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Evaluation of the new AmpliSens multiplex real-time PCR assay for simultaneous detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Mycoplasma genitalium*, and *Trichomonas vaginalis*

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In this study, we performed an evaluation of the new CE-marked multiplex real-time AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT PCR assay compared to APTIMA tests, i.e., APTIMA COMBO 2 assay, APTIMA *Trichomonas vaginalis* assay (FDA-approved), and two different APTIMA *Mycoplasma genitalium* assays (research use only; one of them only used for discrepancy analysis). Vaginal swabs (n = 209) and first-void urine (FVU) specimens from females (n = 498) and males (n = 554), consecutive attendees (n = 1261) at a dermatovenerological clinic in Sweden, were examined. The sensitivity of the AmpliSens PCR assay for detection of *C. trachomatis* (6.3% prevalence), *M. genitalium* (5.7% prevalence), *N. gonorrhoeae* (0.3% prevalence), and *T. vaginalis* (0.08% prevalence) was 97.5% (95% confidence interval (CI): 91.2–99.6%), 81.9% (95% CI: 70.7–89.7%), 100% (95% CI: 40.2–100%) and 100% (95% CI: 16.5–100%), respectively. The specificity of the AmpliSens PCR assay was 100% (95% CI: 99.6–100%) for all agents. The analytical sensitivity and specificity for *N. gonorrhoeae* detection was excellent, i.e., 55 international gonococcal strains detected and 135 isolates of 13 non-gonococcal Neisseria species were negative. In conclusion, the multiplex real-time AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT PCR assay demonstrated high sensitivity and excellent specificity for the detection of *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis*, and excellent specificity but suboptimal sensitivity for *M. genitalium* detection.

Key words: Sexually transmitted infections; AmpliSens; APTIMA COMBO 2 assay; APTIMA *Trichomonas vaginalis* assay; APTIMA *Mycoplasma genitalium* assay.

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Non-viral curable sexually transmitted infections (STIs) remain global public health concerns. In 2008, 105.7, 106.1, and 276.4 million cases of *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, and *Trichomonas vaginalis*, respectively, were estimated globally (1). The *Mycoplasma genitalium* prevalence has been reported to be 1–3% in sexually active adults (2–4), and up to 7.3–47.5% in high-risk populations (5–8). In Europe, during the latest decade the reported numbers of gonorrhoea and

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chlamydial infection have increased in many particularly high-income countries (9, 10), which likely reflects the availability and use of appropriate testing and diagnostic assays in these settings (11–14).

Sensitive and specific laboratory diagnostics is essential for accurate diagnosis of STIs. For detection of *C. trachomatis*, *N. gonorrhoeae*, and *T. vaginalis*, the implementation of nucleic acid amplification tests (NAATs) have revolutionized the diagnostics, due to superior sensitivities, mainly excellent specificities, use of non-invasive specimens, e.g., first-void urine (FVU) and vaginal swabs, rapidity and opportunities of high degrees of automation (15–29). For detection of *M. genitalium*, NAATs are the only feasible methods (2, 20, 30). For *C. trachomatis* and *N. gonorrhoeae* detection, there are several NAATs approved by the US Food and Drug Administration (FDA). For *T. vaginalis*, FDA recently approved the APTIMA *Trichomonas vaginalis* assay (Hologic, San Diego, USA). Unfortunately, no FDA-approved tests for the detection of *M. genitalium* are available yet, however, Hologic provides a Research Use Only (RUO) test. The performances of these APTIMA tests for the detection of *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, and *M. genitalium* have been excellent in many studies (2, 13, 14, 17, 18, 23, 25, 28–34). However, in many less-resourced settings, which frequently suffer from the highest prevalences of non-viral STIs, the thoroughly evaluated FDA-approved NAATs are not affordable (11, 12, 19–22). Accordingly, other NAATs are used and multiplex NAATs can detect etiological agents of multiple STIs in a rapid and cost-effective manner (19–22, 35–44). It is crucial that appropriate evaluations of these NAATs are performed, ideally against some of the thoroughly evaluated FDA-approved tests, and used with a high level of quality assurance.

The AmpliSens singleplex PCR assays for the detection of *C. trachomatis, N. gonorrhoeae*, and *T. vaginalis* have previously demonstrated high sensitivities and specificities, and the ones for *M. genitalium* detection high specificities but slightly suboptimal sensitivities (19–22). Recently, an AmpliSens multiplex CE-marked real-time PCR assay for simultaneous detection of *C. trachomatis, N. gonorrhoeae, T. vaginalis*, and *M. genitalium* was developed.

This study performed a thorough evaluation of the new AmpliSens multiplex real-time PCR assay for simultaneous detection of *N. gonorrhoeae, C. trachomatis, M. genitalium*, and *T. vaginalis* (AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT). As reference methods, the APTIMA COMBO 2 assay (AC2) and APTIMA *Trichomonas vaginalis* assay (ATV) (both FDA-approved), and two different APTIMA *Mycoplasma genitalium* assays (RUO; one only used for discrepancy analysis) were used.

MATERIALS AND METHODS

Patients

Consecutive attendees (n = 1261) at the STI clinic, Örebro University Hospital, Sweden from May 2012 to January 2013 were, after giving their written consent, enrolled in the study. The age range of the females (n = 707) and males (n = 554) was 18–65 years (median age: 29 years) and 21–80 years (median age: 32 years), respectively.

Biological specimens

FVU was collected from all males (n = 554), and FVU (n = 498) or vaginal swabs (n = 209) were collected from all females. All specimens were collected using the APTIMA Urine Collection Kit for Male and Female Urine Specimens (Hologic, San Diego, USA) or the APTIMA Vaginal Swab Specimen Collection Kit (Hologic, San Diego, CA, USA), according to the manufacturer's instructions.

Reference testing

Reference testing was performed within 1 week after specimen collection using AC2 (Hologic) and ATV (Hologic) on the PANTHER platform (Hologic). Specimens were subsequently frozen (−20 °C) prior to testing with the APTIMA *Mycoplasma genitalium* Assay (RUO) on the TIGRIS platform (cut-off level: 50 relative light units), according to the manufacturer's instructions, and the CE-marked multiplex real-time AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT PCR assay (InterLabService, Moscow, Russia). For discrepancy analysis and confirmation of true positive results, repeated testing (using DNA extracts and re-extracted clinical specimens), and the Cobas Taqman CT v. 2.0 test (Roche Molecular Systems, Branchburg, NJ, USA), APTIMA GC assay (Hologic) and an additional APTIMA *Mycoplasma genitalium* RUO assay (Hologic) were used. Samples were considered true positive if (i) the APTIMA test and the AmpliSens multiplex PCR assay were both positive, (ii) both the AC2 and the Cobas TaqMan CT 2.0 test were positive (*C. trachomatis*), (iii) both the APTIMA *Mycoplasma genitalium* RUO assays were positive (*M. genitalium*), and iv) the AmpliSens multiplex PCR assay and the confirmatory APTIMA *Mycoplasma genitalium* RUO assay were both positive (*M. genitalium*). Samples were considered true negative if (i) they were negative in the reference APTIMA test and the AmpliSens multiplex PCR assay or (ii) positive in only one of the screening and confirmative assays used.

Nucleic acid extraction for the AmpliSens multiplex real-time PCR assay

Nucleic acid extraction using 100 μL of specimen for testing by the AmpliSens multiplex PCR assay was performed

using the silica-based manual extraction kit DNA-Sorb-AM (InterLabService), according to the manufacturer's instructions. The nucleic acid extracts were stored at 4 °C prior to PCR amplification.

AmpliSens multiplex real-time PCR assay

PCR amplification was performed according to the manufacturer's instructions (InterLabService). All PCR testing was performed blinded to the results of the reference testing. Positive kit controls for each agents and negative control (sterile nucleic acid-free water) were included in each run. The main characteristics of the CE-marked multiplex real-time AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT PCR assay (InterLabService) are presented in Table 1.

Result analysis

Based on the results of the reference testing and confirmatory analysis, the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the AmpliSens multiplex PCR assay were calculated with 95% confidence intervals (95% CI), determined using the exact binominal distribution method.

Analytical sensitivity and specificity for *Neisseria gonorrhoeae* detection using the AmpliSens multiplex real-time PCR assay

The analytical sensitivity and specificity for the detection of *N. gonorrhoeae* were evaluated using 55 gonococcal strains (including 15 WHO reference strains and geographically and genetically diverse clinical isolates cultured in 1971–2012 globally) and 135 isolates of non-gonococcal Neisseria species (n = 13). The non-gonococcal Neisseria species included *N. meningitidis* (n = 47; serogroups A, B, C, W, Y, 29E, non-groupable), *N. lactamica* (n = 20), *N. sicca* (n = 15), *N. subflava* (n = 12), *N. cinerea* (n = 11), *N. flavescens* (n = 8), *N. mucosa* (n = 7), *N. kochii* (n = 4), *N. flava* (n = 2), *N. animalis* (n = 1), *N. caviae* (n = 1), *N. polysaccharea* (n = 1), *N. weaveri* (n = 1), and non-species identified non-gonococcal Neisseria isolates (n = 5). Prior to testing, the samples were prepared as previously described (31).

Determination of the detection limits of the AmpliSens multiplex real-time PCR assay

The detection limits were determined using *C. trachomatis* DNA control, *M. genitalium* DNA control, *T. vaginalis* DNA control (VIRCELL, Santa Fe, Spain), and

Table 1. Main characteristics of the AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT PCR assay

Characteristics	AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT PCR assay
Genetic targets	*Neisseria gonorrhoeae* (NG): 16S rRNA gene *Chlamydia trachomatis* (CT): cryptic plasmid *Mycoplasma genitalium* (MG): *gyrB* gene *Trichomonas vaginalis* (TV): Tv-E650 repeat
Primers/probes (5′→3′)	NG **(Fam)**-CCG ACA TCG GCC GCC GAT ATT GGC A–**(BHQ1)** GGA CTT TTG TCA GGG AAG AAA AGG CTG TT CAC GTA GTT AGC CGG TGC TTA TTC TTC A CT **(JOE)**-CCC CGC ACG TGC TTC GAG CAA CCG CG-(**BHQ1)** CAA GCA GGA CTA CAA GCT GCA AT CCT AGG CGT TTG TAC TCC GTC A MG (**ROX**)-CGA CGA GTT AAC TCC CTA -GCC TCG TC-(**BHQ2**) CCC ACA AGA AGC AAA CGC TAT CAT CAG A CCA GCA CTA TCA CCC TCA ACA ATG TAA AGT TV **(CY5.5)**- CGC ACT CAT GAC GAA CGA AGA AGG GTG CG-(**BHQ3)** TGT CGA ACA TTG GTC TTA CCC TCA GTT CCA GTA CTT ACG CTT GGA GAG GAC ATG
Internal Control (IC)	Added before DNA extraction
Reaction volume (DNA template)	25 (10) μL
Amplification instrument	Rotor-Gene Q (QIAGEN, Hilden, Germany)
Amplification program	Hold: 95 °C/15 min 5 Cycles: 95 °C/5 s; 60 °C/20 s; 72 °C/15 s 40 Cycles: 95 °C/5 s; 60 °C/20 s (detection); 72 °C/15 s
Fluorophores	CT - JOE GC - FAM MG - ROX TV - Cy5.5 IC - Cy5

N. gonorrhoeae DNA control (Advanced Biotechnologies, Columbia, USA) in six 10-fold dilutions.

Ethics

The study was approved by the Ethics Committee, Uppsala University, Sweden (Dnr2012/249).

RESULTS

In the initial screening, the AmpliSens multiplex real-time PCR assay (APTIMA screening NAATs) detected 78 (80) *C. trachomatis*, 59 (70) *M. genitalium*, 4 (4) *N. gonorrhoeae*, and 1 (1) *T. vaginalis* positive specimens. Concordant positive and negative results by the APTIMA screening NAATs and the AmpliSens multiplex real-time PCR assay were obtained for 100% of samples for *T. vaginalis*, 100% for *N. gonorrhoeae,* 99.8% samples for *C. trachomatis,* and 98.8% for *M. genitalium*. After discrepancy analysis, 80 *C. trachomatis*, 72 *M. genitalium*, 4 *N. gonorrhoeae*, and 1 *T. vaginalis* positive specimens were verified as true positive specimens. Accordingly, the true prevalence of *C. trachomatis* was 5.8% (41/707) among females and 7.0% (39/554) among males; *M. genitalium* – 6.1% (43/707) among females and 5.2% (29/554) among males; *N. gonorrhoeae* – 0.3% (2/707) among females and 0.4% (2/554) among males; and *T. vaginalis* – 0.1% (1/707) among females and no *T. vaginalis* was detected in males. Four cases of mixed infections were detected: *C. trachomatis* plus *N. gonorrhoeae* (n = 1), and *C. trachomatis* plus *M. genitalium* (n = 3).

The results of all the true positive and true negative results, and the results using the evaluated AmpliSens multiplex real-time PCR assay for each type of specimen are presented in Table 2.

Briefly, the AmpliSens multiplex real-time PCR assay missed two true positive *C. trachomatis* specimens and 13 true positive *M. genitalium* specimens, however, the assay detected all the true *N. gonorrhoeae* (n = 4) and *T. vaginalis* (n = 1) positive specimens. No false-positive results using the AmpliSens multiplex real-time PCR assay was obtained for any of the four non-viral STIs (Table 2). The APTIMA Combo 2 assay and the APTIMA *Trichomonas vaginalis* assay detected all the true positive *C. trachomatis* (n = 80), *N. gonorrhoeae* (n = 4), and *T. vaginalis* (n = 1). However, the APTIMA *Mycoplasma genitalium* RUO assay used for screening detected two false-positive specimens and missed four true positive specimens, which were all detected by the AmpliSens multiplex real-time PCR assay.

In total, the concordance of the results obtained by the AmpliSens multiplex real-time PCR assay

Table 2. True positive and negative results and the results obtained using the AmpliSens multiplex real-time PCR assay, divided into specimen type

True results[1]	AmpliSens result	No. of samples			
		Chlamydia trachomatis	*Neisseria gonorrhoeae*	*Mycoplasma genitalium*	*Trichomonas vaginalis*
Vaginal samples					
+	+	12	0	13	0
–	–	197	209	192	209
+	–	0	0	4	0
–	+	0	0	0	0
Total		209	209	209	209
FVU, females					
+	+	28	2	22	1
–	–	469	496	472	497
+	–	1	0	4	0
–	+	0	0	0	0
Total		498	498	498	498
FVU, males					
+	+	38	2	24	0
–	–	515	552	525	554
+	–	1	0	5	0
–	+	0	0	0	0
Total		554	554	554	554
Overall results					
+	+	78	4	59	1
–	–	1181	1257	1189	1260
+	–	2	0	13	0
–	+	0	0	0	0
Total		1261	1261	1261	1261

[1]True negative and positive results after discrepancy analysis.

and the true positive and negative results was 100% for *C. trachomatis,* 100% for *T. vaginalis*, 100% for *N. gonorrhoeae,* and 98.1% for *M. genitalium* in female vaginal swabs; 100% for *N. gonorrhoeae,* 100% for *T. vaginalis*, 99.8% for *C. trachomatis,* and 99.2% for *M. genitalium* in female FVU; and 100% for *N. gonorrhoeae,* 100% for *T. vaginalis*, 99.8% for *C. trachomatis,* and 99.1% for *M. genitalium* in male FVU. Overall concordance was 99.8% for *C. trachomatis*, 99.0% for *M. genitalium*, and 100% for both *T. vaginalis* and *N. gonorrhoeae*.

The sensitivities, specificities, PPVs and NPVs of the AmpliSens multiplex real-time PCR assay are displayed in Table 3.

Briefly, the overall sensitivity of the AmpliSens multiplex real-time PCR assay for detection of *C. trachomatis*, *M. genitalium*, *N. gonorrhoeae*, and *T. vaginalis* was 97.5%, 81.9%, 100%, and 100%, respectively. The specificity of the AmpliSens test was 100% for all the STI agents.

Analytical sensitivity and specificity for detection of *Neisseria gonorrhoeae* using the AmpliSens multiplex real-time PCR assay

The AmpliSens multiplex real-time PCR assay detected all the 55 international gonococcal strains and all the 135 isolates of 13 different non-gonococcal Neisseria species were negative.

Determination of the analytical detection limits of the AmpliSens multiplex PCR assay

The detection limits of the AmpliSens multiplex real-time PCR assay were 1.4 genome equivalents per reaction for *C. trachomatis*, 1.5 genome equivalents per reaction for *N. gonorrhoeae*, 0.16 genome equivalents per reaction for *T. vaginalis,* and 14.5 genome equivalents per reaction for *M. genitalium*.

DISCUSSION

In many countries, there is a need for cheap, strictly validated NAATs for reliable diagnosis of non-viral STIs. In this study, a thorough evaluation of the new CE-marked AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT PCR assay (InterLabService) was performed, as the singleplex AmpliSens PCR assays for detection of these four STIs have previously demonstrated acceptable excellent sensitivities and excellent specificities (19–22). As reference assays, the highly sensitive and specific AC2 and ATV (both FDA-approved), and APTIMA *Mycoplasma genitalium* assays (RUO) were used (2, 13, 14, 17, 18, 23, 25, 28–34).

Concordance with the true positive and negative results and the results using the AmpliSens multiplex PCR were obtained for 100% of samples for *T. vaginalis*, 100% for *N. gonorrhoeae,* 99.8% for C. *trachomatis,* and 99.0% for *M. genitalium*. These concordance levels were on a similar level or higher than in previous studies when other FDA-approved NAATs have been compared to the APTIMA NAATs (17, 18, 23, 24).

Overall, the multiplex AmpliSens PCR assay demonstrated high sensitivity and specificity for *C. trachomatis* (97.5% [95% CI: 91.2–99.6%] and 100% [95% CI: 99.7–100%], respectively), *N. gonorrhoeae* (100% [95% CI: 40.2–100%] and 100% [95% CI: 99.7–100%], respectively), and *T. vagi-*

Table 3. Sensitivities, specificities, PPVs, and NPVs of the multiplex real-time AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTIPRIME-FRT PCR assay

Specimen type	Target	Sensitivity, % [95% CI]	Specificity, % [95% CI]	PPV, % [95% CI]	NPV, % [95% CI]
Vaginal swab (n = 209)	*Chlamydia trachomatis*	100 [73.3–100]	100 [98.1–100]	100 [73.3–100]	100 [98.1–100]
	Neisseria gonorrhoeae	ND	100 [98.2–100]	ND	100 [98.2–100]
	Mycoplasma genitalium	76.5 [50.1–93.0]	100 [98.1–100]	100 [75.1–100]	97.9 [94.8–99.4]
	Trichomonas vaginalis	ND	100 [98.2–100]	ND	100 [98.2–100]
FVU, females (n = 498)	*C. trachomatis*	96.5 [82.2–99.4]	100 [99.2–100]	100 [87.5–100]	99.8 [98.8–100]
	N. gonorrhoeae	100 [19.3–100]	100 [99.2–100]	100 [19.3–100]	100 [99.2–100]
	M. genitalium	84.6 [65.1–95.5]	100 [99.2–100]	100 [84.4–100]	99.2 [97.9–99.8]
	T. vaginalis	100 [16.5–100]	100 [99.2–100]	100 [16.5–100]	100 [99.2–100]
FVU, males (n = 554)	*C. trachomatis*	97.4 [86.5–99.6]	100 [99.3–100]	100 [90.7–100]	99.8 [98.9–100]
	N. gonorrhoeae	100 [19.3–100]	100 [99.3–100]	100 [19.3–100]	100 [99.3–100]
	M. genitalium	82.8 [64.2–94.1]	100 [99.3–100]	100 [85.6–100]	99.1 [97.8–99.7]
	T. vaginalis	ND	100 [99.3–100]	ND	100 [99.3–100]
All samples (n = 1261)	*C. trachomatis*	97.5 [91.2–99.6]	100 [99.7–100]	100 [95.3–100]	99.8 [99.4–100]
	N. gonorrhoeae	100 [40.2–100]	100 [99.7–100]	100 [40.2–100]	100 [99.7–100]
	M. genitalium	81.9 [70.7–89.7]	100 [99.6–100]	100 [92.3–100]	98.9 [98.1–99.4]
	T. vaginalis	100 [16.5–100]	100 [99.7–100]	100 [16.5–100]	100 [99.7–100]

ND, not determined due to lack of positive specimens.

nalis (100% [95% CI: 16.5–100%] and 100% [95% CI: 99.7–100%], respectively). However, importantly only four positive *N. gonorrhoeae* samples and one positive *T. vaginalis* sample were identified. Accordingly, these specificity values can be considered valid, but to verify the sensitivities additional *N. gonorrhoeae* and *T. vaginalis* positive samples are essential to examine. Nevertheless, the singleplex AmpliSens PCR assays for detection of *N. gonorrhoeae* and *T. vaginalis* (using the identical genetic targets as in the multiplex AmpliSens assay) have previously demonstrated 100% sensitivities (19, 21). For detection of *C. trachomatis*, *N. gonorrhoeae,* and *T. vaginalis* using the multiplex AmpliSens PCR assay, the sensitivities and specificities were on similar levels or higher than in previous studies when FDA-approved NAATs have been compared (15–17, 26, 27). The multiplex AmpliSens PCR assay also demonstrated 100% specificity, however, suboptimal sensitivity (81.9%; 95% CI: 70.7–89.7%) for *M. genitalium* detection. These results were in concordance with characteristics of the previously evaluated singleplex AmpliSens *M. genitalium* PCR assay (20). Surprisingly, the lowest sensitivity (76.5%) was obtained for vaginal samples, which is in contrast to many previous studies using other NAATs (20, 45, 46). The lower sensitivity for the detection of *M. genitalium* using the AmpliSens multiplex PCR assay might be explained by the presence of *M. genitalium* in low concentrations in many biological samples (2), combined with the use of only 100 μL specimen for AmpliSens DNA extraction (can be enhanced to 1 mL using another AmpliSens extraction kit) and the single copy *gyrB* gene (47) target in the multiplex AmpliSens PCR assay. The reference APTIMA *M. genitalium* RUO NAATs targets 16S rRNA that can be present in up to thousands of copies in each *M. genitalium* cell. In general, the specimens that were false-negative for *M. genitalium* in the multiplex AmpliSens PCR assay showed also a lower positivity in the APTIMA *M. genitalium* RUO NAATs, which indicate a lower *M. genitalium* load in these specimens. The clinical significance of *M. genitalium* present in extremely low concentrations in biological specimens might also be questionable, that is, because the corresponding patients can lack all symptoms/signs of urethritis (48).

The main limitations of this study were: (i) All samples were collected in APTIMA specimen collection kits, which favored the APTIMA NAATs because these specimen collection kits have never been validated for use with the multiplex AmpliSens PCR assay, (ii) Only 100 μL of clinical specimen was used in the DNA extraction for the multiplex AmpliSens PCR assay, which might have negatively affected the sensitivity of this NAAT. Another AmpliSens DNA extraction kit using 1 mL of clinical specimen is also available, (iii) Only four positive *N. gonorrhoeae* samples and one positive *T. vaginalis* sample were identified. Additional *N. gonorrhoeae* and *T. vaginalis* positive samples are essential to examine, to further verify the sensitivities in the detection of these STIs, (iv) No extra-genital specimens were analyzed, which are frequently crucial to test especially in men-who-have-sex-with-men (MSM) but also, indicated by sexual practice, in many heterosexual women and men (2), and (v) The specimens were freeze-thawed several times during the study, which potentially could have influenced the stability of the genetic targets and/or destroyed some inhibiting compounds present in the samples (45).

CONCLUSIONS

The multiplex real-time AmpliSens N.gonorrhoeae/C.trachomatis/M.genitalium/T.vaginalis-MULTI-PRIME-FRT PCR assay demonstrated high clinical and analytical sensitivity and excellent specificity for the detection of *C. trachomatis, N. gonorrhoeae*, and *T. vaginalis*, and excellent clinical and analytical specificity but suboptimal sensitivity for the detection of *M. genitalium*. Furthermore, the multiplex real-time AmpliSens PCR was simple and quick to perform, as well as cheap compared to many international STI diagnostic NAATs. However, ideally the DNA extraction step should be automated. Further investigations, particularly to optimize the detection of *M. genitalium*, using higher volume of clinical specimen for DNA extraction and appropriate transport media for the multiplex real-time AmpliSens PCR assay are needed. Additional studies also need to include a higher number of *N. gonorrhoeae* and *T. vaginalis* positive samples, as well as extra-genital specimens.

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