosomal RNA have been reported to have a sensitivity and specificity of 100%, but the exact sequences of these probes have not been published. Gonococcal clinical isolates have several plasmids.<sup>6</sup> The smallest of these which is present in about 96% of strains is approximately 4.2 kilobases (kb) in size and its function is unknown. The use of cryptic plasmid-derived probes for DNA hybridisation was thought to have limited application for the detection of gonococci in clinical isolates. It has

recently been shown by Hagblom *et al* that the polypeptide product of the *cppB* gene of the cryptic plasmid pJD1 is also produced by plasmid-free strains and that a copy of this gene was present in the gonococcal chromosome of all strains tested.<sup>7</sup> However the possible cross-reaction with plasmids from *N meningitidis* and *N lactamica* needs to be borne in mind.<sup>8</sup> The problem of low gene copy number has been overcome by the development of the polymerase chain reaction (PCR) where, theoretically, even a single gene copy would be sufficient for amplification.

As the complete nucleotide sequence of the cryptic plasmid is known<sup>9</sup> it was decided to develop a PCR using primers derived from the integrated regions of the *cppB* gene. During the course of the present study, Torres *et al* reported the evaluation of a cryptic plasmid-derived DNA probe in a dot-blot hybridisation assay.<sup>10</sup> The probe, consisting of a 237 bp segment of the plasmid-encoded gene *cppB* labelled with digoxigenin-11-dUTP, was evaluated using both known bacterial isolates and clinical specimens. The present paper describes the development of a PCR assay for *N gonorrhoeae* in which a 390 base pair (bp) DNA fragment of the *cppB* gene was amplified and its performance with that of culture compared using urethral and cervical swabs from patients with acute urethritis.

## Methods

### Bacterial strains

The *N gonorrhoeae* strains used were all clinical isolates recovered from urethral or cervical swabs by inoculation onto modified Thayer Martin medium and subsequent identification by Gram stain, colonial morphology, positive oxidase, and rapid carbohydrate utilisation test.

Bacterial species closely related to *N gonorrhoeae* were used in specificity studies and included *N pharyngis* NCTC 4591, *N flavescens* NCTC 8263, *N caviae* NCTC 10293, *N cinerea* NCTC 10294, *N denitrificans* NCTC 10295, *N canis* NCTC 10296, *N cuniculi* NCTC 10297, *N elongata* NCTC 10660, *N mucosa* var *mucosa* NCTC 10774, *N ovis* NCTC 11018, *N lactamica* ATCC 23970, and a clinical isolate of *N meningitidis*. Additional bacterial strains representing different species that are often found as genital commensals, including members of the genera *Corynebacterium*, *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Acinetobacter*, *Moraxella*, *Branhamella*, and members of the family Enterobacteriaceae were also used. The NCTC strains were kindly provided

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by the Gonococcus Reference Unit, Public Health Laboratory, Bristol and the other strains were obtained from the Queen Mary Hospital Clinical Microbiology Laboratory, Hong Kong.

#### Clinical specimens

Clinical specimens were collected from men and women with acute urethritis attending a sexually transmitted disease clinic in Guangzhou, China. Two urethral swabs from the male and two cervical swabs from the female were taken with phosphate buffered saline pre-wet swabs. One swab was inoculated directly onto modified Thayer Martin medium, incubated in CO<sub>2</sub>, and any suspected organisms were identified following the above procedure. The other swab was stored frozen at -20°C except during transport to Hong Kong.

#### Specimen processing for DNA amplification

Bacterial DNA from *N gonorrhoeae* strains, other *Neisseria* species, and genital commensals was extracted as described below. A bacterial suspension in water was made from an overnight cultured plate to give about 10<sup>8</sup> cfu/ml. Of the bacterial suspension, 50 µl was used for each extraction and the following reagents were added sequentially to the suspension giving a final concentration of 1 M NaCl, 1 M NaOH and 1% sodium dodecyl sulphate. The contents were mixed and boiled for one minute, then neutralized with two volumes of 1 M TRIS pH 7. The DNA was extracted once with phenol-TRIS, once with phenol-chloroform, and then precipitated with ethanol. The extracted DNA was resuspended in 30 µl of sterile distilled water or TRIS-EDTA. For clinical specimens from China, each was suspended in 2 ml phosphate buffered saline and vortexed for one minute to release the material from swab. The swab was removed and the suspension centrifuged for five minutes at 2–3000 rev/min to pellet cells. After removing the supernatant by aspiration, the cells were resuspended in 100 µl 1 × PCR buffer with non-ionic detergent Tween 20 0.45% and proteinase K (200 µg/ml). The cell suspension was incubated at 50–60°C for one hour, then heated to 95°C for ten minutes to inactivate the proteinase K.

#### Selection of PCR primers

Sequence data on the *cppB* gene carried on both the chromosome and the 4.2 kb cryptic plasmid of *N gonorrhoeae* was used to select two 20-mer oligonucleotide primers designated HO1 (5'GCTACGCATACCCGCGTTGC3') and HO3 (5'CGAAGACCTTCGAGCAGACA3'). The expected length of the amplified product of the target sequence with these primers was 390 bp. It was known from the restriction map of the target sequence that the restriction enzyme *MspI* would cut within the amplified product to give two fragments of 250 and 140 bp respectively and so it was used to confirm the specificity of the amplified product.

#### PCR amplification

Amplification was performed in 100 µl reaction volumes containing: 50 pmol of each oligonu-

cleotide primer (HO1 and HO3); 200 µM each of dATP, dCTP, dTTP, and dGTP; 1 × PCR reaction buffer (50 mM KCl, 10 mM TRIS pH 8.3, 100 µg/ml bovine serum albumin, 1.5 mM MgCl<sub>2</sub>); and a 10 µl volume of DNA. For DNA from clinical swabs, 50 µl of proteinase K treated suspension was used and a 100 µl reaction volume was made up with the same constituent as described for DNA from bacteria. The tubes were mixed before overlaying with a drop of mineral oil. Forty cycles of amplification were performed in a DNA thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of a 30 second denaturation step at 94°C, a one minute annealing step at 55°C, and a 30 second extension step at 74°C.

Amplified product (10 µl) was analysed by electrophoresis in a 2% agarose gel using standard methods.<sup>11</sup> The ethidium bromide stained gel was examined under ultraviolet light for the presence of a 390 bp fragment. If a fragment of the expected size was present, 5 µl of the product was digested with 5 U of *MspI* (Boehringer) at 37°C for one to two hours, each in a final volume of 20 µl of 1 × reaction buffer provided by the manufacturer. The digested product was again electrophoresed and examined under ultraviolet light for two fragments of sizes 250 and 140 bp respectively.

#### Results

To evaluate the specificity of the selected primers (HO1 and HO3), DNA was extracted from 12 non-gonococcal *Neisseria* species, 18 genital commensal strains and 33 *N gonorrhoeae* strains (table 1). After a 40-cycle PCR reaction, only *N gonorrhoeae* DNA was amplified (fig 1) to give a product of the expected fragment size (390 bp). *N denitrificans* DNA also gave an amplified product, but the size was about 190 bp (fig 2). Apart from *N denitrificans*, none of the non-gonococcal

Table 1 Bacteria used in specificity test for the PCR assay of *N gonorrhoeae*

Organism	No of isolates tested	No with positive PCR
<i>N gonorrhoeae</i>	33	33
<i>N meningitidis</i>	1	0
<i>N lactamica</i> ATCC 23970	1	0
<i>N pharyngis</i> NCTC 10293	1	0
<i>N flavescens</i> NCTC 8263	1	0
<i>N caviae</i> NCTC 10293	1	0
<i>N cinerea</i> NCTC 10294	1	0
<i>N denitrificans</i> NCTC 10295	1	0
<i>N canis</i> NCTC 10296	1	0
<i>N cuniculi</i> NCTC 10297	1	0
<i>N elongata</i> NCTC 10660	1	0
<i>N mucosa</i> var <i>mucosa</i> NCTC 10774	1	0
<i>N ovis</i> NCTC 11018	1	0
<i>Cornibacterium</i> sp	1	0
<i>Lactobacillus</i> sp	1	0
<i>S. aureus</i>	1	0
<i>S. epidermidis</i>	2	0
<i>S. agalactiae</i>	1	0
Alpha-haemolytic <i>Streptococcus</i> spp.	1	0
<i>Acinetobacter amitratus</i>	5	0
<i>Acinetobacter lwoffii</i>	1	0
<i>Moraxella</i> sp	1	0
<i>Branhamella catarrhalis</i>	1	0
<i>Klebsiella</i> sp	1	0
<i>Enterobacter cloacae</i>	1	0
<i>Escherichia coli</i>	1	0

Positive PCR reaction is indicated by the presence of a 390 bp amplified fragment and on digestion with *MspI* gives two fragments of sizes 250 and 140 bp respectively.

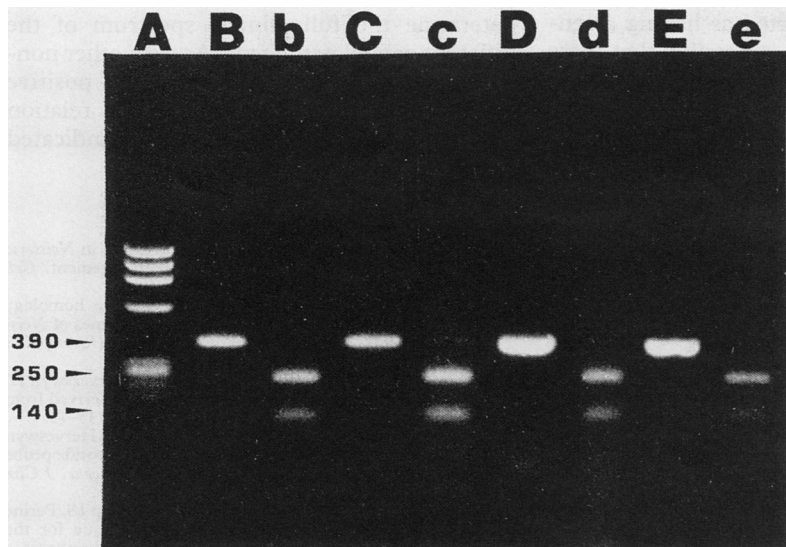


Figure 1 PCR of representative samples from patients with a positive gonococcal culture result (lanes B-E). (A) Size marker  $\phi$ X174 digested with HaeIII. (B-E) PCR products (390 bp) of clinical swabs containing *N gonorrhoeae*. (b-e) corresponding DNA fragments (250 bp and 140 bp) of *MspI* digested PCR product.

*Neisseria* strains and genital commensals showed any amplified product. All the 33 *N gonorrhoeae* strains tested were amplified and the product contained the predicted internal restriction enzyme site for *MspI*, yielding a 250 bp and a 140 bp fragment after digestion (fig 1).

For the sensitivity of the reaction, DNA was extracted from serial dilutions of a bacterial suspension prepared from overnight plate culture and an amplified product of 390 bp was detected at approximately 500 cfu of *Neisseria gonorrhoeae*.

To demonstrate the detection of *N gonorrhoeae* in clinical specimens, urethral swabs were collected from 52 patients. Of these, 34 were culture and PCR positive, two were only PCR positive, and 16 were both culture and PCR negative, giving a sensitivity of 100% and

specificity of 88.9% respectively. Digestion of the PCR amplified product with *MspI* yielded fragments of the predicted sizes.

**Discussion**

Selection of two primers which will amplify a specific sequence of the organism is a crucial step in the success of the PCR assay. In the present study we targeted the *cppB* gene since a separate copy was present in the genome of all *N gonorrhoeae* strains tested. The *cppB* gene originates from the 4.2 kb cryptic plasmid of *N gonorrhoeae* which was found in whole or part, integrated in the chromosome of all gonococcal strains tested.<sup>7</sup> There are two variants of the cryptic plasmid, one had a deletion of 54 bp within the *cppB* gene. Both the deleted and the undeleted version of the *cppB* gene may be found as a separate copy in the gonococcal genome, including the strains lacking plasmid. Therefore, when selecting for the primer sequence, the possibly deleted region of *cppB* gene was not included in the amplified region and the two chosen 20-mer oligonucleotide primers almost marked the entire length of the remaining *cppB* gene sequence.

For confirmation of the specificity of the amplified product, the presence of a restriction site within the amplified sequence was shown. This protocol was used instead of Southern hybridisation with a specific probe because of the possibility of clinical application of this assay and the use of hybridisation for confirmation may lengthen the time for diagnosis by another day.

Optimal conditions for the PCR were determined using crude DNA extracted from serial dilutions of gonococcal culture and it was shown that sensitivity of the assay was 500 cfu of *N gonorrhoeae*. Using the cryptic-derived DNA probe, Torres *et al* demonstrated a similar sensitivity of 500 cfu.<sup>10</sup> Sensitivity of the PCR assay can be further increased if the method of detection is by probe hybridisation instead of visualisation with the ethidium bromide stained gel.

The specificity test for the chosen primers indicated that the assay would only give a fragment of expected size with *N gonorrhoeae*. Although the cryptic plasmid of *N gonorrhoeae* cross-reacts with plasmids from *N meningitidis* and *N lactamica*, this was not detected with our PCR assay. However, only one strain of each species was tested. There was no cross-reaction with any of the non-gonococcal *Neisseria* strains or other genital flora tested.

For application of this assay to clinical samples, only urethral swabs and cervical swabs have so far been tested. Using culture as the "gold standard", the sensitivity, specificity, PPV, and NPV for this test would be 100, 88.9, 94, and 100% respectively (table 2). The 'gold

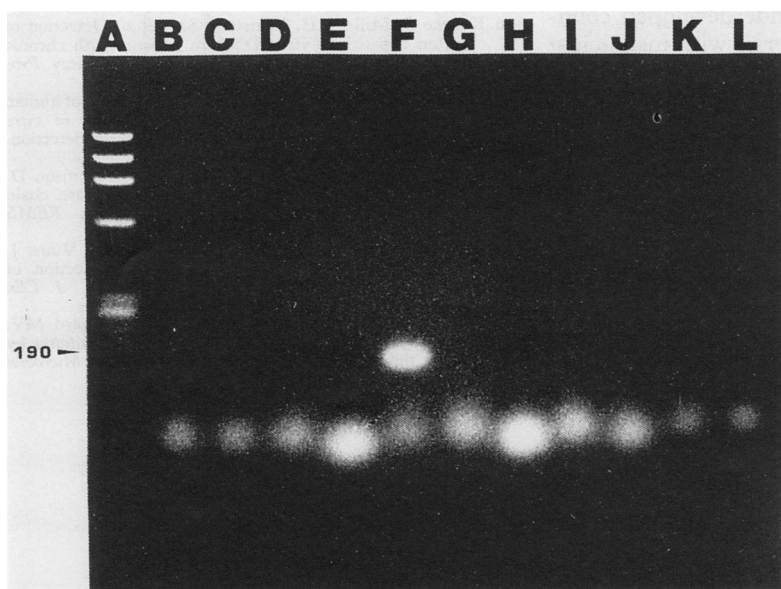


Figure 2 PCR results of DNA extracted from cultures of non-gonococcal *Neisseria* species. (Lanes A-L)  $\phi$ X174-HaeIII size marker, *N pharyngis*, *N flavescens*, *N caviae*, *N cinerea*, *N denitrificans*, *N lactamica*, *N cuniculi*, *N elongata*, *N mucosa*, *N ovis*, *N meningitidis* respectively.

Table 2 PCR results of clinical swabs compared with culture

	Culture positive	Culture negative
PCR positive	34	2
PCR negative	0	16

PCR assay: sensitivity 100%, specificity 88.9%, positive predictive value 94.4%, negative predictive value 100%.

standard' has been reported as having a sensitivity of 85–95%<sup>12</sup> so it is possible that there were false negatives for the culture method which would lead to an underestimation of the specificity of the PCR. The two specimens which were PCR positive and culture negative were found to be positive with Gonozyme (Abbot Laboratories) (results not shown). Though Gonozyme is well known to have false positive results and PCR may also give false positives due to contamination, it is unlikely in these cases. As PCR is detecting the gonococcal DNA and Gonozyme is detecting its antigen, if two tests are positive concurrently it is likely that the results in question are genuine detection of gonococci in specimens. Nevertheless, the possibility of these two specimens giving false positive results with both tests should not be overlooked.

This *cppB* gene sequence was chosen as primer due to its presence even on the chromosome of plasmid-free strains, thus overcoming the limitation of using cryptic plasmid as probe in DNA hybridisation for diagnosis of gonococcal infection. In this study, we were unable to test this hypothesis as plasmid-free strains have so far not been encountered in Asia<sup>13</sup> and we have failed to obtain plasmid-free strains from other workers. We are thus unable to say that this PCR assay is universal for all gonococci. More plasmid-free strains will have to be tested with this assay before it can be used as a reliable diagnostic tool, but our results are promising. It should be investigated whether this PCR method is more useful than the existing enzyme immunoassay (Gonozyme, Abbott Laboratories) or other DNA probes in the accurate assessment of asymptomatic and symptomatic gonococcal infections.

Frozen swabs were used and also swabs which had been stored frozen for one month, and the PCR result was not affected. With the durability of DNA under such conditions, it is possible for specimens to be sent to distant laboratories for investigation. This would be particularly useful for underdeveloped countries or for small laboratories with insufficient facilities.

With the recent development of the PCR, more sensitive DNA probes have been produced and used to detect many of the common agents of sexually transmitted disease, namely *Chlamydia trachomatis*,<sup>14</sup> human papillomavirus,<sup>15</sup> hepatitis B virus,<sup>16</sup> human immunodeficiency virus,<sup>17</sup> *Mycoplasma genitalium*<sup>18,19</sup> and *Treponema pallidum*.<sup>20</sup> Publication of primer sequences and the evaluation of primers for these organisms could result in multiple probing systems which can be used to

determine the full clinical spectrum of the different genital pathogens. As with other non-cultural methods the significance of positive results must be carefully assessed in relation to the activity or otherwise of the indicated organism.

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