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A comparison of three real-time PCR assays for the confirmation of *Neisseria gonorrhoeae* following detection of *N. gonorrhoeae* using Roche COBAS AMPLICOR

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ABSTRACT

Three different real-time PCR assays were evaluated as confirmatory tests for *Neisseria gonorrhoeae* after initial screening using the COBAS AMPLICOR *Chlamydia trachomatis* and *N. gonorrhoeae* duplex assay. The target genes used for the confirmation were the *gyr*, *cppB* and 16S rRNA genes. Analytical specificity was determined by testing 60 strains belonging to different bacterial species and/or serogroups. The primers chosen from the 16S rRNA gene for confirmation of *N. gonorrhoeae* were highly specific, showed no cross-reactivity with other bacteria included in the study, and had an analytical sensitivity of 1 CFU. Of 192 clinical specimens that were positive for *N. gonorrhoeae* according to the COBAS AMPLICOR assay, 42 were confirmed as positive using the 16S rRNA gene target, 26 were confirmed using the *cppB* target, and 30 were confirmed using the *gyr* target. It was concluded that the real-time PCR assay targeting the 16S rRNA gene is a useful confirmatory assay to complement the COBAS AMPLICOR screening test for *N. gonorrhoeae*.

Keywords COBAS AMPLICOR, confirmation, diagnosis, *Neisseria gonorrheae*, real-time PCR, rRNA gene

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INTRODUCTION

Neisseria gonorrhoeae infection is one of the most prevalent sexually transmitted diseases (STDs) that affect both genders [1]. Diagnosis of *N. gonorrhoeae* in the early stage of infection can be difficult, since a significant proportion of gonococcal infections are asymptomatic or have non-specific symptoms [2,3]. It is essential that the detection methods used can confirm the presence of the organism so that appropriate antibiotic therapy can be initiated. Culture is still considered to be the reference standard for the diagnosis of *N. gonorrhoeae* infection. Under optimal laboratory conditions, culture has sensitivity in the range of 85–95% for acute infection [4]. However, factors such as specimen type and quality, as well as transport conditions, need to be stringently controlled to achieve this level of sensitivity, because of the fragility of the organism [5].

Nucleic acid amplification tests (NAATs), either in-house or commercial assays, targeting *N. gonorrhoeae* have been shown to have varying degrees of specificity and sensitivity [6–11]. NAATs do not require viable bacteria, and can therefore circumvent the problems of specimen collection and transportation for this fragile organism, especially when specimens are collected in remote areas. A commercial NAAT that is widely used in the diagnosis of STD is the COBAS AMPLICOR assay; this is an automated multiplex PCR that detects both *Chlamydia trachomatis* and *N. gonorrhoeae*. However, a major drawback of this assay with respect to the detection of *N. gonorrhoeae* is the cross-reactivity seen with other *Neisseria* spp. [12–15]. To overcome this problem, a confirmatory assay for *N. gonorrhoeae* should be used before the results are reported definitively [12,16].

The objectives of the present study were to evaluate the *N. gonorrhoeae* gyrase (*gyr*) gene, the *cppB* gene located on the *N. gonorrhoeae* cryptic

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plasmid [17] and the *N. gonorrhoeae* 16S rRNA gene as potential genetic targets for confirmation of an initial positive screening result for *N. gonorrhoeae* according to the COBAS AMPLICOR test, and to adapt these assays to the real-time LightCycler (LC) platform.

MATERIALS AND METHODS

Bacterial strains and analytical specificity

The analytical specificity of the three confirmatory assays was determined by assaying 60 bacterial strains that were selected on the basis of being either closely related phylogenetically to *N. gonorrhoeae* or present in the urogenital tract. These bacteria were *Actinomyces naeslundii* (one clinical isolate), *Candida albicans* (ATCC 10231, ATCC 90028), *Enterococcus durans* (ATCC 6065), *Enterococcus faecalis* (ATCC 29212, ATCC 5199), *Enterococcus faecium* (ATCC 35667), *Escherichia coli* (ATCC 25922, ATCC 35218), *Fusobacterium nucliatom* (ATCC 25586), *Gardnerella vaginalis* (ATCC 14018), *Haemophilus ducreyi* (one clinical isolate), *Lactobacillus confuses* (one clinical isolate), *Moraxella catarrhalis* (ATCC 25240, ATCC 25238), *Mycoplasma hominis* (two clinical isolates), *Neisseria cinerea* (ATCC 3308, one clinical isolate), *Neisseria flavescens* (ATCC 13120), *N. gonorrhoeae* (ATCC 43069, one clinical isolate), *Neisseria lactamica* (ATCC 23970, ATCC 23971), *Neisseria meningitis* (ATCC 13102), *N. meningitidis* serogroup 29E (one clinical isolate), *N. meningitidis* serogroup A (one clinical isolate), *N. meningitidis* serogroup B (ATCC 13090, one clinical isolate), *N. meningitidis* serogroup C (ATCC 13102, one clinical isolate), *N. meningitidis* serogroup W135 (two clinical isolates), *N. meningitidis* serogroup X (two clinical isolates), *N. meningitidis* serogroup Y (two clinical isolates), *N. meningitidis* serogroup Z (two clinical isolates), *Neisseria mucosa* (ATCC 19695, one clinical isolate), *N. mucosa heidelbergensis* (ATCC 25999), *Neisseria polysaccharea* (AATCC 43768, one clinical isolate), *Neisseria sicca* (one clinical isolate), *Neisseria subflava* (one clinical isolate), *N. subflava* biovar *subflava/flava* (Roche 6122), *N. subflava* biovar *subflava/flava* (Roche 6115), *N. subflava* biovar *flava* (Roche 6129), *Peptostreptococcus* sp. (one clinical isolate), *Propionibacterium lymphophilum* (ATCC 27520), *Propionibacterium* sp. (one clinical isolate), *Staphylococcus epidermidis* (ATCC 12228, ATCC 14990), *Staphylococcus saprophyticus* (ATCC 15305, one clinical isolate), *Streptococcus agalactiae* (ATCC 12386), *Streptococcus bovis* (ATCC 49147, ATCC 9809) and *Vagococcus* sp. (one clinical isolate). All of the clinical isolates were identified according to phenotypic criteria. All bacterial cultures were standardised to a 30% transmittance and 200 μL of suspension were used for DNA extractions.

Analytical sensitivity assays for the three targets

To determine the analytical sensitivity of the three confirmatory assays, six strains of *N. gonorrhoeae* (four clinical isolates and two reference strains, ATCC 49226 and ATCC 43069) were standardised to a 30% transmittance and ten-fold dilutions were made from 10^{-1} to 10^{-9}. An aliquot of 5 μL was inoculated in duplicate on chocolate agar plates (Oxoid; Nepean, Ontario, Canada) and colonies were counted after incubation for 48 h at 37°C to determine the number of CFU/mL used in the PCR assays. DNA extraction was performed using 200-μL aliquots of the cell suspensions.

DNA extraction and PCR

DNA was extracted using the automated MagNA Pure LC instrument (Roche Diagnostics, Laval, Quebec, Canada) in association with DNA Isolation Kit I (Roche Diagnostics), as specified by the manufacturer. Amplification using the COBAS AMPLICOR system was also performed as recommended by the manufacturer. A 200-μL aliquot of the samples that were positive according to the COBAS AMPLICOR assay was used in an extraction with a final elution volume of 200 μL; 5-μL portions of the eluted DNA were then used in the PCR assays for the *gyr*, *cppB* and 16S rRNA genes. Amplification of the *gyr* and *cppB* [17] genes was performed in capillaries using the LC instrument (Roche Diagnostics), with 20-μL reactions containing 0.5 μM each primer (Table 1), 1 × LC FastStart DNA Master SYBR Green 1 (Roche Diagnostics), and 2.5 mM $MgCl_2$. For the PCR targeting the 16S rRNA gene, the amplification mixture contained 1 × LC FastStart DNA Master Hybridization Probes Reaction Mix (Roche Diagnostics), 0.5 μM each primer (Table 1), 2.5 mM $MgCl_2$ and 0.2 μM hybridisation probes in a total volume of 20 μL. A positive control (ATCC 49226) and a negative control (water only) were included in each LC-PCR run. Primers, probe sequences and amplification conditions are summarised in Table 1.

For specimens that were positive according to the 16S rRNA gene real-time PCR confirmation assay, DNA sequencing was performed to confirm that the amplified product was the expected gene target. The PCR products were separated by electrophoresis on agarose 1.5% w/v gels, excised from the gels and purified using a QIAquick gel purification kit (Qiagen, Valencia, CA, USA), and then sequenced using an ABI 3700 sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The

Table 1. PCR primer sequences and conditions for different targets

Target	Position	Primer and probe sequences	PCR conditions
gyr (this study)	168–187	5′-ACTGTACGCGATGCACGAGC	94°C/10 min, followed by 45 cycles of 94°C/10 s, 61°C/10 s, 72°C/15 s.
	615–635	5′-TCGGTTTTGGGTTCGTCCAAA	Melting curve: 95°C, 51°C/10 s, increase temperature to 95°C at a rate of 0.2°C/s with continuous acquisition
cppB [17]	3141–3160	5′-GTCACGCATACCCGCGTTGC	94°C/10 min, followed by 45 cycles of 94°C/10 s, 45°C/10 s, 72°C/15 s.
	3512–3531	5′-CGAAGACCTTCGAGCAGACA	Melting curve: 95°C, 35°C/10 s, increase temperature to 95°C at a rate of 0.2°C/s with continuous acquisition
16S	124–144	5′-TATCGGAACGTACCGGGTAGC	95°C/10 min, followed by 45 cycles of 95°C/15 s, 59°C/10 s, 72°C/17 s.
rRNA [30]	517–537	5′-GTATTACCGCGGCTGCTGGCA	Melting curve: 95°C, 45°C/20 s, increase temperature to 95°C at a rate of 0.2°C/s with continuous acquisition
16S rRNA	440–461	TTGTCAGGGAAGAAAAGGCTGT-FL	
probes (this study)	463–478	LC Red640-GCCAATATCGGCGGCC-PH	

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sequences were compared with those for *N. gonorrhoeae* strain ATCC 49226 using DNA STAR Megalign software (DNASTAR Inc., Madison, WI, USA). The sequences obtained were also screened against GenBank using BLAST (http://www.ncbi.nlm.nih.gov/Genbank/GenbankSearch.html) to confirm that the PCR products were those expected from the *N. gonorrhoeae* genes targeted. These samples were further confirmed using a conventional PCR targeting the *orf1* gene [18], followed by *Hin*dIII restriction (PCR–restriction fragment length polymorphism analysis). The forward and reverse primer sequences were 5′-CAACTATTCCCGATTGCGA (221–239) and 5′-GTTATACAGCTTCGCCTGAA (461–239), respectively. The PCR comprised 94°C for 10 min, followed by 45 cycles of 94°C for 30 s, 52°C for 30 s and 72°C for 1 min, with a final extension at 72°C for 10 min. Each 50-μL reaction contained 40 pmol each primer, 100 μM each dNTP (Invitrogen Life Technologies, Burlington, Ontario, Canada), 1 U Ampli*Taq* Gold (Roche Diagnostics) and 2 μL template. Following amplification, 3 μL of the product was subjected to *Hin*dIII digestion, according to the manufacturer's specification, and was then analysed by electrophoresis on agarose 2% w/v gels.

Clinical specimens

The clinical samples were collected from patients attending the Sexually Transmitted Disease Clinics or Family Planning Clinics in Edmonton, Alberta, Canada between 1 January 2003 and 30 June 2004. Urine from males was collected in sterile containers, while cervical swabs from females were inserted in dry collection tubes; both types of specimen were transported to the laboratory at room temperature. Upon receipt in the laboratory, urine samples were vortexed and a 200-μL aliquot of the supernatant was used for DNA extraction in the MagNA Pure LC instrument. Cervical swabs were inserted into 1 mL of sterile saline, vortexed and then removed from the tube, after which 200 μL was used for extraction, as for the urine samples. For each sample processed, the internal control provided in the COBAS AMPLICOR kit was added to the reaction mixture and was co-amplified with the target DNA to monitor potential inhibition. Positive and negative controls supplied by the manufacturer, as well as in-house positive and negative controls, were included in each run.

For the COBAS AMPLICOR assay, results were interpreted according to the product insert. An optical density at A_{660} of ≥0.2 units was interpreted as a presumptive positive result. If the IC and the *N. gonorrhoeae* target A_{660} values were <0.2, the assay was interpreted as showing inhibition; in such instances, the test was repeated after diluting specimens 1:10. Collection of a follow-up specimen was requested if the repeated test still showed evidence of inhibition. According to the algorithm established in the laboratory at the time of the comparison study, all samples that were positive according to the COBAS AMPLICOR assay were re-extracted within 24 h and confirmed using a different gene target. This protocol also included an extra step involving cross-checking by two laboratory assistants to ensure that the correct specimen was identified for confirmation. During the validation period of 1.5 years, all specimens that were positive for *N. gonorrhoeae* according to the COBAS AMPLICOR assay were tested by real-time PCR for the *gyr*, *cppB* and 16S rRNA gene targets, as well as by the standard laboratory confirmation assay using PCR in a 96-well format and probe hybridisation for the 16S rRNA with the primers and probes provided by Roche Diagnostics. A positive *N. gonorrhoeae* result was determined by comparing the melting temperature in the SYBR Green assays (*gyr* and *cppB* real-time PCRs) with the positive control; a positive result was recorded for the real-time 16S rRNA assay on the basis of an analysis of the amplification curve and the melting temperature.

Statistical analysis

Differences among the results obtained using the 16S rRNA gene, the *gyr* gene and the *cppB* gene as the target were analysed using the McNemar method.

RESULTS

Analytical specificity

The COBAS AMPLICOR assay for *N. gonorrhoeae* cross-reacted with *N. subflava* biovar *subflava/flava* and *N. subflava* biovar *flava*. *N. mucosa* and *N. mucosa heidelbergensis* were also misidentified as *N. gonorrhoeae* when the *N. gonorrhoeae gyr* gene was the target in the confirmatory assay and melting temperature was used for the analysis. The melting temperatures using the positive control strains of *N. gonorrhoeae* were 88°C and 86°C for the *gyr* and *cppB* genes, respectively, with ±1°C tolerance for the melting curves in both assays. The melting temperatures for *N. mucosa*, *N. mucosa heidelbergensis* and *N. gonorrhoeae* were 88–89°C, and the sizes of the *gyr* amplicons from these isolates were identical when analysed using agarose gel electrophoresis. Sequencing revealed that there was >90% homology between the amplicons for *N. gonorrhoeae* and *N. mucosa*, but only 35% homology for *N. gonorrhoeae* and *N. mucosa heidelbergensis*. No misidentifications were obtained with primers targeting the *cppB* and 16S rRNA genes for any of the bacterial strains assayed. Detection of 16S rRNA gene amplicons used a hybridisation probe, but the melting curve was always checked for confirmation. The melting temperature of all positive samples was 60 ± 1°C, with a cycle cut-off (C_t) value of 36 cycles.

Analytical sensitivity

The detection limit for the COBAS AMPLICOR assay was 10 CFU/mL, while that for detection of the *gyr* gene by LC PCR was 100 CFU/mL. Both the *cppB* and 16S rRNA LC assays had a detection limit of 1 CFU/mL, and were ten-fold and 100-fold more sensitive than the COBAS AMPLICOR and *gyr* assays, respectively.

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Clinical specimens

Of 7731 clinical specimens that were screened using the COBAS AMPLICOR assay, 192 (2.5%), comprising 156 swabs and 36 urine samples, were positive according to the manufacturer's criteria. These samples were further analysed using the three LC confirmatory assays targeting the *gyr*, *cppB* and 16S rRNA genes of *N. gonorrhoeae*. The number of positive PCR results confirmed using the different targets, together with the corresponding A_{660} values from the COBAS AMPLICOR assay, are summarised in Table 2. In total, 32 of the 42 samples that were positive according to the 16S rRNA gene PCR were confirmed by at least one of the other confirmatory PCR assays (*gyr* or *cppB*). The remaining ten samples were only confirmed as *N. gonorrhoeae* according to the 16S rRNA assay; the C_t values for these ten samples are shown in Table 3, with an average C_t value for these samples of 28.48. The amplified products were purified, sequenced and checked against sequences contained in GenBank, and were also confirmed using PCR to amplify the *orf1* gene. The latter yielded a PCR product of 260 bp (Fig. 1a), which generated two fragments of 107 and 153 bp following digestion with *Hin*dIII (Fig. 1b).

Table 2. Correlation of optical density (OD) values generated with the COBAS AMPLICOR assay and positive results obtained according to PCRs for different targets

COBAS AMPLICOR OD values	Total no. of specimens	*gyr*	*cppB*	16S rRNA
>0.2–2.0	94	1	0	1
>2.0–3.5	32	1	0	3
>3.5	66	28	26	38
Total	192	30	26	42

Table 3. Crossing point (C_t) values for ten specimens confirmed as positive only by the 16S rRNA gene assay

Specimen no.	Type of specimen	Real-time PCR C_t value[a]
3389	Swab	32.30
5077	Swab	30.14
5758	Swab	26.54
3772	Swab	30.68
7636	Urine	23.83
4615	Urine	27.80
5224	Urine	35.66
4150	Urine	28.12
1143	Urine	26.18
7091	Urine	23.72

[a]The C_t value is the number of cycles after which the real-time PCR indicated a positive result.

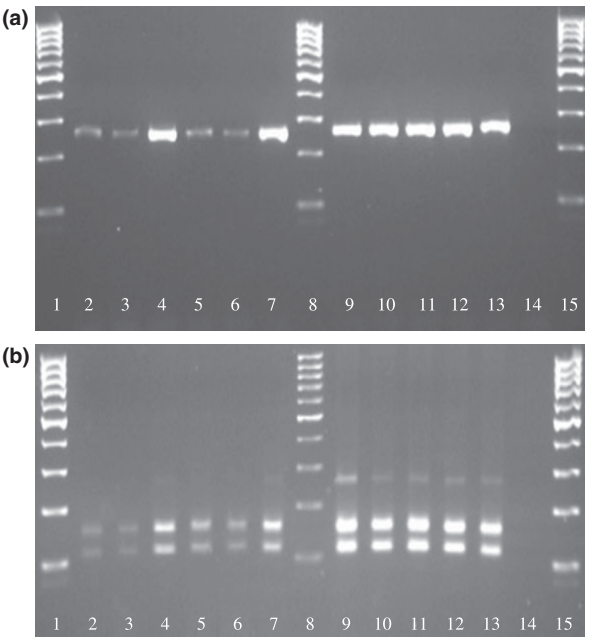

Fig. 1. (a) Amplified products (260 bp) generated by PCR using the *orf1* gene as target. (b) *Hin*dIII fragments (107 bp and 153 bp) generated from the PCR products shown in (a). Lanes: 1, 8 and 15, 100-bp marker; 2–7 and 9–12, clinical isolates; 13, *Neisseria gonorrhoeae* positive control strain ATCC 49226; 14, negative control (water).

Two specimens confirmed as *N. gonorrhoeae*-positive according to PCR for the *cppB* and 16S rRNA genes, but not according to PCR for the *gyr* gene, had A_{660} values in the COBAS AMPLICOR assay of >3.5. Twenty-four of the 42 samples were confirmed as *N. gonorrhoeae*-positive by PCR for all three targets; each of these had an A_{660} value in the COBAS AMPLICOR assay of >3.5. Six (14%) specimens were confirmed as positive according to PCR for the *gyr* and 16S rRNA genes, but not according to PCR for the *cppB* gene; this result is in line with the findings of earlier studies of *N. gonorrhoeae* isolates from the same STD population, which indicated that 12% of isolates lacked the *cppB* cryptic plasmid (L. Chui, J. Kakulphimp and W. Albritton, unpublished results). However, overall, there were significant differences in the confirmation of *N. gonorrhoeae*-positive specimens according to PCRs targeting the 16S rRNA gene and the *gyr* ($p < 0.001$) and *cppB* ($p < 0.001$) genes.

DISCUSSION

All of the clinical specimens included in this study were initially assayed by the COBAS

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AMPLICOR duplex assay for *C. trachomatis* and *N. gonorrhoeae*. This assay has been validated for urethral and endocervical swabs and for urine specimens, with sensitivities of 92.4–100% for swabs and of 42.3–94.1% for urine, and with specificities of 95.9–99.9% for all three types of specimen [8,9,13,15,19–23]. However, a major drawback of this assay is its reported cross-reactivity with other *Neisseria* spp. and lactobacilli [5,12,14,20,21]. The present study provides further evidence of this continuing problem.

To address this problem, an alternative algorithm, designed to expand the equivocal zone for the COBAS AMPLICOR assay, that increases the specificity of the assay has been proposed [9,21]. In a multicentre trial using an expanded equivocal zone [21], it was observed that 53% of the specimens with A_{660} values of 0.2–3.5 were negative according to the 16S rRNA LC assay. Using the same approach, the present study found that only 42 (22%) of 192 positive samples were confirmed as positive for *N. gonorrhoeae* according to two of the three real-time confirmation assays tested, by *orf1* gene PCR–restriction fragment length polymorphism, or by sequencing the amplified 16S rRNA PCR products. When the expanded equivocal zone was applied, 57.5% (38/66) of the samples with A_{660} values >3.5 were confirmed according to 16S rRNA PCR, 9.4% (3/32) with values >2.0–3.5, and 1.1% (1/94) with values >0.2–2.0. Therefore, the majority of the confirmed positive samples had A_{660} values >3.5. This finding is in accord with previous studies [5,14,16,21]. Based on the results of the present study, if the original cut-off A_{660} value of 0.2 recommended by the manufacturer was used, 2.5% (192/7731) of the samples would have been identified as positive for *N. gonorrhoeae*, compared with 0.54% (42/7731) based on positive results with at least two PCR confirmation assays or PCR and sequencing of the amplified products. There was also 100% concordance between the 16S rRNA gene LC assay and the established standard confirmation assay using the 16S rRNA gene target provided by Roche Diagnostics (results not shown).

When multi-assay consensus was used as the criterion for *N. gonorrhoeae* confirmation, 14% of the samples that were positive according to COBAS AMPLICOR were confirmed by the *gyr* and 16S rRNA LC assays, but gave a negative result with primers targeting the cryptic plasmid *cppB* gene. A failure to amplify the *cppB* gene has been documented in previous studies [12,14,24–26]. In one study [12], 5.7% of positive *N. gonorrhoeae* clinical samples collected in The Netherlands failed to yield a *cppB* amplicon, and 5.8% of *N. gonorrhoeae* isolates from patients in Amsterdam also showed this characteristic [24]. However, *cppB*-negative strains were distributed unevenly throughout The Netherlands, suggesting that *N. gonorrhoeae* isolates lacking the *cppB* gene may be associated with particular patient populations [12]. Palmer *et al.* [14] demonstrated that 18 of 20 *N. gonorrhoeae* isolates of the PA°U auxotype, which are typically plasmid-free strains [27], could not be detected by PCR targeting the *cppB* gene. Other studies have revealed that the *cppB* gene is not only found in the cryptic plasmid [28], but is also integrated into the chromosome of some strains [25,29]. Chromosomal integration of the *cppB* gene was the rationale that supported the use of the *cppB* gene as a target for detecting *N. gonorrhoeae.* However, the present study, as well as previous studies, revealed that significant numbers of isolates do not possess this target, and the *cppB* gene can therefore not be recommended for use as a target in the confirmation of *N. gonorrhoeae* [12,14,24].

False-positive results have also been observed when using the *cppB* gene as a target, and these have been attributed to cross-reactions with *N. cinerea*, *N. flavescens*, *N. lactamica*, *N. subflava* and *N. sicca* [14]. Homology has been observed between the cryptic plasmid of *N. gonorrhoeae* and plasmids in other *Neisseria* spp. [14]. Nevertheless, although these species were included in the present study, no amplified *cppB* DNA was detected. Similarly, the *gyr* gene would not be the target of choice for *N. gonorrhoeae*, because of cross-reactions with *N. mucosa* and *N. mucosa heidelbergensis*. The *gyr* LC assay had the least sensitivity of the assays tested. It was not possible to explain why the ten samples that were positive only according to the 16S rRNA assay were not detected by the *gyr* assay, but a possible explanation might be a change in the sequence of the *gyr* gene target. According to McNemar analysis, there was a significant difference ($p < 0.001$) between the results obtained using the 16S rRNA and *gyr* assays. Similarly, there was a significant difference ($p < 0.001$) between the results obtained using the 16S rRNA and *cppB* assays.

On the basis of these results, the COBAS AMPLICOR *C. trachomatis/N. gonorrhoeae* assay

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can be used as a preliminary screening test for *N. gonorrhoea*, provided that positive results are confirmed using the 16S rRNA assay. By using the MagNa Pure LC instrument, DNA can be extracted from clinical specimens and amplified on an LC platform with minimal handling. The 16S rRNA LC PCR assay was more sensitive and specific than the *gyr* and *cppB* LC assays, and the entire confirmation process can be completed within 10 h.

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