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Multicenter Validation of the *cppB* Gene as a PCR Target for Detection of *Neisseria gonorrhoeae*

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The *cppB* gene is often used as a target for detection of *Neisseria gonorrhoeae* by PCR. Using a coded panel of 500 DNA samples, we determined that the *cppB* gene is missing in 5.8% of *N. gonorrhoeae* strains, and therefore we consider the *cppB* gene to be an unsuitable target.

The laboratory diagnosis of gonorrhea depends on identification of *Neisseria gonorrhoeae* in urogenital samples. Amplification using the Roche COBAS AMPLICOR (CA) assay has been found highly suitable for screening asymptomatic subjects as well as diagnosing gonorrhea in populations with a high prevalence of sexually transmitted infections (STI) (4, 6). Specimens containing other species of the *Neisseriaceae*, probably members of the normal pharyngeal flora, but also non-*Neisseria* bacterial strains, were reported to produce false-positive results in the CA PCR test, which targets the cytosine DNA methyltransferase gene (4, 8, 12).

Confirmatory tests using the 16S rRNA genes and the *cppB* gene have been reported (2, 5, 13, 14); however, the cryptic plasmid on which the *cppB* gene is located is suspected to be missing in some clinical isolates (11). Therefore, we determined the frequency of the *cppB* gene in well-characterized *N. gonorrhoeae* strains cultured from STI patients by using real-time PCR technologies as developed in different diagnostic laboratories in The Netherlands (Table 1).

From September 2002 to April 2003, patients with complaints indicative of gonorrhea visited the STI clinic in Amsterdam, The Netherlands, where clinical and epidemiological data were registered and samples were taken. Urethral, cervical, rectal, or tonsil specimens were used to inoculate GC-Lect agar plates (Becton Dickinson) at the Gemeentelijke Geneeskundige en Gezondheidsdienst (GG&GD). Culture conditions and biochemical determination of *N. gonorrhoeae* were as described previously (1), including Gram staining, positive catalase reaction, cytochrome *c* oxidase, and sugar metabolism tests. DNA was isolated from a few colonies by isopropanol precipitation, and the pellet was dissolved in 50 μl of T10 buffer (10 mM Tris-HCl, pH 8.0) (9). In the context of a communal epidemiology study, we typed these *N. gonorrhoeae* strains by PCR-restriction fragment length polymorphism analysis of the *opa* and *por* genes, confirming further that true *N. gonorrhoeae* strains were used for DNA isolation (7, 10). A coded panel of 500 samples was composed by distributing 50 negative samples randomly among 450 DNA preparations of confirmed *N. gonorrhoeae* strains. The *N. gonorrhoeae*-positive samples were prepared by dilution of the original DNA solution to ensure an easily detectable target load. The 50 negative controls consisted of 26 samples containing different buffers, namely, 10× T10 buffer, 5× H_2O, 5× phosphate-buffered saline, and 6× AMPLICOR resuspension buffer; 20 samples containing bacterial DNAs derived from non-*Neisseria* species; and 4 DNA samples from the nongonococcal species *N. meningitidis, N. mucosa, N. lactamica*, and *N. subflava*. For each diluted DNA and control sample, five aliquots of 200 μl were prepared and distributed to the participating laboratories by regular mail at room temperature. The samples were analyzed by real-time PCR tests targeting the *cppB* or the 16S rRNA genes by use of an input of 5 μl of the DNA solution, along with primers, probes, and hardware as outlined in Table 1. The Academisch Medisch Centrum (AMC) performed discrepancy analysis (see below).

After submission of all test results that were obtained in a blinded fashion to the GG&GD, the codes were broken and reported back to the participating laboratories. Each laboratory reset the values for cycle threshold (C_T) (for the ABI and RotorGene hardware) and crossing point (C_p) (for the LightCycler), with the results of the negative control samples taken into account. Initially, 459 of 500 (91.8%) results were fully concordant and consisted of 48 negative and 411 *N. gonorrhoeae*-positive samples. The 41 samples with discordant test results plus 7 negative controls were tested by the AMC to establish whether a sample was truly lacking the *cppB* gene. If both 16S rRNA tests were positive for a sample but at least three of the four *cppB* tests were negative, this was taken as proof that the *cppB* target was lacking.

Two *N. gonorrhoeae* DNA samples proved to be negative in all tests, probably because they were diluted excessively and were counted as negatives in addition to the 50 negative samples. The SLGD reported two false-positive 16S rRNA test results for the *N. meningitidis* and *N. lactamica* samples, yielding 96% specificity. However, when the two samples were retested in the CA test at the Streeklaboratorium voor de Volksgezondheid in Groningen en Drenthe (SLGD), they proved to

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TABLE 1. Real-time PCR systems used for analysis

Laboratory	Target gene	Chemistry of probes	Primers and probes (reference)[a]	Hardware (company)
GG&GD	*cppB*	TaqMan	HO1, HO2, CppBNB (4)	RotorGene (Corbett Research)
SLGD	*cppB*	TaqMan	cppB2-1, B2-2, cppB-pr	ABI 7700 (Applied Biosystems)
SLGD	16S rRNA	TaqMan	16S-f1, 16S-r2, GO-207T	ABI 7700 (Applied Biosystems)
PAMM	16S rRNA	FRET	SL67, 16S-Rev, FL+LC	LightCycler (Roche Diagnostics)
LVF	*cppB*	FRET	Ngon3, -4, -5FL, -6LC	LightCycler (Roche Diagnostics)
AMC	*cppB*	FRET	Ngon3, -4, -5FL, -6LC	LightCycler (Roche Diagnostics)

[a] Sequences and PCR conditions are as published and available on request from each laboratory.

be negative. Laboratorium voor Pathologie en Medische Microbiologie (PAMM) also reported low C_p values (less than 30) in the 16S rRNA test for these strains, suggesting positivity, but melting-curve analysis with fluorescent resonance energy transfer (FRET) probes showed that these samples were not *N. gonorrhoeae*. The specificity was 100% for all other tests. The number of discrepant *N. gonorrhoeae* samples was thus reduced from 41 to 37 (41 minus 2 minus 2) for the six tests (Table 2). For 11 of these 37 samples, a negative result was reported by the 16S rRNA assays and/or by one or two *cppB* assays, suggesting either a low DNA load or a false-negative result. Thus, for these 11 samples, two to four *cppB* tests were positive, indicating the presence of a *cppB* gene. In contrast, in the other 26 *N. gonorrhoeae* samples, the 16S rRNA assays were positive at both laboratories. In 3 of these 26, only one positive *cppB* gene result was reported with C_T values just below the cutoff, and in the other 23, all four *cppB* gene tests were negative. These 26 samples were thus discovered to be *cppB*-lacking *N. gonorrhoeae* strains, resulting in a proportion of 5.8% (26 of 448) of the *N. gonorrhoeae* strains tested.

The clinical sensitivities were 98.9 and 99.6% for the 16S rRNA targets at the SLGD and PAMM laboratories, respectively. For the *cppB* assays, the sensitivities without correction for identifying strains lacking the *cppB* gene were 92.4, 93.7, and 93.9% at the GG&GD, Laboratorium voor de Volksgezondheid in Friesland (LVF), and SLGD laboratories, respectively, and are thus obviously affected by the lack of a *cppB* gene in 5.8% of the strains. In a recent CA PCR study in The Netherlands performed directly with clinical samples, a similar proportion of 6% of *N. gonorrhoeae* strains lacking *cppB* was found (D. S. Luijt, P. A. J. Bos, A. A. van Zwet, P. C. van Voorst Vader, and J. Schirm, submitted for publication). The present study quantified for the first time the frequency of the *cppB* gene in a well-defined group of *N. gonorrhoeae* strains. This gene was found on the cryptic plasmid but may also be present chromosomally in the *N. gonorrhoeae* genome in cases where the plasmid is integrated. The cryptic plasmid was found to be missing in 4% of *N. gonorrhoeae* strains collected 25 years ago in the United States and Europe (11) and appeared to be correlated to the PA°U auxotype (3, 8).

Since the sensitivity of a PCR test targeting the *cppB* gene can never surpass 94%, we conclude that either assays targeting 16S rRNA or alternative PCR assays for detection of *N. gonorrhoeae* that targets, for example, the highly specific *opa* genes need to be evaluated in future studies.

TABLE 2. Samples with discrepant test results (n = 37)

No. of strains in panel	No. of tests positive		*cppB* gene interpretation[c]
	16S rRNA target[a]	*cppB* gene target[b]	
3	2	3	Yes
1	2	2	Yes
3	1	4	Yes
3	1	3	Yes
1	1	2	Yes
23	2	0	No
3	2	1	No

[a] The PAMM and SLGD laboratories produced 16S rRNA test results (n = 2).
[b] The SLGD, GG&GD, LVF, and AMC laboratories produced *cppB* gene test results (n = 4).
[c] The cutoff C_p or C_t values were as follows: for the PAMM laboratory, 38; for SLGD, 35 (both tests); for GG&GD, 37; for LVF, 33; for AMC, 40. In case of FRET technologies (see Table 1), a melting curve was also used for specificity. Yes, positive for *cppB* gene; No, negative for *cppB* gene. There were totals of 11 results positive and 26 results negative for the *cppB* gene.

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