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Original Article

Comparison of two commercial multiplex PCR assays for the detection of sexually transmitted infections

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Abstract

Introduction: Multiplex molecular panels are replacing conventional methods for the detection of sexually transmitted infections. In the current study, we evaluated the performance of two commercial multiplex assays, EUROArray STI and Allplex STI essential assays, for detecting six sexually transmitted infections.

Methodology: The diagnostic performance of the EUROArray STI and Allplex STI essential assays was evaluated against a panel of 105 positive DNA samples identified by in-house real-time PCR assays including *Ureaplasma parvum*, *Ureaplasma urealyticum*, *Mycoplasma hominis*, *Trichomonas vaginalis*, *Chlamydia trachomatis*, and *Neisseria gonorrhea*. Samples from healthy subjects, negative for any microorganism, were used as negative controls.

Results: Of the 105 positive specimens, 103 (98%) were tested positive by Allplex and 102 (97%) by EUROArray. Among the 51 negative samples that were tested by in house assay, 48 (94%) were tested negative by Allplex assay and 43 (84%) by EUROArray assay. The overall sensitivity of EUROArray and Allplex were 97.1% and 98.1% with an accuracy of 92.9% and 96.7%, respectively. The overall assay specificity was 94.1% for Allplex assay and 84.3% for EUROArray assay, The sensitivity of both kits to all targeted microorganisms ranged from 55.6% to 100%, with the lowest sensitivity noted for *Trichomonas vaginalis.*

Conclusions: Diagnostic performance varies depending on the method used to detect the targeted pathogens, the assay manipulation, and the cost. This study showed sensitivity, specificity, and accuracy characteristics for two kits commonly used to detect STIs, which will guide the choice for an appropriate multiplex PCR platform.

Key words: STI; commercial assays; sensitivity; specificity.

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Introduction

Sexually transmitted infections (STI) have a well-documented impact on the public health sector since they cause medical, social and economic sequelae [1–5]. The importance of STI lies in the fact that they can cause complications in infected individuals [4,6]. Unspecific symptoms and subclinical infections may lead to challenges in the clinical diagnosis. Laboratory-confirmed etiological diagnosis is therefore the most reliable approach that should be used in the management of such infections [7,8].

Several pathogens may cause STI ranging from bacteria, viruses, fungi and parasites; conventionally, various diagnostic assays were used to identify them including wet mount, gram stain, cell culture and serological assays [9–18] (Supplementary Table 1). However, some of the fastidious microbes may not be easily identified by any of those methods such as *Mycoplasma* and *Ureaplasma* species. In the last decades, these techniques were replaced by nucleic acid amplification tests (NAAT) [19–24], which are currently recommended for screening and clinical diagnosis; they proved to be more sensitive and more specific than conventional assays.

The new era of molecular diagnostics is not only expected to accelerate detection but to also replace traditional methods and enter all disciplines and diagnostic fields. As demonstrated in the ongoing improvements of the new syndromic panel-based platforms to enhance assays, save time, make specimen preparation easier, and utilize multiplex platforms for the simultaneous detection of pathogens along with automation [25].

Several commercial assays or systems based on nucleic acid amplification have been developed to increase the sensitivity and specificity to detect simultaneously the most prevalent sexually transmitted pathogens [26–28].

The aim of the study was to evaluate the performance of two commercial multiplex assays,

EUROArray STI and Allplex STI essential assays, for the detection of six pathogens: *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (NG), *Trichomonas vaginalis* (TV), *Ureaplasma urealyticum* (UU), *Ureaplasma parvum* (UP), *Mycoplasma hominis* (MH), and *Mycoplasma genitalium* (MG). This unprecedented comparison will provide essential information for a better choice of diagnostic tools.

Methodology

Study specimens

Two genital flocking swabs (endocervical and vaginal) were collected by healthcare practitioners from women seeking gynecological checkup (N = 505) for a period of one year and placed in a single tube containing universal transport media (Copan Diagnostics Inc, Murietta, USA). DNA from 200 µL of each sample was extracted using a QIAamp DNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The samples were tested by an in-house multiplex real time PCR assay and a diagnosis was given accordingly [29]. For cost limits, only 105 positive samples for *Chlamydia trachomatis* (CT*), Neisseria gonorrhea* (NG), *Mycoplasma genitalium* (MG), *Ureaplasma urealyticum* (UU), *Urealplasma parvum* (UP), *Trichomonas vaginalis* (TV), and *Mycoplasma hominis* (MH) were selected for further testing by commercial assays along with 51 negative specimens.

STI Commercial detection assays

Two commercial assays were used for sensitivity/specificity analysis: the commercial multiplex real time PCR assay (Allplex STI essential assay, Seegene, Seoul, Korea) and the commercial fluorescence-based DNA microarray assay (EUROArray STI, EUROIMMUN, Lübeck, Germany). Allplex STI Essential Assay is an in vitro qualitative test that detect seven microorganisms: *Chlamydia trachomatis, Neisseria gonorrhea, Mycoplasma genitalium, Ureaplasma urealyticum, Urealplasma parvum, Trichomonas vaginalis,* and *Mycoplasma hominis.* EUROArray STI is a molecular genetic detection kit designed to identify the presence of eleven sexually transmitted infections among which are: *Chlamydia trachomatis, Neisseria gonorrhea, Mycoplasma genitalium, Ureaplasma urealyticum, Urealplasma parvum, Trichomonas vaginalis, Mycoplasma hominis,* Herpes simplex virus types 1 and 2, *Haemophilus ducreyi*, and *Treponema pallidum.*

Both assays were performed according to the manufacturer's protocol using the CFX96 real time thermocycler (Bio-Rad- Hercules, CA, USA). A second step is however required for the EUROArray STI to detect the amplified products using an oligonucleotide DNA probe ship which is then read on a EUROArrayScan software (EUROIMMUN).

Statistical analysis

In house PCR was considered as the gold standard procedure for testing the investigated pathogens upon which the two commercial kits were compared.

The sensitivity and specificity of each test and for each microorganism was calculated. The positive predictive value (PPV) and negative predictive value (NPV) were calculated for all specimens using JMP®, Version 15. SAS Institute Inc., Cary, NC, 1989-2019. The threshold for significance was set at 0.05. This study was approved by the Institutional Review Board of the Saint Georges Hospital University Medical Center (Number: 009, Date: 2016).

Results

Of the 105 positive specimens, 103 (98%) were positive by Allplex and 102 (97%) by EUROArray. Among the 51 negative samples by in house assay, 48 (94%) were shown to be negative by Allplex and 43 (84%) by EUROArray. The overall performance of the two assays was compared for their accuracy, sensitivity, specificity, PPV and NPV (Table 1).

The overall sensitivity of EUROArray and Allplex were 97.1% and 98.1% with an accuracy of 92.9% and 96.7%, respectively. As for the specificity, Allplex Seegene was seen to be more specific than EUROArray, 94.1% versus 84.3%, respectively.

The positive predictive value (PPV) and negative predictive value (NPV) were slightly higher in Allplex compared to EUROArray reaching a PPV of 97% and 93% and a NPV of 96% and 94% respectively. No significant differences in the overall performance of the two assays was detected.

We then calculated the inter-rater reliability, the Cohen's kappa coefficient ($\boldsymbol{\kappa}$), for both tests. Our results showed a very good agreement in the two assays when testing for the 6 microorganisms (Table 1).

The performance of both assays for six targets was compared: UU, UP, CT, TV, MH, MG and NG. The overall sensitivity of both kits to all targeted microorganisms ranged from the lowest rate of 55.6% to 100% (Table 1). Both assays exhibited a poor sensitivity for TV. High number of false positives were detected in EUROArray for MH (N = 9, PPV = 64%), TV (N = 3, PPV = 62.5%), CT (N = 2, PPV = 60%), NG and MG (N = 1, PPV = 50%), which is likely

attributed to the low PPV of the tested microorganism. The PPV of MH was also low (57.1%) when tested by Allplex assay. The overall specificity of Allplex assay was high when detecting the targeted microorganisms ranging from 79% to 100%. The accuracy of the assays targeting the microorganisms ranged from 82.3% to 100% in both kits (Table 1).

Discussion

In the last decades, new diagnostic molecular tools have been implemented in diagnostic laboratories including monoplex PCR, multiplex PCR and real time PCR assays [21–24,27,28,30–32]. For the application of these assays, various factors are usually considered including hands on time, targets coverage, sensitivity, specificity, degree of automation, and cost. These assays are now considered the test of choice for the diagnosis of infection; they replaced the need for traditional testing methods, improved diagnostic performance and can now detect the presence of multiple organisms in one tube [19].

Multiplex assays that target more than three organisms causing STI's in one assay have been developed commercially. The currently available CE market multiplex assays include: FilmArray STD Panel (BioFire Diagnostics), FTD STD9 (Fast-track Diagnostics), Allplex™ STI Essential (Seegene), STD finder (PathoFinder), STI EUROArray (Euroimmun), STI multiplex array (Randox Biosciences) and VIASURE sexually transmitted diseases (CerTest Biotec). With the increase availability of assays, comparison between commercial kits should be performed to assess their performance including their workflow. Therefore, the current study attempted to

Table 1. Accuracies, sensitivities, specificities and positive and negative predictive values of Euroimmune STI and Allplex STI assays.

Pathogen	Total number of samples tested	Performances	EUROImmune STI		Allplex STI Seegene		Inter-rater reliability	
			Estimate	95% CI	Estimate	95% CI	κ	*p*
In-house	105	Accuracy	**92.9**	83.8- 97.1	**96.7**	89.5-99.1		
		Sensitivity	**97.1**	91.9-99	**98.1**	93.3-99.5		
		Specificity	**84.3**	72-91.8	**94.1**	84.1-98		
		PPV	**92.7**	86.3-96.3	**97.2**	92-99		
		NPV	**93.5**	82.5-97.8	**96**	86.5-98.9		
UP	78	Accuracy	**93.6**	83.8-97.7	**94.5**	85.6-97.9	0.81	0.1317
		Sensitivity	**97.4**	91.1-99.3	**94.9**	87.5-98		
		Specificity	**87.8**	75.8-94.3	**93.9**	83.5-97.9		
		PPV	**92.7**	84.9-96.6	**96.1**	89.2-98.7		
		NPV	**95.6**	85.2-98.8	**92**	81.2-96.8		
UU	23	Accuracy	**100**	90.1-100	**100**	90.1-100	1	1
		Sensitivity	**100**	85.7-100	**100**	85.7-100		
		Specificity	**100**	91.6-100	**100**	91.6-100		
		PPV	**100**	85.7-100	**100**	85.7-100		
		NPV	**100**	91.6-100	**100**	91.6-100		
MH	17	Accuracy	**86.4**	72.6-94	**82.3**	67.6-91.3	0.73	0.3173
		Sensitivity	**94.1**	73-99	**94.1**	73-99		
		Specificity	**84.2**	72.6-91.5	**78.9**	66.7-87.5		
		PPV	**64**	44.5-79.8	**57.1**	39.1-73.5		
		NPV	**98**	89.3-99.6	**97.8**	88.7-99.6		
TV	9	Accuracy	**87**	77.1-92.9	**94.4**	86.9-96.5	0.67	0.3173
		Sensitivity	**55.6**	26.7-81.1	**66.7**	35.4-86.9		
		Specificity	**93.3**	82.1-97.7	**100**	92.1-100		
		PPV	**62.5**	30.6-86.3	**100**	61-100		
		NPV	**91.3**	79.7-96.6	**93.8**	83.2-97.9		
CT	3	Accuracy	**95.7**	83.9-98.9	**100**	90.9-100	0.73	0.1573
		Sensitivity	**100**	43.9-100	**100**	43.9-100		
		Specificity	**95.9**	86.3-98.9	**100**	92-100		
		PPV	**60**	23.1-88.2	**100**	43.9-100		
		NPV	**100**	91.6-100	**100**	92-100		
NG/MG	1	Accuracy	**97.7**	87.6-99.6	**100**	91.5-100	0.66	0.3173
		Sensitivity	**100**	20.7-100	**100**	20.7-100		
		Specificity	**97.7**	87.9-99.6	**100**	91.8-100		
		PPV	**50**	9.5-90.5	**100**	20.7-100		
		NPV	**100**	91.6-100	**100**	91.8-100		

UP:*Urealplasma parvum;* UU:*Ureaplasma urealyticum;* MH:*Mycoplasma hominis;* TV:*Trichomonas vaginalis;* CT:*Chlamydia trachomatis;* PPV: Positive predictive value; NPV: Negative predictive value.

evaluate the performance of two commercial assays in terms of sensitivity, specificity and accuracy. Table 2 shows the workflow analysis of Allplex™ STI Essential and STI EUROArray assays when compared for hands-on time, turnaround time, number of steps, number of target cost, along with other variables.

Both assays were sensitive, however, Allplex achieved the best performance in term of specificity, accuracy and efficiency in many aspects ranging from turnaround time, number of step and simultaneous detection of samples. EUROArray can only test 25 samples at a time while Allplex assay may detect 96 samples in one run and require one instrument. EUROArray was found to be a more technically sensitive since the assay is light, heat and bubble sensitive. However, the turnaround time of the EUROArray is longer and needs 5 hours to complete the test. It also requires expertise in assay mixing and application along with special care to avoid any laboratory contamination. Nonetheless, the turnaround time of both assays does not have an impact on patient's treatment as the results are provided on the same day.

Allplex STI is one step assay; it reduces hands on time and prevents possible contamination along with having semi-quantitative result while EUROArray provides qualitative results. The Allplex STI assay detects a single channel multiple fluorophores values while EUROArray PCR products are hybridized to biochip microarray slides containing immobilized complementary DNA probes. The positivity of the samples is detected by their fluorescence signals read on an additional scanner instrument required in the laboratories.

When the three assays including the in-house were compared for their sensitivity, specificity and accuracy to detect 6 pathogens, both commercial assays had poor sensitivity to detected TV. The low specificity and PPV witnessed for MH in both kits may be due to the probes used in the assays that are affecting the detection of the microorganism. Both assays performed similarly and efficiently in detecting UU and UP.

The study had few limitations including the limited number of patients infected with MH (N = 17), CT (N = 3), TV (N = 9), NG (N = 1) and MG (N = 1) which resulted in a wider confidence interval and prevented meaningful calculations to compare the performance of the two assays in the detection of these microorganisms. In addition, we did not assess the performance of additional targets in the study such as microorganisms causing genital ulcer due to the absence of positive samples.

In conclusion, it is recommended that before the use of any routine STI diagnostic platform, comparative analysis should be done. Various factors should be evaluated, the sensitivity and specificity of the assay to detect various microorganisms, the assay manipulation, and the cost that may vary between countries. Additional studies are also needed to compare the performance of all assays available in the market.

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Author's Contributions

MEC supervised and analyzed the experiments and was responsible for writing up the manuscript. RY and JH performed and analyzed the experiments in addition to data analysis. RY and JH have equal contribution. REB helped in

Table 2. Profile of assays based on analysis of workflow parameters.

Parameters (per run)	In-house PCR	EUROArray STI	Allplex STI Seegene
Turnaround time	2-3 hours	4-5 hours	2-3 hours
Hands-on time	≤ 1 hours	2 hours	≤ 1 hours
Instrument time	1-2 hours	2-3 hours	1-2 hours
Number of steps	3	5	3
Characteristics of assays	Light sensitive	Light, heat and bubble sensitive	Light sensitive
Sample type	Genital swab& urine	Genital swab & urine	Genital swab, urine & liquid-based cytology
Pre-extraction required	Yes	Yes	Yes
Throughput*	96 samples	25 samples	96 samples
Number of targets	4 per tube	11 per tube	7 per tube
Laboratory contamination	Low risk of contamination (Closed system)	Risk of contamination during the washing step of the slide	Low risk of contamination (Closed system)
Internal Control	Yes	Yes	Yes
Data interpretation software	Bio-Rad CFX software	EUROArray Scanner and software	Seegene Viewer and Bio-Rad CFX softwares
Date interpretation	Semi-quantitative	Qualitative	Semi-quantitative

*Throughput: is the total number of samples possible to be tested simultaneously per run (Time calculation excludes extraction procedure).

the statistical analysis of the data. JM helped in the writing up of the manuscript. All authors reviewed the manuscript.

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Annex – Supplementary items

Supplementary Table 1. Conventional methods used for the identification of sexually transmitted infections and their limitations.

Type of infection	Disease Notation	Common Transmission	Specific Causative Agents	Conventional method for detection	General limitations of conventional diagnostic method	Sensitivity/ Specificity compared to molecular methods	Ref
Bacterial	*Bacterial vaginosis (BV)*	Sexual contact that leads to an imbalance in the normal vaginal microbiota. Direct sexual contact.	*Gardnerella vaginalis* *Bacteroides* spp. *Fusobacterium* spp. *Mycoplasma* spp. *Ureaplasma* spp.	Presence of milky homogenous watery discharge which may be gray or yellowish in color. "Whiff test": the presence of a fishy odor after adding 10% potassium hydroxide (indicating the presence of aromatic amines). The PH of the vaginal secretion must be > 4.5. Gram stain of vaginal secretion smear: normal lactobacilli population is replaced by anaerobes and *G. vaginalis* bacteria and detection of clue cells. No culture is required for *Gardenerella vaginalis*. *Mycoplasma* spp and *Ureaplasma* spp are not stained. Culture on New York City Agar for *Mycoplasma spp* (Prolonged incubation period).	Some microscopic findings can be misinterpreted due to lack of experience, skills, and credibility. No complete picture of universal standards in diagnosis. Lack of objectivity in testing. Not routinely tested in laboratories. Needs special agar medium and long incubation time.	Varying sensitivity. Low specificity.	[10-11]
	Gonorrhea	Direct sexual contact.	*Neisseria gonorrhea*	Gram-negative diplococci under Gram-stain. Culture on modified Thayer-Martin medium (selective medium). Identification by glucose and maltose biochemical tests.	Relatively timely and labor intensive. Needs specialized media, specimen handling, collection methods, and transportation conditions. Fastidious pathogen (difficult to grow).	Relatively low sensitivity. Low specificity.	[9-12]
	Chlamydia	Direct sexual contact. During birth.	*Chlamydia trachomatis*	Direct detection: Antigen detection by immunochromatographic tests. *Chlamydia* isolation by cell line cultures. Serological tests: used to diagnose invasive infections or chronic ones by detecting serum antibodies.	Cell cultures are labor intensive and technique dependent.	Low sensitivity. Low specificity.	
	Syphilis	Direct sexual contact. During birth.	*Treponema \pallidum*	Dark filed microscopy on clinical specimens that reveal silver staining spirochetes with a corkscrew motility. Bacteria cannot be isolated. Direct fluorescent antibodies essays. Rapid plasma regain (RPR) titer. The Venereal Disease Research Laboratory (VDRL) result.	RPR false positive results can occur due to biological interferences and cross reactions. Dark field microscopy false positive and negative results due to lack of experience in distinguishing microscopic morphologies of *Treponema pallidum* and other *treponemas*. Endobiotic property of the bacterium makes direct identification tests technically hard to perform. Conventional tests cannot distinguish syphilis stages and severity.	Low sensitivity in the early stage of disease. Low specificity.	[25]
	Chancroid	Direct sexual contact. Contact with lesions or discharge.	*Hemophilus ducreyi*	Culture on *H. ducreyi* selective agar. Direct fluorescent antibodies essays.	Fastidious pathogen (difficult to grow). Special transport systems and media must be used for optimal recovery.	Low sensitivity.	[13]
Viral	Genital herpes	Direct sexual contact. Contact with open lesions.	HSV-2/ HSV-1	Cell viral cultures. Serology testing. Direct fluorescent antibodies essays (DFA).	Cell cultures and DFA techniques are labor intensive and technique dependent. Cell culture need a high-quality specimen with proper transportation conditions to preserve viral infectivity. Serological tests are dependent on the time elapsed after initial infection. Antibody response varies between different populations and regions affecting the specificity of serological tests.	DFA has a lower sensitivity than PCR. DFA has high specificity. ELISA have low specificity.	[14-15]
	Neonatal herpes	Exposure to the virus in the birth canal. Transplacental infection in some cases.	HSV-1 or HSV-2				
	Human papillomas/ Cervical cancer	Direct sexual contact. Any direct contact.	HPV of various strains.	No culture on cell lines. Pap smear test to check for changes in cervical cells.	False positive results can happen. Need highly trained cytotechnologists to give the correct interpretation. Need optimal smears to be taken by clinicians for best diagnosis.	Variable sensitivity and specificity.	[16]
Fungal	Vaginal candidiasis	Direct sexual contact. Immunosuppression. Disruption of the normal flora.	*Candida* spp.	Culture on sabouraud and blood agars. Blastoconidia detection under gram stain. Germ tube test.	Long turnaround time for species identification.	Highly sensitive and specific.	[17]
Parasitic	Trichomoniasis	Direct sexual contact.	*Trichomonas vaginalis*	Wet mounts to detect *Trichomonas* under the microscope. InPouch TV Culture System (a kit that combines between culture and wet mount).	TV Culture System is expensive and not readily available in laboratories and timely (identification can need from 2 to 7 days). Wet mount needs high expertise, fast specimen transport, and quick processing since the organism can easily loose motility and viability.	Wet mount has low sensitivity. TV culture system has higher sensitivity than wet mount yet less than PCR techniques. Very high specificity.	[18]