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The performance of pooled three anatomic site testing for *Chlamydia trachomatis* and *Neisseria gonorrhoeae* among men who have sex with men and transgender women.

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Abstract

Background—While molecular testing for *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG) is highly sensitive, the cost can be prohibitive. Those high costs are amplified when the recommended screening approach is used, which requires separate testing of specimens from three anatomic sites (rectal, pharyngeal and urogenital). While individual molecular testing is standard of care (SOC), pooled testing may offer a cost-saving alternative.

Methods—Using the Xpert® CT/NG assay (Cepheid, Sunnyvale, CA) we tested urine, rectal and pharyngeal swabs for CT and NG in a high-risk cohort of participants assigned male at birth who reported sex with other persons who were assigned male at birth. Remnant specimens (0.34 mL from each anatomic site) were combined to perform a single 'pooled' test. We calculated positive and negative percent agreement between the pooled testing results with SOC Xpert CT/NG test results as the reference.

Results—We conducted 644 pooled tests. Of those, 598 (92.3%) gave CT and NG results. The CT positive and negative percent agreement were 90.1% (95% CI: 80.7%, 95.9%) and 99.2% (98.1%, 99.8%), respectively. The NG positive and negative percent agreement were 96.2% (95% CI: 86.8%, 99.5%) and 99.8% (95% CI: 99.0%, 100%), respectively. Pooled testing identified 4 CT and 1 NG infections that were negative at all anatomic sites by individual testing.

Conclusions—Three-site pooled CT and NG testing performs similarly to single anatomic site testing among tests providing a valid result. Future cost analyses should evaluate the cost effectiveness of pooled three-site testing to determine if such a strategy improves the feasibility and accessibility of molecular STI testing.

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Short Summary:

We assessed the performance of standard of care versus pooled three-anatomic site testing (one test per person versus three) for *Chlamydia trachomatis* (CT) and *Neisseria gonorrhoeae* (NG).

Keywords

pooled testing; extra-genital; Chlamydia trachomatis; Neisseria gonorrhoeae; nucleic acid amplification test

Introduction

Chlamydia trachomatis (CT) and *Neisseria gonorrhoeae* (NG) infections, the two most common reportable bacterial sexually transmitted infections (STIs), are increasing in the United States and remain a persistent problem worldwide. CT and NG were responsible for over 2.3 million infections reported to the U.S. Centers for Disease Control and Prevention (CDC) in 2018(1).

Testing for CT and NG infections requires specimens from the anatomic sites of possible infection (pharynx, rectum and genitalia) and then each of those specimens is typically tested individually by a nucleic acid amplification test (NAAT)(2). CT and NG infections are frequently asymptomatic(3) and therefore go undetected and untreated if screening tests are not performed and may result in continued transmission. In the absence of appropriate 3-anatomic site screening, extragenital sites may be important reservoirs for CT and NG in a population, which can serve to perpetuate the spread of these infections.(4–7) The majority of extragenital CT and NG infections among men (65% to 85%) (8–10), and 14% to 44% among women are detected in the absence of urogenital infection, which may warrant routine screening at extragenital sites in addition to urethral screening for some populations.(11–14)

STI screening is one of the core strategies to STI prevention. Barriers to STI screening include the high costs of testing and limited test supplies and testing capacity. While individual testing of samples from all three anatomic sites is currently recommended(2), *pooled* testing may offer a cost-saving and resource saving alternative. Three-anatomic site pooled testing involves combining specimens from one individual's three anatomic sites into one test. Such a strategy would reduce the need for sampling and testing resources, likely resulting in significant reduction in the cost of screening. Furthermore, NAAT platforms can only perform a finite number of tests per day; in busy labs, three tests per patient may be a larger burden than can be accommodated. In September 2020, due to the COVID-19 pandemic, CDC issued a statement about shortages of STI test kits and laboratory supplies(15). Pooled testing could allow for continued triple site screening, even when testing supplies or capacity is limited.

Three anatomic site pooled testing has been evaluated in some prior studies using various NAAT assays(16–26), however there is no consensus on whether pooled testing has sufficient validity for clinical implementation. The objective of our study was to assess

the performance of routine pooled three-anatomic site testing (one test vs. three tests per person) for CT and NG using the Xpert CT/NG assay (Cepheid, Sunnyvale, CA, USA).

Materials and Methods

Study Setting and participants

Participants for this study were recruited at 'Good to Go', a public health campaign that provides HIV and STI testing to those meeting specific eligibility criteria in San Diego, CA. Those who are eligible to take part in the 'Total Test' program at Good to Go are those who are 18 years of age or older, assigned male at birth having sex with other persons who were assigned male at birth, available for follow-up for at least 2 weeks after testing, HIV status unknown or negative as of their last test, and have not participated in the Total Test in the past 3 months. Good to Go and the Total Test are supported by San Diego Primary Infection Resource Consortium (PIRC), a longitudinal cohort study of acute and early HIV infected (AEH) individuals in the United States (R24AI106039).

Specimen collection and testing

Participant specimens from the Total Test included: (1) a tube containing a rectal swab with stabilizing reagent (Xpert swab collection kit, Cepheid), (2) a tube containing a pharyngeal swab with stabilizing reagent (Xpert swab collection kit, Cepheid), (3) a tube containing urine with stabilizing reagent (Xpert urine collection kit, Cepheid). All specimens were self-collected. Testing on the individual specimens was done within 24 hours after specimen collection using the Xpert® CT/NG assay (Cepheid, Sunnyvale, CA) on the GeneXpert instrument.

Pooled testing was performed using three remnant specimens described above (rectal swab, pharyngeal swab and urine) per participant. Those specimens selected for inclusion in the pooled testing evaluation were those that had valid results from the individual testing. Aliquots of 0.34 mL from each of the swab collection kits (pharyngeal and rectal) were extracted and put into a dry tube. A 0.34 mL aliquot of the urine/stabilizing reagent mixture was extracted to combine into the dry tube containing the combined rectal and pharyngeal specimens. The 0.34 mL volume was chosen as ~1 mL is the manufacturer's recommended volume for testing using the Xpert CT/NG cartridge. The tube containing the combined specimens was vortexed for 30 seconds and then inoculated into the Xpert CT/NG test cartridge and put into the GeneXpert instrument for testing. Remnant specimens were refrigerated and on a bi-weekly basis, the pooled testing was conducted in bulk. Pooled testing was conducted within 2 weeks of specimen collection. If a pooled test result was not valid, the test was not repeated. The Xpert CT/NG is not labeled for the use with pooled specimen types and the pooled test results were not used for patient management.

The Xpert CT/NG assay amplifies one unique chromosomal gene target for the detection of CT, and two unique chromosomal gene targets for detection of NG. Both NG targets need to be positive for the Xpert® CT/NG assay to return a positive NG result. The amplification of those targets is indicated by a pathogen-specific cycle threshold value for each target. A lower pathogen-specific cycle threshold value indicates an earlier cycle target detection and

more pathogen target in the specimen. The pathogen-specific cycle threshold values in the GeneXpert platform have been explored as a measure of bacterial load in some of the other assays manufactured for the GeneXpert, including that for MRSA and tuberculosis.(27–30) A failure detection mode included in the assay is the Sample Adequacy Control (SAC), which targets a single copy human gene that should be present in each specimen. The SAC controls for false negative results where no human cells are present by confirming adequate patient sample has been collected and appropriate testing conditions have occurred. In the Xpert® CT/NG assay, the SAC is quantified by its cycle threshold, the number of cycles required to detect the presence of 1 human gene target, hydroxymethylbilane synthase. A lower SAC cycle threshold value indicates an earlier cycle detection threshold and more human cellular target in the specimen. If a test does not give a valid result, it will give a result of 'Error', 'Invalid', or 'No Result' each of which indicate different potential control failures or testing problems.

Data analysis

We used descriptive statistics to summarize the results from individual and pooled testing. Positive percent agreement was determined by the percent of pooled test positive of those positive on at least one of the individual tests. Negative percent agreement was determined by the percent of pooled test negative results of those negative on all individual tests. We also calculated positive and negative predictive values and Cohen's kappa. In addition, we calculated positive and negative percent agreement by anatomic site of infection. We calculated 95% confidence intervals (CIs) using the binomial exact method. Mean CT, NG and SAC cycle threshold values and standard deviation (SD) were calculated for the pooled and individual test for each result category. We averaged the two NG target cycle threshold values to create one summary cycle threshold value for NG. We used Student's t-test to compare mean NG, CT and SAC cycle threshold values between concordant and discordant results for the pooled and individual tests. All analyses were conducted using STATA version 16 (College Station, TX).

Results

We had a sample size of 644 participants of whom each had a valid individual test results for three anatomic sites: rectum, pharynx and genitalia (urine). Of the rectal specimen test results, there were 52 positive for CT, 25 positive for NG and 11 that were positive for both CT and NG. Of the pharyngeal specimen test results, there were 9 positive for CT, 35 positive for NG and 1 that was positive for both CT and NG. Of the urine specimen test results, there were 17 positive for CT, 4 positive for NG and 1 that was positive for both CT and NG. Of the 644 individuals, 64 tested positive for CT in at least one anatomic site and 42 that tested positive for NG in at least one anatomic site and 14 that were coinfected with both CT and NG in at least one anatomic site.

Of the 644 pooled tests, 594 (92.3%) gave CT and NG results (Table 1). Of the indeterminate results, 41 (6.4%) were due to error, 4 (0.6%) were invalid, and 5 (0.8%) gave no result. Of the 50 pooled tests for which a valid result was not obtained, 6 were

positive for CT, 3 were positive for NG and 1 was positive for both CT and NG on individual tests.

The CT positive and negative percent agreement were 90.1% (95% CI: 80.7%, 95.9%) and 99.2% (98.1%, 99.8%), respectively (Table 2). The NG positive and negative percent agreement were 96.2% (95% CI: 86.8%, 99.5%) and 99.8% (95% CI: 99.0%, 100%), respectively (Table 3). Pooled testing identified 4 CT and 1 NG infections that were negative at all anatomic sites by individual testing. The positive percent agreement stratified by anatomic site are shown in Table 4.

Table 5 shows the mean cycle threshold values for each specimen type and each target: CT, NG and the sample adequacy control. All of the 7 specimens that were negative for CT on the pooled test but had a positive for CT on individual tests were infected at only 1 anatomic site (3 urine, 1 pharyngeal, 3 rectal). Of those 7, the mean CT cycle threshold value for the positive individual results was 35.07 (SD: 3.02) compared with 27.85 (SD: 5.01) for those that were CT positive on both the pooled and at least one individual test (p=0.0004). For the 4 specimens that were CT positive on the pooled test but had negative individual tests the mean pooled cycle threshold value was significantly higher than the mean pooled CT cycle threshold value for those that were positive on both pooled and at least one individual test (38.53 (SD:0.75) vs 28.90 (SD:4.98); p=0.0003). Both of the 2 specimens that were negative for NG on the pooled test but had a positive for NG on individual testing had NG infection at only one anatomic site (both pharyngeal infections); the mean NG cycle threshold value for the positive individual results was 37.53 (SD: 0.18) compared with 25.20 (SD: 4.22) for those that were NG positive on both the pooled and at least one individual test (p=0.0002). For the one specimen that was NG positive on the pooled test but NG negative on all individual tests the pooled NG cycle threshold was 29.15 compared with a mean of 23.93 (SD: 4.51) pooled NG cycle thresholds for those positive on both pooled and at least one individual test.

Discussion

We conducted an evaluation of three-anatomic site pooled CT and NG testing compared to individual anatomic site testing and we found that pooled testing performs similarly to single anatomic site testing among those tests that gave a valid result.

Discordant results where the pooled test (4 CT and 1 NG) was positive while individual tests were negative did occur and were associated with higher CT and NG cycle threshold values than those observed in concordant specimens. While low pathogen volume is a potential explanation for the discordance, another potential explanation is that pooling of specimens actually diluted inhibitory material. Further research could be done to assess whether inhibitors are playing an important role. This same trend of high cycle threshold values for discordant specimens was also observed for discordant specimens where the individual test was positive and the pooled test was negative (7 CT and 2 NG).

We found that some of the pooled tests did not yield valid results (7.8%), those were due to three different reasons, the most common being a result of ‘error’. An error result indicates

a control failure leading the assay to abort. This could be due to the reaction tube being improperly filled, a reagent probe integrity problem, pressure limits being exceeded or a valve positioning error. Optimizing the pooled testing protocol (e.g. using a single elution buffer for all 3 specimens) may further enhance a pooled testing approach.

Three-anatomic site pooled testing has been evaluated in some prior studies using various NAAT assays(16–26). Similar to our findings, those studies found sensitivities that ranged from 78%-96% for CT detection and 82%-100% for NG detection, however the precision around those estimates tended to be low because of limited sample sizes of positive participants. In addition, those studies used varying reference tests and patient infection status definitions. Those studies used assays for pooled testing including the Xpert CT/NG, Abbott Real Time, and Aptima Combo 2 with no one assay that had especially low or high performance compared to the others. Meta-analyses may provide more precise estimates of positive and negative percent agreement. In addition, future studies should evaluate pooled testing with multiple reference tests to allow for a more precise infection status determination. Cost analyses should also be conducted to evaluate the cost effectiveness of pooled three-site testing to determine if such a strategy improves the feasibility and accessibility of molecular STI testing in both domestic and international settings.

Our study had some limitations. Every diagnostic assay is subject to some error and we used only one assay as a comparison to pooled testing, thus our reference test may be subject to some limited misclassification. We had a large sample size for calculation of negative percent agreement, however we had a modest sample size of CT and NG infections which was even more limited when we stratified by anatomic site of infection. We did not conduct a second test when the pooled test did not give a valid result and thus, we do not know if this step would have improved the proportion of valid pooled tests. In addition, our study was limited to participants that were assigned male at birth and therefore the results may not be generalizable outside of this population. Prior studies have included female participants in pooled testing evaluations with confidence intervals for positive and negative percent agreement that overlap with the results from our study.(21, 25, 26)

Three-anatomic site pooled testing has the potential to improve diagnostic efficiency. In some cases, it may be necessary to know the anatomic site of a CT or NG infection to optimize the treatment plan. Thus, protocols should be developed for when and how to conduct reflex individual anatomic site tests to confirm pooled results or identify the anatomic site of infection.

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Table 1.

Pooled test results from rectal swab, pharyngeal swab, and urine pooled specimens on the Xpert CT/NG.

Test Result	Frequency	Percent
CT DETECTED; NG DETECTED	14	2.2
CT DETECTED; NG NOT DETECTED	54	8.4
CT NOT DETECTED; NG DETECTED	37	5.8
CT NOT DETECTED; NG NOT DETECTED	489	75.9
ERROR	41	6.4
INVALID	4	0.6
NO RESULT	5	0.8
Total	644	100

Table 2.

Performance for detection of *Chlamydia trachomatis* using pooled specimens from 3 anatomic sites on the Xpert CT/NG assay compared to individual test results.

	Number of samples		Total	Positive percent agreement (95% CI)	Negative percent agreement (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	Kappa (95% CI)
	Individual test +	Individual test −						
Pooled test POS	64	4	68	90.1% (80.7%, 95.9%)	99.2% (98.1%, 99.8%)	94.1% (85.6%, 98.4%)	98.7% (97.3%, 99.5%)	0.910 (0.858, 0.963)
Pooled test NEG	7	519	526					
Total	71	523	594					

Table 3.

Performance for detection of *Neisseria gonorrhoeae* using pooled specimens from 3 anatomic sites on the Xpert CT/NG assay compared to individual test results.

	Number of samples		Total	Positive percent agreement (95% CI)	Negative percent agreement (95% CI)	Positive predictive value (95% CI)	Negative predictive value (95% CI)	Kappa (95% CI)
	Individual test +	Individual test −						
Pooled test POS	50	1	51	96.2% (86.8%, 99.5%)	99.8% (99.0%, 100%)	98.0% (89.6%, 100%)	99.6% (98.7%, 100%)	0.968 (0.932, 1.000)
Pooled test NEG	2	541	543					
Total	52	542	594					

Table 4.

Positive percent agreement for detection of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* using pooled specimens from 3 anatomic sites on the Xpert CT/NG assay compared to individual test results stratified by anatomic site of infection.

	TP/TP+FN	Positive percent agreement (95% CI)
Chlamydia trachomatis		
Urine	14/17	82.4 (56.6, 96.2)
Pharyngeal	9/10	90.0 (55.5, 99.7)
Rectal	53/56	94.6 (85.1, 98.9)
Neisseria gonorrhoeae		
Urine	5/5	100 (47.8, 100)
Pharyngeal	31/33	93.9 (79.8, 99.3)
Rectal	35/35	100 (90.0, 100)

TP=true positive, FN=false negative

Table 5.

Cycle threshold from 3 anatomic site specimens and a pooled specimen of all 3 on the Xpert CT/NG assay.

	Number of positive results	Mean cycle threshold	Standard deviation
Chlamydia trachomatis			
Urethral	18	29.92	3.21
Pharyngeal	10	30.38	7.46
Rectal	63	27.96	5.69
Pooled specimens	68	29.46	5.34
Neisseria gonorrhoeae[*]			
Urethral	5	22.97	6.98
Pharyngeal	36	28.41	5.25
Rectal	36	23.23	5.11
Pooled specimens	51	24.03	4.53
Sample Adequacy Control[**]			
Urethral	644	26.80	2.33
Pharyngeal	644	22.66	2.07
Rectal	644	24.75	4.03
Pooled specimens	594	22.20	1.76

[*] Took an average for each individual of both NG target cycle thresholds to generate this summary cycle threshold. Note that both targets had to be met in order to be included.

[**] We included the sample adequacy control cycle thresholds for only those that had valid results