Sylvain Robinet* and François Parisot

Performance assessment of the Allplex™ STI Essential real-time PCR assay for the diagnosis of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections in genital and extra-genital sites

<https://doi.org/10.1515/labmed-2019-0030>

Received February 27, 2019; accepted June 18, 2019; previously published online August 2, 2019

Abstract

Background: Commercial kits performing *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) nucleic acid amplification tests (NAATs) for genital samples are recommended in association with culture, but the majority of real-time polymerase chain reaction (PCR) methods have not received regulatory approval for diagnostics in extra-genital sites. Since 2017, only the Hologic® Aptima Combo2 assay has an *in vitro* diagnostic (IVD) certification from the European Medicine Evaluation Agency.

Methods: We assessed the Allplex™ STI-Essential Assay (EA) for the diagnosis of NG and CT in both genital and extra-genital sites. The performance of the extraction step was studied by means of a standard curve between the concentration of expected cultivable gonococci and the cycle threshold (C_t). Three later-generation NAATs were used as comparators, particularly to assess the specificity (Sp).

Results: A relation between the gonococcal concentration, expressed as colony-forming unit (CFU) per milliliter logarithm, and the C_t was shown to be linear irrespective of the matrices (95% confidence interval [CI]). The detection limit was 10 CFU/mL, contrasting with the relatively poor sensitivity of culture due to inhibitory effects such as pH and the overgrowth of the commensal flora. NG molecular diagnostic is complex and the method comparisons showed some discrepancies when C_t was above 34. We decided to include interpretative comments on our reports on the basis of the C_t result. For CT, comparisons displayed a satisfactory agreement, and the detection limit was 50 copies/mL.

Conclusions: The Seegene Allplex™ STI-EA showed acceptable performance characteristics for the detection of genital and extra-genital NG and CT.

Keywords: *Chlamydia trachomatis*; extra-genital specimens; *Neisseria gonorrhoeae*; real-time PCR; sexually transmitted diseases.

Brief summary: A performance survey of the Allplex™ STI Essential PCR assay for the detection of *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) in genital and extra-genital sites. Relevance of culture as a tool for diagnosis of gonococcal infections. Interpretative comments proposal for positive polymerase chain reaction (PCR) results for NG in a context of low prevalence.

Introduction

Neisseria gonorrhoeae (NG) and *Chlamydia trachomatis* (CT) serovars D through K are the two most prevalent bacterial sexually transmitted infections worldwide [1].

Although gonorrhea is an ancient disease that has affected humans for centuries and effective therapy has been available since the early twentieth century, this sexually transmitted disease (STD) remains prevalent: gonorrhea is the second most commonly reported disease in the United States and 555,608 cases were recorded by the Centers for Disease Control and Prevention (CDC) [2]. As with other STDs, the reporting of gonorrhea is incomplete and the CDC estimates that approximately 820,000 infections of gonorrhea occur yearly in the United States, whereas estimations amount close to 2,860,000 for CT. The annual incidence of the latter in France is shown to be nearly 6 times higher than NG [3], and the literature review reveals that co-infection with CT/NG represents less than 6% of positive samples for CT in asymptomatic patients [4]. We found similar statistics within the laboratory for the year 2018: among the 11,300 clinical specimens tested by polymerase chain reaction (PCR),

*Correspondence: Sylvain Robinet, PhD, Eurofins – Clinical Diagnostics, Laboratory of Medical Microbiology, 2 rue Eugène Coste, 06300 Nice, France, E-Mail: sylvainrobinet@eurofins.com
François Parisot: Eurofins – Clinical Diagnostics, Laboratory of Medical Microbiology, Nice, France

5.6% were positive for CT, 1.4% for NG and 0.3% for both CT/NG.

The cornerstone of public health control is the detection and treatment of these STDs, so as to prevent sequelae and limit disease transmission [5, 6]. Even though gonococcal culture remains the “gold standard” to monitor developing resistance to current antibiotic treatments, published data support the fact that PCR is more sensitive than culture for the diagnosis of NG and CT infections [7, 8].

Commercial nucleic acid amplification tests (NAATs) for CT/NG diagnosis have received regulatory approval for *in vitro* diagnostic (IVD) from various world health authorities such as the Food and Drug Administration (FDA) or the European Medicine Evaluation Agency (EMA). The validated specimens are female endocervical or self-collected and clinician-collected vaginal swab specimens, male urethral swab specimens, and male and female urine specimens. Allplex™ STI-Essential Assay (EA) allows simultaneous detection of seven pathogens, among which some are systematically responsible for sexually transmitted infections [9]. However, the assay is not validated for oropharyngeal and ano-rectal specimens, while recommendations, driven by a relatively poor sensitivity of culture, show that NAATs for CT/NG are indicated for such sites [10–12]. So far, only the Aptima Combo2 method is EMA-IVD cleared for extra-genital specimens [13].

The clinical situations include symptomatic and asymptomatic subjects with an increased risk: young population sexually active, history of STD, HIV positive, men-who-have-sex-with-men, use of drugs and penitentiary population [14]. In addition, analysis from extra-genital sites should also be realized with treated subjects, symptomatic evolving favorably or asymptomatic, as a test for microbiological cure after a period of 2 weeks post cure. Lastly, recent guidelines also underline the importance of screening within an assessment of reactional arthritis (for subjects with sexual risk behavior or symptomatic), and for subjects with a behavior of gonococcal disease as a test of new late contamination after a period of 3–6 months post cure [15].

Surveys are thus necessary to assess the Allplex™ STI-EA for extra-genital matrices, which can be complex due to the presence of various inhibitors and commensal *Neisseria* leading to false-positives and false-negatives [16, 17]. It is also worth recalling that performance surveys for qualitative and semi-quantitative methods are compulsory for their accreditation in clinical laboratories.

The goal of this study was to (i) assess the efficiency of the extraction step and establish a standard curve with intervals of prediction between the concentration of expected cultivable bacteria and the cycle threshold (C_t); (ii) discuss the relevance of culture as a tool for detecting NG at both genital and extra-genital sites; (iii) study the specificity (Sp) of the kit by comparing its performances with that of the well-characterized Aptima Combo2, Abbott *m2000* and Roche cobas 4800 assays for CT/NG detection; (iv) propose interpretative comments for routine NG-detected specimens, as it is strongly encouraged by quality assurance programs.

Materials and methods

Molecular testing and culture

Surveys were realized between June 2017 and October 2018. The multi-channel automated liquid handling system Microlab NIMBUS™ (Hamilton, Bonaduz, Switzerland) operates with the STARMag™ Universal Cartridge kit (Seegene, Seoul, South Korea) for DNA extraction. The latter begins with a cell lysis with proteinase K to make DNA free, which is then bound to magnetic beads (Seegene) using a sodium perchlorate 20–40% and ethanol 35–55% buffer. DNA is purified with three washes with a buffered solution (sodium perchlorate 5–20% and ethanol 20–35%) before elution from magnetic beads at 56 °C.

Using the Allplex™ STI-EA kit, the analyzer CFX96™ (Biorad, Marnes-la-Coquette, France) allows a particular real-time multiplex method, called Tagging Oligonucleotide Cleavage Extension™. This technology enables the identification of multiple target analytes simultaneously in a single fluorescent channel. The signal can be measured in real-time thanks to the catcher melting temperature.

For preliminary assays, real-time PCR was carried out with two serial dilutions of a NG strain (ATCC® 19424, Microbiologics®, St. Cloud, MN, USA), previously cultured for 24 h and 96 h, respectively, on chocolate agar/PolyVitest™ medium. Each dilution sample was made with a brain-heart-infusion broth (bioMérieux, Marcy l’Etoile, France).

To study the extraction step and to establish a predictive model between the concentration of expected cultivable bacteria and the C_t , we used a dilution panel of each of the following matrices: genital, oropharyngeal, ano-rectal, sperm, urethral and first-pass urine. The latter were made with pools of female and male

specimens (eSwab™, Copan, Brescia, Italy) or urine collection tubes (Becton Dickinson, Le Pont de Claix, France), previously tested negative for NG. For sperm, 300 µL of the genital secretion was added to 1 mL of liquid Amies. A 3.0 ± 0.1 MacFarland ($n=48$; 95% confidence interval [CI]) mother suspension of NG ATCC® 19424 enabled to prepare the serial dilutions. It is important to note that every sample was amplified with specific primers of human beta-globin, a universal cellular gene, to ensure that amplifiable DNA has been correctly extracted from specimens.

Standardized cultures were performed alongside PCR for all samples using a PREVIIsola® (bioMerieux). Eighteen microliters of each specimen were plated on a culture medium, made of chocolate agar and PolyVitek™ (bioMerieux).

For testing the proposed predictive model, analysis of 52 clinical samples was performed. Specimens were obtained from patients using either eSwab™ or the previously mentioned urine collection tubes. In some cases, additional plating on a selective medium was realized. Identifications were performed using a VITEK-MS device (bioMerieux).

Method comparisons

The Allplex™ STI-EA was compared to the ribosomal RNA (rRNA)-based Aptima Combo2 assay (Hologic®, Marlborough, MA, USA), and to the DNA-based RealTime CT/NG assay that uses the Abbott m2000 molecular platform. Clinical specimens were collected in duplicate for the former, according to the required Aptima collection Kit and following the specified tubes by Seegene. All Aptima collection tubes were stored at 2–8 °C and transported to the Biomnis Laboratories (Lyon, France) where they were stored at 2–8 °C and tested within 5 days of receiving. For the latter, specimens detected for NG and/or CT were stored at –20 °C and sent as a confirmatory test to the Reference Center for Gonococcal Infections (CNRNG, Saint-Louis hospital, Paris, France). Overall, 95 and 110 clinical samples were compared for NG and CT, respectively. Sequencing analysis were also performed by Seegene to our confirmatory request for 20 discrepancies.

Moreover, the laboratory subscribes to external quality programs such as the Association de Biologie Praticienne (ABP, France) and The Royal College of Pathologists of Australasia Quality Assurance Program (RCPA-QAP, Australia) for molecular diagnostics. Thereby, comparisons with the DNA-based Roche cobas 4800 CT/NG assay were also carried out.

Statistical analysis

Analysis of variance (ANOVA) followed by an F-test was performed using the spreadsheet application Microsoft Excel®. The latter was also used for linear regressions where the coefficient of determination r^2 allows to appreciate the quality of the adjustment.

One of the purposes of linear regression is to propose predictions for the variable to be explained (Y). The latter can be predicted using the following estimated model:

$$\widehat{y}_{n+1}^p = \widehat{b}_0 + \widehat{b}_1 x_{n+1}$$

The interval of predication at the $(1 - \alpha)$ level is given in the following equation and this determination for all the points of the linear regression line leads to a hyperbola, where t represents the quantile $(1 - \alpha/2)$ from a t -distribution with $n - 2$ degrees of freedom:

$$IP_{\widehat{y}_{n+1}^p}^{1-\alpha} = \left[\widehat{y}_{n+1}^p - t_{1-\alpha/2} \widehat{\sigma} \sqrt{1 + \frac{1}{n} + \frac{(x_{n+1} - \bar{x})^2}{\sum (x_i - \bar{x})^2}}, \right. \\ \left. \widehat{y}_{n+1}^p + t_{1-\alpha/2} \widehat{\sigma} \sqrt{1 + \frac{1}{n} + \frac{(x_{n+1} - \bar{x})^2}{\sum (x_i - \bar{x})^2}} \right]$$

Residual values $e_i = y_i - \widehat{y}_i$ are independent random variables having a normal distribution $\mathcal{N}(0, \sigma)$.

A non-parametric test Kappa allowed to measure the inter-rater agreement for categorical items. Statistical significance has been characterized in the literature [18, 19].

Sensitivity, Sp, accuracy and predictive values were calculated using the software MedCalc®.

Ethical approval

The conducted research is not related to either human or animal use.

Results and discussion

Preliminary assays

In a natural sample, microbiota contain dead, live and viable but non-cultivable cells. All of them are detected by PCR, whereas by culture only live cells are detected and expressed as colony-forming unit (CFU). To establish the best standard curve between the concentration

of cultivable bacteria and the measured C_t , the influence of culture age was considered by preliminary essays on both measured C_t and amount of live cells. Dilutions were prepared by using a matrix without any kind of potential inhibitor. No difference for the measured C_t was noted between 24-h and 96-h cultures (95% CI; data not shown), but the observed levels for cultivable bacteria were found to be significantly lower for the 96-h culture than those measured for 24 h (Figure 1). As detailed in Table 1 and illustrated in Figure 1, a dilution panel prepared with NG cultured for 24 h enabled to establish the best sensitive calibration between live bacteria and C_t . No culture was found to be positive at the highest dilution (D1).

PCR performance and relevance of culture

The extraction step was assessed by using dilution panels of an NG strain in various matrices, and the levels of C_t were associated with their corresponding cultivable

bacterial concentrations established in the preliminary assays (Table 1). ANOVA was performed with PCR results of each gonococcal dilution from all matrices. ANOVA enabled to test for differences among the C_t means of each dilution by examining the amount of variation within each of these samples, relative to the amount of variation between the samples. At a given dilution setting, ANOVA showed no statistical difference concerning the measured C_t between the matrices (p -value > 0.05; Table 2). A single standard curve with intervals of prediction between the expected gonococcal concentration and the C_t was therefore established with 252 measures, and was shown to be linear when C_t ranging from 12.7 ± 2.2 to 34.9 ± 1.8 (95% CI; Figure 2). The average efficiency of the PCR was $94.5 \pm 4.5\%$ (95% CI). The constants of the predictive model were not significantly different from those of the 24-h-BHI/eSwab™ regression line (slope: 3.4 ± 0.3 and intercept: 40.5 ± 1.7 ; 95% CI), which demonstrated the extraction effectiveness to prevent natural and synthetic inhibitors.

Figure 1: Comparison between observed levels of cultivable *N. gonorrhoeae*.

Table 1: Dilution panels with concentrations of cultivable *N. gonorrhoeae*.

Dilution	D 8	D 7	D 6	D 5	D 4	D 3	D 2	D 1
Volume, μ L	120 of MS ^a	67 of D 8	67 of D 7	67 of D 6	67 of D 5	67 of D 4	67 of D 3	67 of D 2
Volume of matrix, μ L	600	600	600	600	600	600	600	600
Concentration of <i>N. gonorrhoeae</i> ATCC® 19424 in Log CFU/mL								
Average of six replicates	7.95	6.95	5.95	4.95	3.95	3.03	1.76	–
Min–max	7.70–8.00	6.70–7.00	5.70–6.00	4.70–5.00	3.70–4.00	2.60–3.21	0–2.35	–

^aMS, mother suspension.

Table 2: ANOVA followed by F-test^a.

Dilution	D 8	D 7	D 6	D 5	D 4	D 3	D 2	D 1
Inter-group variations								
SS	8.73	11.60	6.47	5.90	13.15	16.85	14.49	4.26
df	5	5	5	5	5	5	5	5
Intra-group variations								
SS	30.59	46.87	39.46	30.41	40.03	59.94	67.36	1.70
df	30	30	30	30	30	30	27	5
F	1.71	1.48	0.98	1.16	1.97	1.69	1.16	2.50
p-Value	0.16	0.22	0.44	0.35	0.11	0.17	0.35	0.17

^aSS, sum-of-square; df, degrees of freedom.

For the vaginal matrix, 50% of D1 replicates were positive with C_t reaching values between 33.5 and 35.1. Although no isolate was shown, they corresponded to a theoretical concentration of 10 CFU/mL, D1 being a dilution in the tenth of D2. Analysis of D1 CI (95%) so showed that the hypothesis of linearity between 10^1 and 10^8 CFU/mL could not be rejected.

For culture, NG strains were only identified on 18 samples among the 48 tested and there was no positive dilution with less than or equal to 10^4 CFU/mL of gonococci. In addition, the bacterial concentration often remained lower than predicted for a given C_t . As expected, all culture

media showed the presence of abundant flora, mostly *Lactobacillus*, probably having an inhibitory effect on NG. It is well known that the anaerobic metabolism of the glycogen by vaginal flora and/or the epithelial cells themselves cause the vagina to become acidic between menarche and menopause, with a normal pH level between 3.6 and 4.5. The effect of pH on survival of gonococcal clinical isolates has been studied, and the lower limit of survival for stationary and log-phase cultures is typically 4.5. Moreover, it has been shown that no survivors could be detected with a pH value of 4 or lower [20]. This is consistent with the fact that endocervix is a better location than vagina for gonococcal growth, often leading to asymptomatic cervicitis, and should therefore be a site of choice for testing particularly when repeat sampling is appropriate.

The PCR performances for urethral specimens were similar to the ones of vaginal matrix with a reduced sensitivity for C_t values near 35. In contrast, an acceptable agreement between PCR and culture was achieved and almost all of the 30 positives results were within the 99% prediction interval. These observations suggested a relatively suitable environment for bacterial growth and could explain the success of the microorganism as a pathogen of the urethral tract with often symptomatic presentations.

Low numbers of gonococci were noticed for the oropharyngeal (19/48) and ano-rectal (1/48) samples, whereas a

Figure 2: Relation between C_t and concentration of *N. gonorrhoeae* for all matrices.

normal oral flora with α -*Streptococci* and commensal *Neisseria* could be seen for the former and an abundant intestinal flora for the latter (mostly *Enterobacteriaceae* and *Enterococci*). Commensal flora had an inhibitory effect on gonococcal growth, and its abundant character made bacterial isolation difficult. For clinical specimens, it would be justified to perform additional plating with a selective medium leading to antibiotic susceptibility testing as the antimicrobial resistance is facilitated through genetic exchange between oral commensal and pathogenic bacteria in the oropharynx [21, 22]. Additionally, recommended drug regimens such as ceftriaxone may not reach sufficient concentration levels in the oropharyngeal tissue [23]. PCR offered a reproducible tool for detecting NG up to 10^2 CFU/mL, but a relative lack of sensitivity at 10 CFU/mL was observed.

For semen, molecular testing showed a perfect linearity up to 33.6 ± 6.6 (95% CI), whereas cultures were poorly sensitive. The number of gonococci was far below that predicted, which seemed surprising at first as far as the examined media were poor. However, two compounds from prostatic secretions are known to have an antimicrobial effect for protecting spermatozoa against infection risk [24]. A diamine-oxidase acts on spermine and spermidine and produces oxidized derivatives which are responsible of a potent anti-bacterial effect, while zinc displays

a bactericidal activity for many Gram-positive and Gram-negative pathogens. In addition, it should be noted that gonococcal prostatitis and epididymo-orchitis are not very common as they occur in case of treatment failure or untreated urethritis which become asymptomatic in more than 95% of men within 6 months [25]. But gonococcal infection persists as long as there is no treatment and creates conditions for complications and sequelae.

The standard curve demonstrated perfect linearity up to $C_t = 33.9 \pm 2.0$ (95% CI) for urine and the highest dilution showed an average sensibility of 50%. With only 15/48 positives, all the culture results were outside the predicted interval, and this low sensitivity could be explained by the bactericidal properties of urine. It is well known that ordinary urinary pathogenic bacteria can grow well in most urines, but it was shown that human urine could kill NG rapidly and that the main determinant of killing was urinary pH. Additionally, urea and sodium chloride at physical concentration in urine also contribute to the killing of gonococci [26]. As each of these lethal factors is increased, concentrated urine can thus rapidly sterilize a large inoculum of bacteria. Besides, cystitis caused by NG has not been reported in the literature.

We tested the predictive model by studying specimens collected from various anatomical sites (Figure 3).

Figure 3: Predictive model tested with clinical specimens.

This model could help technicians and biologists by improving the bacterial location on cultures, quite particularly in case of small amounts, and additional plating on selective agar could also be decided.

With a majority of urethral swabs, 80% of the samples collected with a positive culture were within the predicted limits. In some cases, the number of colonies was far below what we had planned. Less than 10 gonococci were found, for instance, from a pelvic-peritoneal effusion, whereas the C_t was 14.5. But in this case, the patient had received an empiric antibiotic therapy with 1 g intramuscular ceftriaxone per day 24 h before sampling. Furthermore, the predictive model provided some help about an anal positive detection with C_t near 25. At this level, no more than 20–30 colonies could be ideally isolated but it was also necessary to bear in mind the negative impact of commensal flora. A selective medium was thus plated which allowed to identify two colonies of gonococci, while only digestive flora were found on standard medium. The same case occurred with an oral specimen for which only the selective medium was positive.

Overall, however, cultures often remained negative in our experience. But despite its poor sensitivity, the culture of NG should always be realized alongside PCR to evaluate suspected cases of gonorrhea treatment failure and to monitor developing resistance to current antibiotic treatments.

Method comparisons with focus on specificity

Although NAATs duplex CT/NG become widespread all over the world to the detriment of gonococcal culture, their main limit is cross-binding. For a given Sp and a same number of tested persons, the proportion of false-positives increases when the prevalence decreases [27, 28]. This combination between low prevalence and significant number of false-positives with NAAT-NG has led most guidelines to recommend assays targeting two different genes, in particular with the expected prevalence less than 5% or predictive positive value (PPV) less than 90% [29, 30]. The Sp can be undermined by ongoing genetic exchange between species within the *Neisseria* genus, and many strains have been described that give either false-positives or false-negatives in different commercial PCR systems. For instance, it was shown that commensal *Neisseria* of the throat could acquire the targeted DR-9 sequences of NG, and that the latter was able to replace large segments or its entire targeted *porA* pseudogene with a *Neisseria meningitidis porA* gene [31].

Using a dual-target approach for both CT and NG components, the Allplex™ STI-EA was compared with three later-generation NAAT assays (Table 3). The aim

Table 3: Performance of molecular detection by Allplex STI-EA for *N. gonorrhoeae* and *C. trachomatis* compared to those by Abbott RT CT/NG, Aptima Combo 2 and Roche cobas 4800 CT/NG assays^a.

Comparison	Number of assays	Positive samples with NG/CT STI-EA		Sensitivity	Specificity	Positive predictive value	Negative predictive value	Accuracy	Kappa	
		n	$\bar{x}_{ct}$							$\hat{\sigma}_{ct}$
NG STI-EA vs. NG Aptima Combo 2	38	13	24.5	7.2	100 (66.4–100)	86.2 (68.3–96.1)	69.2 (47.5–84.8)	100 (ns)	89.5 (75.2–97.1)	74.8 (50.7–98.9)
NG STI-EA vs. NG Abbott <i>m</i> 2000	39	22	28.0	7.7	100 (79.4–100)	73.9 (51.6–89.77)	72.7 (57.3–84.1)	100 (ns)	84.6 (69.5–94.1)	69.9 (47.1–92.7)
NG STI-EA vs. NG Roche cobas	18	11	32.3	3.6	84.6 (ns)	100 (ns)	100 (ns)	71.4 (ns)	88.9 (ns)	75.4 (na)
CT STI-EA vs. CT Aptima Combo 2	51	22	25.7	7.5	100 (79.4–100)	82.9 (66.4–93.4)	72.7 (56.3–84.7)	100 (ns)	88.2 (76.1–95.6)	75.2 (56.6–93.8)
CT STI-EA vs. CT Abbott <i>m</i> 2000	39	8	28.6	7.2	87.5 (ns)	96.8 (83.3–99.9)	87.5 (ns)	96.8 (82.7–99.5)	94.9 (82.7–99.4)	84.4 (62.6–100)
CT STI-EA vs. CT Roche cobas	20	9	28.5	6.3	88.9 (ns)	90.9 (ns)	88.9 (ns)	90.9 (ns)	90 (ns)	79.8 (na)

^aValues are % (95% confidence interval); n, number of positives samples; $\bar{x}_{ct}$, threshold cycle average; $\hat{\sigma}_{ct}$, threshold cycle standard deviation; na, non-applicable when statistical analysis cannot be performed; ns, non-significant.

was to focus on specimens obtained from asymptomatic patients with a small amount of pathogen which tested negative for gonococcal culture. Similar statistical

distribution (C_t average and standard deviation) was respected in order to improve the relevance of each comparison group.

Figure 4: Specificity.

Table 4: Sequencing analysis.

Samples (year 2017)	Laboratories	PCR Allplex™ STI-EA (threshold cycle value)	Sequencing			Identification
			Match	Strain	GenBank accession number	
1	Eurofins Seegene (South Korea) for confirmatory	33.27	100%			<i>C. trachomatis</i>
		38.87		SB013321	CP016427.1	
				SB013112	CP016425.1	
				SB008107	CP016423.1	
2	Eurofins Seegene (South Korea) for confirmatory	38.94	99%	SB006930	CP016421.1	<i>C. trachomatis</i>
		37.35		SB013321	CP016427.1	
				SB013112	CP016425.1	
				SB008107	CP016423.1	
3	Eurofins Seegene (South Korea) for confirmatory	38.76	100%	SB006930	CP016421.1	<i>C. trachomatis</i>
		34.33		QH111L	CP018053.1	
				SB013321	CP016427.1	
				SB013112	CP016425.1	
4	Eurofins Seegene (South Korea) for confirmatory	34.37	100%	SB008107	CP016423.1	<i>N. gonorrhoeae</i>
		–		SB006930	CP016421.1	
				FDA ARGOS 205	CP020418.1	
				FDA ARGOS 204	CP020415.1	
5	Eurofins Seegene (South Korea) for confirmatory	36.00	100%	NCTC 13819	LT592163.1	<i>N. gonorrhoeae</i>
		30.64		NCTC 13821	LT592161.1	
				NCTC 13800	LT906472.1	
				NCTC 13798	LT906440.1	
				NCTC 13799	LT906437.1	
				RIVM0640	CP019467.1	
				RIVM0610	CP019466.1	

For CT detection, method comparisons showed a satisfactory agreement with excellent kappas, and the analytical Sp remained above 94% regardless of the threshold cycle (Figure 4). The limit of detection (LOD) was assessed by means of external quality programs for molecular diagnostics and the lower concentration was detected at 50 copies/mL, the claimed performance by the manufacturer. On the basis of kappas for NG comparisons, hypothesis of excellent agreement could not be rejected. Considering the threshold cycle, the Sp and PPV remained between 95% and 100% for NG when C_t was below 34. However, supposed false-positives were identified and these performances decreased at least 10% for PPV and by nearly 15% for Sp when C_t met 39. It should be stressed that Sp and predicted positive values were apparent percentages as each of the three comparative methods was viewed as a reference. But as previously seen, these assays could also have flaws and the patient status was unknown. Overall, an increased risk of false-positives arose when C_t was above 34 (120 CFU/mL).

We sought to explain discrepant samples that were amplified with target-specific PCR for NG (108, 297 bp) and CT (143, 218 bp) and were analyzed by sequencing. The study demonstrated that five false-positives out of 20 were in fact true positives. For two specimens, the GenBank Nucleotide Basic Local Alignment Search Tool (BLAST) analysis provided a 100% match with four and five NG sequences, respectively (Table 4). Similarly, analysis of two other isolates have shown a 100% match with four and five CT sequences, respectively, while the highest match with four sequences of the pathogen was found at 99% for a fifth specimen. These false-negatives were observed with the Aptima™ Combo2 assay and could be explained by RNA degradation, although the sensitivity of such a method is usually increased by the target capture of rRNA genes that are present in higher copy numbers in the bacterial cell.

Interpretative comments

Many bacteria, fungi and viruses are tested by laboratories to assess the analytical Sp for regulatory approval, but molecular diagnostics for NG are complex and hypothesis of a false-positive is always possible. Despite additional cost for testing and institution of a useless antibiotic treatment, false-positives for gonococci can have a really negative psychosocial impact on the patient and his close friends, leading to disruption of the relationships and loss of confidence. In a context of low

prevalence (close to 1%), clinical laboratories should therefore take precautions and provide interpretative comments on the basis of their PCR results. When the C_t is higher than 34, we decided to add the following comments on the report: “Equivocal. A recollection for repeat testing may be appropriate”. Furthermore, taking into account the prevalence of CT in our laboratory which is 5% or higher, we indicate “Positive” on the report when detected.

Acknowledgments: The authors thank N. Auloy, J. Delaroché, D. Elise, L. Espinet, A. Heddebaut, N. Portal-Truche, A.M. Ranc and E. Sajot for their technical help. We also wish to thank the CNRNG, headed by B. Berçot, for testing performed on the Abbott m2000 molecular platform.

Author contributions: All the authors have accepted responsibility for the entire content of this submitted manuscript and approved submission.

Research funding: None declared.

Employment or leadership: None declared.

Honorarium: None declared.

Competing interests: The funding organization(s) played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

References

1. World Health Organisation. Global strategy for the prevention and control of sexually transmitted infections: 2006–2015. Breaking the chain of transmission. Available at: <https://www.who.int/reproductivehealth/publications/rtis/9789241563475/en/> Accessed: 8 Jun 2017.
2. Center for Disease Control and Prevention. Sexually Transmitted Disease Surveillance. Atlanta, GA: Department of Health and Human Services, 2017. Available at: <https://www.cdc.gov/std/stats17/default.htm>. Accessed: 9 Oct 2018.
3. Ndeikoundam N, Viriot D, Fournet N, De Barbeyrac B, Goubard A, Dupin N, et al. Bacterial sexually transmitted infections in France: recent trends and characteristics in 2015. *Bull Epidemiol Hebd* 2016;41–42:738–44.
4. French National Authority for Health (HAS). Evaluation of nucleic acid amplification tests (NAATs) for detecting *Neisseria gonorrhoeae*. 2015. Available at: http://www.has-sante.fr/portail/jcms/c_2035591/fr/evaluation-des-tests-d-amplification-des-acides-nucleiques-taan-recherchant-neisseria-gonorrhoeae. Accessed: 4 Dec 2015.
5. Bignell C, Unemo M. 2012 European guideline on the diagnosis and treatment of gonorrhea in adults. *Int J STD AIDS* 2013;24:85–92.
6. Bolan GA, Sparling PF, Wasserheit JN. The emerging threat of untreatable gonococcal infection. *N Engl J Med* 2012;366: 485–7.

7. Boyadzhyan B, Yashina T, Yatabe JH, Patnaik M, Hill CS. Comparison of the APTIMA CT and GC assays with the APTIMA combo 2 assay, the Abbott LCx assay, and direct fluorescent-antibody and culture assays for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. *J Clin Microbiol* 2004;42:3089–93.
8. Ota KV, Tamari IE, Smieja M, Jamieson F, Jones KE, Towns L, et al. Detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in pharyngeal and rectal specimens using the BD Probetec ET system, the gen-probe Aptima combo 2 assay and culture. *Sex Transm Infect* 2009;85:182–6.
9. Robinet S, Parisot F. Accreditation of a multiplex real time PCR assay for detection and semi-quantitative determination of pathogens responsible of sexually-transmitted infections. *Ann Biol Clin* 2018;76:459–76.
10. Sonnenberg P, Clifton S, Beddows S, Field N, Soldan K, Tanton C, et al. Prevalence, risk factors, and uptake of interventions for sexually transmitted infections in Britain: findings from the National Surveys of Sexual Attitudes and Lifestyles (Natsal). *Lancet* 2013;382:1795–806.
11. Kent CK, Chaw JK, Wong W, Liska S, Gibson S, Hubbard G, et al. Prevalence of rectal, urethral, and pharyngeal chlamydia and gonorrhea detected in 2 clinical settings among men who have sex with men: San Francisco, California, 2003. *Clin Infect Dis* 2005;41:67–74.
12. Patton ME, Kidd S, Llata E, Stenger M, Braxton J, Asbel L, et al. Extragenital gonorrhea and chlamydia testing and infection among men who have sex with men-STD surveillance network, United States, 2010–2012. *Clin Infect Dis* 2014;58:1564–70.
13. Venter JM, Mahlangu PM, Müller EE, Lewis DA, Rebe K, Struthers H, et al. Comparison of an in-house real-time duplex PCR assay with commercial Hologic® APTIMA assays for the detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in urine and extra-genital specimens. *BMC Infect Dis* 2019;19:6.
14. Centers for Disease Control and Prevention. Recommendations for the laboratory-based detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* 2014. *MMWR Recomm Rep* 2014;63:1–19.
15. Carlin EM, Ziza JM, Keat A, Janier M. 2014 European Guideline on the management of sexually acquired reactive arthritis. *Int J STD AIDS* 2014;25:901–12.
16. Field N, Kennedy I, Folkard K, Duffell S, Town K, Ison CA, et al. Screening for gonorrhea using samples collected through the English national Chlamydia screening programme and risk of false positives: a national survey of local authorities. *Br Med J Open* 2014;4:e006067.
17. Luijt D, Di Lorenzo C, van Loon AM, Unemo M. Most but not all laboratories can detect the recently emerged *Neisseria gonorrhoeae* porA mutants – results from the QCMD 2013 *N. gonorrhoeae* external quality assessment programme. *Euro Surveill* 2014;19:pii = 20711.
18. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977;33:159–74.
19. Fleiss JL. Measuring nominal scale agreement among many raters. *Psychological Bulletin* 1971;76:378–82.
20. Pettit RK, McAllister SC, Hamer TA. Response of gonococcal clinical isolates to acidic conditions. *J Med Microbiol* 1999;48:149–56.
21. Unemo M, Shafer WM. Antimicrobial resistance in *Neisseria gonorrhoeae* in the 21st century: past, evolution, and future. *Clin Microbiol Rev* 2014;27:587–613.
22. Golparian D, Ohlsson A, Janson H, Lidbrink P, Richtner T, Ekelund O, et al. Four treatment failures of pharyngeal gonorrhea with ceftriaxone (500 mg) or cefotaxime (500 mg), Sweden, 2013 and 2014. *Euro Surveill* 2014;19:pii = 20862.
23. Barry PM, Klausner JD. The use of cephalosporins for gonorrhea: the impending problem of resistance. *Expert Opin Pharmacother* 2009;10:555–77.
24. Soufir JC. Secretions of the male reproductive system and fertility. *Med Reprod* 2010;12:216–24.
25. Scrivener Y, Cribier B. Infections urogénitales à gonocoques et à *Chlamydia* (en dehors de la maladie de Nicolas-Favre): épidémiologie, diagnostic, évolution, traitement [Urogenital gonococcal and chlamydial infections (Lymphogranuloma venereum excepted): epidemiology, diagnosis, evolution, treatment]. *Rev Prat* 2001;51:453–8.
26. Allen McCutchan J, Wunderlich A, Braude AI. Role of urinary solutes in natural immunity to gonorrhea. *Infect Immunity* 1977;15:149–55.
27. Field N, Clifton S, Alexander S, Ison CA, Hughes G, Beddows S, et al. Confirmatory assays are essential when using molecular testing for *Neisseria gonorrhoeae* in low-prevalence settings: insights from the third National Survey of Sexual Attitudes and Lifestyles (Natsal-3). *Sex Transm Infect* 2015;91:338–41.
28. Katz AR, Effler PV, Ohye RG, Brouillet B, Lee MV, Whitticar PM. False-positive gonorrhea test results with a nucleic acid amplification test: the impact of low prevalence on positive predictive value. *Clin Infect Dis* 2004;38:814–9.
29. Chow EP, Fehler G, Read TR, Tabrizi SN, Hocking JS, Denham I, et al. Gonorrhoea notifications and nucleic acid amplification testing in a very low-prevalence Australian female population. *Med J Aust* 2015;202:321–3.
30. Fifer H, Ison CA. Nucleic acid amplification tests for the diagnosis of *Neisseria gonorrhoeae* in low-prevalence settings: a review of the evidence. *Sex Transm Infect* 2014;90:577–9.
31. Upton A, Bromhead C, Whitley DM. *Neisseria gonorrhoeae* false-positive result obtained from a pharyngeal swab by using the Roche cobas 4800 CT/NG assay in New Zealand in 2012. *J Clin Microbiol* 2013;51:1609–10.