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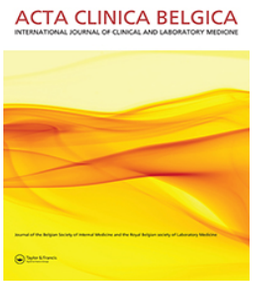

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Take three, test one: a cross-sectional study to evaluate the molecular detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* in pooled pharyngeal, anorectal and urine samples versus single-site testing among men who have sex with men in Belgium

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ABSTRACT

Objectives: To investigate the efficacy of performing a pooling strategy of triple-anatomical site samples (pharyngeal, anorectal and urine samples) for simultaneous *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) nucleic acid amplification detection.

Methods: A total of 117 specimen sets (pharyngeal, anorectal and urine) were collected from 98 men between 2014 and 2016. Double sampling of pharyngeal, anorectal and urine samples allowed for pooled and unpooled analyses using a multiplex Abbott Real Time CT/NG assay, together with confirmatory PCR testing in case of CT/NG positivity. Clinical and demographic data were analyzed.

Results: The positivity rate for the triple-site pooled testing for CT and NG was 8.5% (10/117) and 6.8%, (8/117), respectively, compared to the single-site testing total positivity rate, which was 9.4% (11/117) and 4.3% (5/117) for CT and NG, respectively. Pooled analysis missed one CT-positive urine sample and one CT-positive anorectal sample could not be confirmed. In addition, less PCR inhibition was reported for the pooled sample (PS) testing and ERV-3 qPCR testing revealed ineffective sampling of self-collected anorectal swabs in two cases. No pharyngeal samples were positive for CT, nor were any urine samples positive for NG.

Conclusion: This small study showed that PS testing is a possible testing strategy for screening high-risk men who have sex with men attending pre-exposure prophylaxis (PrEP) clinics. However, due to the low positivity rate of CT/NG in this study, larger evaluations are needed to confirm the effectiveness of CT/NG screening with multiple-site PS nucleic acid amplification test (NAAT) screening practices.

KEYWORDS
Chlamydia trachomatis; *Neisseria gonorrhoeae*; diagnostics; screening; pooling

Introduction

The use of pre-exposure prophylaxis (PrEP) in Men who have Sex with Men (MSM) has been associated with increased diagnoses of Sexually Transmitted Infections (STIs) [1]. In fact, a PrEP demonstration study in Belgium showed a high prevalence for *Chlamydia trachomatis* (CT) (11.7%) and *Neisseria gonorrhoeae* (NG) (12.2%) [2]. Guidelines for delivering PrEP to MSM include 6-monthly screening for CT/NG [3,4]. High prevalence rates of extra-genital CT and NG have been reported among MSM [5]. Thus, screening for CT and NG should preferably be performed on three sites: pharynx, urethra and anorectum. One way to reduce the cost of biannual three-site testing is to pool the three samples per patient. Using the Aptima Combo 2 assay, Sultan et al. [6] have recently demonstrated that pooled three-site testing in MSM has a similar sensitivity to single-site testing for CT (92% vs. 96%) and a slightly poorer sensitivity for NG (90% vs. 99%) in a high-risk population characterized by a high CT (16 %) and NG (27 %) prevalence.

We conducted an exploratory study to evaluate the appropriateness of pooled triple-site (pharyngeal, urethral, anorectal) testing using nucleic acid amplification tests (NAATs) for CT and NG in a cohort of high-risk MSM. In contrary to the method used by Sultan et al., we designed a strategy that allowed individual testing of the samples after pooled testing.

Materials and methods

Design and evaluation of the pooling strategy

The pooling strategy was designed so that individual testing of the three samples after unpooling was still feasible.

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Study cohort

Between August 2014 and May 2016, participants were recruited in an observational cohort sub-study-related syphilis diagnostic tests (ClinicalTrials.gov Registration Number: NCT02059525) conducted at the Institute of Tropical Medicine (ITM), Antwerp, Belgium. Inclusion of participants was based on a diagnosis of syphilis. Aside from the systematic screening of all individuals at the 6-month follow-up visit, individuals were additionally tested if they developed symptoms suggestive of a CT or NG infection or reported recent sexual contact with a CT- or NG-infected individual. The Institutional Review Board of ITM and the Ethics Committee of the University Hospital Antwerp approved this study (13/44/426). All participants provided written consent to this sub-study.

Patient involvement

Participants were not involved in the design and conduct of the study.

Clinical specimen collection

The study physician collected two pharyngeal samples by rubbing both tonsillar pillars and the posterior oropharynx for approximately 10 seconds using regular flocked swabs (Copan Flock Technologies S.R.L., Brescia, Italy). Two flocked swabs were provided to the participants for anorectal self-sampling. Participants were verbally instructed to insert the swab 3 cm into the anus and rotate three times. All swabs were stored at −20°C (±5°C) within two hours of sampling. First, void urine collected in a sterile container were aliquoted into 2 mL microtubes within two hours and either refrigerated (2–8°C) and tested within three days for the separate NAAT analysis, or frozen at −20°C for later pooled NAAT analyses, as outlined below.

Single-site sample testing for C. trachomatis and N. gonorrhoeae

One set of urine, pharyngeal and anorectal samples (randomly chosen), henceforth referred to as 'Separate Samples' (SSs), were analyzed according to the routine testing algorithm at the ITM. Pharyngeal and anorectal swabs were eluted by adding 1.2 mL of diluted phosphate-buffered saline (dPBS) (pH 7.4–1:9, PBS:saline) directly onto the swab. After vortexing the swab, 500 µL of the sample eluate was immediately analyzed using the Abbott Real-Time (RT) CT/NG assay (DNA extraction and sample preparation using Abbott m2000sp and the Abbott m2000rt system for amplification and detection of CT/NG, Abbott Molecular Inc. Des Plaines, IL, USA) according to the manufacturer's instructions. The remainder of the eluates/urine was stored at −20°C. In case of positivity, the same DNA extracts were tested by in-house real-time PCR (RT-PCR) assays both(Chlamydia trachomatis and Neisseria gonorrhoeae) based on previously published primer sets [7,8].

We defined 'true positives' when positive in both the Abbott and in-house RT-PCR assay. An initial positive Abbott assay result followed by a negative confirmatory NAAT result was defined as 'not confirmed'. Inhibition of the NAAT was defined as 'inhibition'.

Triple-site pooled sample (PS) testing for C. trachomatis and N. gonorrhoeae

All pooled samples (PSs) were batch-tested according to the same test algorithm as the SSs. Results of the SSs were not known by the performer. In short, pharyngeal and anorectal swabs were eluted in 600 µL of dPBS. A total of 170 µL from each of the anorectal and pharyngeal swab eluates were added to 170 µL of urine. The total volume of 510 µL was loaded onto the Abbott instrument. Leftover eluates of the swabs and urine aliquots were stored separately at −20°C. When the PS was positive for CT and/or NG, unpooled analysis followed using 500 µL urine and 200 µL of swab eluate diluted with 300 µL dPBS. These samples are referred to as 'unpooled samples' (USs) from hereon and were tested as outlined above.

Data analysis

In case of discrepant results between the SS and PS testing, human DNA concentration was assessed using an ERV-3 quantitative PCR [9]. Values are summarized as medians and interquartile ranges (IQR). Fisher's exact test was used to compare CT and NG positivity rate following the different testing methods. All analyses were performed in Stata 13 (StataCorp LP, College Station, TX, USA).

Results

Study sample collection and cohort characteristics

A total of 120 sample sets from 98 individuals were collected, 3 sample sets were incomplete and excluded from this analysis. The median age of the participants was 40 (IQR 31–47). All except three participants reporting being MSM and 87% ($N = 85$) were HIV infected. At the time of collection, one patient reported symptoms compatible with CT or NG (dysuria). Twenty-two individuals were screened because one of their recent sexual partners recently tested positive for NG/CT. Sixteen (13.3%) participants reported antibiotic use during the previous 3 months before testing.

Table 1. Results of separate sample/single-site (SS) and pooled triple-site sample (anal/pharyngeal/urine) (PS/US) NAAT analyses for *C. trachomatis* and *N. gonorrhoeae* detection among cohort of 98 individuals (N = 117 specimen sets analyzed).

			Pooled Samples (PSs) including unpooled samples (USs) NAAT results												
			Chlamydia trachomatis (CT)												
			CT+			CT−			Inhibition			Not confirmed			
Separate samples (SS) NAAT results			A	P	U	A	P	U	A	P	U	A	P	U	Total
	CT+	**A**	7			0			0			**1**			8
		P		0			0			0			0		0
		U			2			**1** DC = 0,5			0			0	3
	CT-	**A**	**1**[a]			104			0			0			105
		P		0			117			0			0		117
		U			0			114			0			0	114
	Inhibition	**A**	0			**3**			1			0			4
		P		0			0			0			0		0
		U			0			0			0			0	0
	Not confirmed	**A**	0			0			0			0			0
		P		0			0			0			0		0
		U			0			0			0			0	0
		Total	8	0	2	107	117	115	1	0	0	1	0	0	351
			Neisseria gonorrhoeae (NG)												
			NG+			NG−			Inhibition			Not confirmed			
			A	P	U	A	P	U	A	P	U	A	P	U	Total
	NG+	**A**	4			0			0			0			4
		P		1			0			0			0		1
		U			0			0			0			0	0
	NG-	**A**	**1**[a]			106			0			0			107
		P		0			114			0			**1**		115
		U			0			117			0			0	117
	Inhibition	**A**	0			**2**			1			0			3
		P		0			0			0			0		0
		U			0			0			0			0	0
	Not confirmed	**A**	**2** DC = 0,4 DC = 2,1			**1** DC = 1,5			0			0			3
		P		0			**1** DC = 0,2			0			0		1
		U			0			0			0			0	0
		U			0			0			0			0	0
		Total	7	1	0	109	115	117	1	0	0	0	1	0	351

Legend: A – Anorectal, P – Pharyngeal, U – Urine, [a]low or no presence of human cells (ERV-3 qPCR), DC = delta cycle value Abbott assay of the separate samples.

Detection of Chlamydia trachomatis

CT was detected in eight anorectal, three urine and no pharyngeal samples (Table 1). Out of these, PS testing detected seven of the anorectal and two of the urine infections, one CT-positive anorectal sample was missed due to inhibition in the confirmatory RT-PCR. One anorectal CT infection (confirmed by in-house RT-PCR to be LGV) was detected with the pooled but not the SS analysis. An ERV-3 qPCR performed on this SS anorectal swab was negative, indicating the lack of human cells. PCR inhibition was detected in four anorectal SSs; three of these were not inhibited in the PS testing and tested negative. One CT infection in urine was missed by PS testing; however, the SS testing revealed a weak positive value (Delta cycle value of the Abbott assay (DC) = 0.49). There was no statistically significant difference in the overall positivity rates of CT ascertained by SS (9.4%) or PS (8.5%) testing.

Detection of Neisseria gonorrhoeae

In the SS protocol, four anorectal, one pharyngeal and no urine samples tested positive for NG (Table 1). All of these samples were detected using PS and US analyses. An additional three NG-positive anorectal samples were detected using PS and US testing. Two of them were initially positive in the SS testing by Abbott, but could not be confirmed. One of the anorectal samples was collected from a participant taking amoxicillin treatment for pharyngitis. ERV-3 qPCR testing performed on the SS related to the third PS positive case revealed a low amount of human material (25 cells/PCR vs. 8123 cells/PCR). Further, of the three anorectal SSs-containing inhibitors, two were reported as negative with the PS testing. One pharyngeal sample was negative in the SS testing but was weakly positive in the PS testing (DC = 0.89), however the NG could not be confirmed by the in-house RT-PCR. The contrary was observed for another pharyngeal sample, which was weakly positive in the SS testing (DC = 0.18) but was not further confirmed and which tested negative in the PS testing. There was no statistically significant difference in the positivity rates of NG as determined by SS (4.3%) or PS (6.8%) testing.

Discussion

Although larger evaluations are needed to confirm these findings using the Abbott RT-CT/NG assay, the results of this pilot study are concordant with Sultans' et al. large study using the Aptima assay for Chlamydia and a smaller pooling study that was recently performed by Thielemans' et al. using the Abbott assay for CT/NG [10]. In addition, the design of the pooling strategy allowed us to determine with good sensitivity the biological site of infection. No additional samples will therefore need to be taken nor does the participant needs to come for an additional visit in case prescription is send by mail which will return in an additional cost-saving.

We found less NAAT inhibition in the PS strategy. This could in part be explained by the additional freezing step or by the smaller amount of volume, and thus inhibitors, used to prepare the PSs. We used self-sampling for anorectal specimens in this study because of its proven effectiveness [11]. However, our study that requested the collection of two subsequent anorectal samples showed that patient sampling error can occur, as demonstrated by the two SSs that contained no or few human cells and were likely poorly collected.

Limitations to this study include the low sampling number and the overall low CT/NG positivity rates in urine and pharyngeal samples. Furthermore, the SSs were subjected to immediate PCR testing in the context of routine clinical care, whereas the PS/US testing occurred between 1 month and 1 year after sampling, including subjection to a freeze thaw cycle which could cause DNA degradation in addition to elimination of inhibitors.

In conclusion, this small study contributes to the evidence that PS testing works and that it could be an appropriate testing strategy for screening high-risk MSM in PrEP clinics. It will be up to the end users of the testing to decide whether the site of infection provides valuable information for treatment or surveillance purposes and if this should be required.

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Author contribution

IDB, KO, CK and TC contributed equally. IDB and KO wrote the first draft of the manuscript and performed the analysis. HS and TC created the pooling strategy. HS performed the testing. CK and TC reviewed the manuscript. All authors approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

Data sharing Statement

The data that support the findings of this study are available on request from the ITM's data curator Jef Verellen e-mail:

jverellen@itg.be. The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

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