Diagnostic value of molecular confirmation assays for Neisseria gonorrhoeae

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Diagnostic Value of Molecular Confirmation Assays for *Neisseria gonorrhoeae*

The COBAS Amplicor system (Roche Diagnostics, Rotkreuz, Switzerland) is a widely used screening test for infections with *Neisseria gonorrhoeae* due to its excellent sensitivity and ease of use. However, it has been found to cross-react with certain commensal *Neisseria* species (1, 3, 4). Therefore, an independent confirmation assay is mandatory.

In the May 2007 issue of the *Journal of Clinical Microbiology*, Mangold et al. (2) presented a new confirmation assay which is capable of distinguishing between *N. gonorrhoeae* and *Neisseria* spp. in comparison to confirmation assays targeting the *porA* pseudogene and the *cppB* gene. They obtained positive results in their in-house assay for 102/181 (56%) COBAS Amplicor-positive samples; the *porA* confirmation assay was positive for 69/181 (38%) samples, and the *cppB* assay detected 115/181 (64%) positive samples. Additionally, Mangold et al. reported 15%, 2%, and 4% positive results for COBAS Amplicor-negative samples with the *cppB*, *porA*, and in-house assays, respectively. The presented data are discrepant from observations at our institution. Upon optimization of published detection protocols, we have compared the performances of three assays targeting the *porA* pseudogene (5), the *cppB* gene (6), and the 16S rRNA gene (in-house) for quality assurance. In samples positive for all three targets, the *cppB* confirmation assay was the most sensitive (mean crossing point [C_p], 25.9), followed by the *porA* assay (mean C_p, 26.2) and the 16S rRNA gene assay (mean C_p, 32.8).

Over a period of 6 months, 398 samples yielded a positive COBAS Amplicor result (optical density [OD] of >0.2, as recommended by the manufacturer). Of these 398 samples, 258 samples (64.8%) were negative in all three confirmation assays. The remaining 140 samples (35.2%) yielded a positive result in at least two of the confirmation assays, with the majority being positive in all three assays (128/140 [91.4%]). Eight of 140 samples (5.7%) were negative in the 16S rRNA gene assay, probably due to small DNA amounts in the sample, as this assay is less sensitive than the other two confirmation assays, which is corroborated by the fact that 2/8 samples showed an OD value lower than 2.0 when initially tested in the COBAS Amplicor system. Four specimens were positive in the 16S rRNA gene and *porA* detection assays only and were interpreted as gonococci lacking the *cppB*-harboring plasmid (2.9%); the COBAS Amplicor value was above 2.0 OD in all four samples. Taken together for 136/140 (97.1%) specimens, the *porA* and *cppB* confirmation assays were concordantly positive.

In contrast to the findings of Mangold et al., we did not obtain positive results for the confirmation assays when applied to COBAS Amplicor-negative samples.

The conflicting results might in part be explained by an epidemiological distinct pool of *N. gonorrhoeae* isolates examined and an unfortunate study design, as modified interpretation guidelines for the COBAS Amplicor assay were used by Mangold et al. Samples were considered positive if two out of three runs gave a positive signal above an OD of 2.0, as opposed to one sample above an OD of 0.2 being sufficient. This decreases the number of positive samples and inevitably has a negative impact on the sensitivity of the screening assay. Presumably, this protocol accounts for the "false"-positive results of the *cppB* plasmid assay (33/227). It would be interesting if these samples were initially positive in the COBAS Amplicor system (OD of >0.2) by use of the manufacturer's interpretation criteria. Our analysis further cannot confirm the low sensitivity of the *porA* detection assay, as the majority of our specimens yielded positive results in all confirmation assays. Regarding the in-house NsppID assay published by Mangold et al., the design of the test itself is not completely conclusive: *N. gonorrhoeae* displays 100% homology with strains of *Neisseria polysaccharea* at the binding site of the detection probe (referred to as *Npolysaccharea1* in the publication). *N. polysaccharea* was not tested for cross-reactivity in the NsppID assay. By use of an NCBI BLAST search, strains of *Neisseria meningitidis* also display 100% homology in this region of the 16S rRNA gene. Additionally, coamplification in PCRs should be avoided if no important information is obtained by the coamplified product, as it is likely to decrease sensitivity. A confirmation assay for *N. gonorrhoeae* that coamplifies apathogenic *Neisseria* spp. does not provide any additional information of clinical relevance.

The studies indicate that the use of molecular diagnostic assays for the detection of *N. gonorrhoeae* infection remains challenging and that the characterization of the prevalence of target genes in distinct epidemiological pools is important to ensure accurate diagnosis.

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Authors' Reply

We appreciate the comments forwarded by Dr. Peter-Getzlaff and colleagues regarding our recent publication of a confirmatory assay for *Neisseria gonorrhoeae*. All are in agreement that confirmation of positive results from the Roche COBAS *N. gonorrhoeae* assay (Roche Diagnostics, Indianapolis, IN) is necessary; however, several important points must be made.

TABLE 1. NsppID and COBAS CT/NG results: relationship to initial A_{450} value

Assay	Species amplified	No. (%) of assays with inital A_{450} result of:		
		0.2–1.999	2.0–3.499	≥3.5
NsppID	No amplification	112 (30)	30 (8)	20 (5)
	N. cinerea	2 (1)	5 (1)	6 (2)
	N. meningitidis, *N. perflava*, or *N. subflava*	27 (7)	14 (4)	29 (8)
	N. gonorrhoeae	17 (5)	10 (3)	103 (27)
COBAS CT/NG	Presumptive *N. gonorrhoeae* with positive repeat testing	9 (6)	37 (63)	148 (94)
	Presumptive *N. gonorrhoeae* with positive repeat testing; confirmed *N. gonorrhoeae* by NsppID	4 (3)	9 (15)	101 (64)

First, there are significant differences in the interpretive guidelines specified for the COBAS Amplicor CT/NG assay between the United States and Europe. The European guidelines indicate that a single ≥ 0.2 A_{450} result is a presumptive positive for *N. gonorrhoeae*, with a recommendation for retesting for confirmation. However, in the U.S. product insert, recommendations define an equivocal zone and call for repeated testing of specimens reading between 0.2 and 3.5 A_{450}; at least two of three results (of the initial and the duplicate repeats) must measure ≥ 2.0 A_{450} in order to call the sample presumptively positive for *N. gonorrhoeae*. These were the guidelines used, appropriately, in our study (3). The U.S. recommendations were at least partially based on a study by Van Der Pol et al. (5), which showed that employing the equivocal zone retesting algorithm increased specificity to 98.6 to 99.9%, with little effect (0.1 to 4.9% decrease) on sensitivity in most specimen types. Our own data support this conclusion, as indicated by Table 1.

As indicated, a significant number of specimens that resulted in an initial A_{450} value of 0.2 or greater do not repeat with a value of 2.0 or greater. The percentage of specimens that are positive with repeat testing increases with the initial A_{450} value, with 94% of specimens with an initial value of 3.5 or greater remaining presumptive positive upon repeat testing; 64% of these will ultimately be positive for *N. gonorrhoeae*, as indicated by our 16S-based speciation assay (NsppID).

Although the equivocal zone retesting algorithm improves the specificity of the COBAS *N. gonorrhoeae* assay, significant numbers of false-positive results are still generated during testing of low-prevalence populations, leading to the recommendation by the Centers for Disease Control and Prevention that presumptive *N. gonorrhoeae* infection should be confirmed using independent testing for a different *N. gonorrhoeae*-specific target (2). We developed the NsppID assay to function as this confirmatory test. Once again, our results (as listed in Table 1) identifying nongonorrheal *Neisseria* spp. in the presumptive positive specimens after repeat testing reinforce the value of a second independent test.

In their letter, Peter-Getzlaff and colleagues indicated that the 16S assay used in their studies was less sensitive than either the *cppB* or the *porA* assay. Most published 16S assays (and possibly theirs as well) for the confirmation of *N. gonorrhoeae* were designed to amplify only *N. gonorrhoeae*, whereas our assay was designed to amplify many different *Neisseria* spp., with mismatches under the probe differentiating the various species. As described in our paper, the sensitivity of our assay was four genome copies of *N. gonorrhoeae* per reaction, even in the presence of 40-fold excess concentrations of the nongonorrheal *Neisseria* spp. Indeed, due to the melt curve analysis that differentiates between the *Neisseria* spp., we were able to detect dual infections in six clinical specimens. In comparison, the optimized *porA* assay had an analytical sensitivity of 20 genome copies of *N. gonorrhoeae* per reaction. In addition, although the optimized *cppB* assay detected more of the COBAS-positive samples than NsppID, we question the specificity of this assay, as a significant number of the discrepant specimens did amplify a nongonorrheal *Neisseria* sp. in the absence of any indication of *N. gonorrhoeae* in our NsppID assay. Several other laboratories have also found the *cppB* assay to be less specific than first thought (1, 4).

At the beginning of this project, there was only a single GenBank entry of a nongonorrheal *Neisseria* strain (one of several *N. polysaccharea* strains entered) that was identical in sequence to *N. gonorrhoeae* in the fluorescein-labeled probe region, and the assay was designed based on that information. Since that time, additional sequences have been entered into the GenBank database, and the list of entries with the 30-base sequence identical to that of *N. gonorrhoeae* in the fluorescein-labeled probe region includes some, but not all, strains of two additional *Neisseria* spp. (*N. meningitidis* and *N. lactamica*). Of these three nongonorrheal *Neisseria* spp., only *N. lactamica* has been shown to produce a false-positive result in the COBAS Amplicor CT/NG assay. Neither *N. meningitidis* nor *N. polysaccharea* has been shown to cross-react in a similar fashion. In our analytical validation, we tested a single strain of *N. lactamica* (ATCC 23970); the melting temperature peak observed in the melt curve analysis was over 6°C lower than that observed for all of the *N. gonorrhoeae* strains tested and was lower than that for any of the other *Neisseria* specimens tested as well. For these reasons, after analytical validation of the NsppID assay, we continued with the validation by using clinical specimens (endocervical swabs, urogenital swabs, or male urine). A specimen that was presumptive *N. gonorrhoeae* positive after repeat testing using the COBAS Amplicor CT/NG assay and that amplified in the NsppID assay with a resultant melting temperature of approximately 58°C was called *N. gonorrhoeae* positive. As pointed out by Peter-Getzlaff and colleagues, there remains the small possibility that *N. polysaccharea*, *N. meningitidis*, or *N. lactamica* could give the same NsppID result, but the likelihood of this occurring is very small if used as a confirmatory test of the COBAS Amplicor CT/NG assay with the more restrictive U.S. guidelines on the type of specimens described.

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