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Use of real-time PCR as an alternative to conventional genotyping methods for the laboratory detection of lymphogranuloma venereum (LGV)

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Abstract

Lymphogranuloma venereum (LGV) can be differentiated from non-LGV chlamydial infection using Sanger sequencing or molecular assays, including those that are commercially-available internationally. Here, we describe the performance of a rapid real-time PCR (RT-PCR)-based strategy in differentiating *Chlamydia trachomatis* infections associated with LGV or non-LGV serovars. One hundred three rectal swabs, previously genotyped using Sanger sequencing of the *ompA* gene as a reference method, were tested in the RT-PCR assays. All non-LGV specimens were correctly identified, but the RT-PCR failed to detect 1 LGV specimen, resulting in a sensitivity of 87.5% for the non-LGV/LGV RT-PCR assay. Additional performance characteristics (e.g., specificity, accuracy, and reproducibility) were all between 93% and 100% with a limit of detection ≤100 copies/reaction. Thus, this rapid RT-PCR method for LGV detection in clinical specimens is comparable to the reference method.

Keywords

Lymphogranuloma venereum; *Chlamydia trachomatis* ; Real-time PCR; Outer membrane protein A (*ompA*)

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Authors' contributions

Evonne Woodson performed data collection, data analysis, and wrote the manuscript. Samantha Katz contributed to experimental design, performed data collection, and assisted with data analysis and manuscript review. Sheree Mosley performed data collection. Damien Danavall helped with training for automated DNA extraction platforms. Katherine Bowden contributed to experimental design and performed data collection. Kai-Hua Chi designed primers and probes. Brian Raphael contributed to experimental design and helped with manuscript review.

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Declaration of competing interest

The authors report no conflicts of interest relevant to this article.

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1. Introduction

Chlamydia trachomatis (CT) is the etiologic agent of the most commonly reported sexually transmitted infection (STI) (Centers for Disease, 2021). Based on variations in specific epitopes of the major outer membrane protein (MOMP), CT is classified into at least 19 serovars (Lesiak-Markowicz et al., 2019; Mohseni et al., 2021). While most localized chlamydial infections (e.g., ocular, urogenital, and oropharyngeal) are attributable to serovars A–K, infection with L1–L3 can cause a specific type of chlamydial infection known as lymphogranuloma venereum (LGV). Classical presentation of LGV is characterized by a self-limited ulcer or papule, but more recently LGV has become one of the leading causes of proctitis and proctocolitis in men who have sex with men (Stoner and Cohen, 2015). The recommended treatment for LGV is 100 mg doxycycline, twice daily for 21 days. This recommendation is based on established clinical practice; no clinical trials have been conducted to evaluate the ideal duration of treatment for LGV (Workowski et al., 2021).

Although rare in western countries, clusters of LGV are periodically detected in the United States. These sporadic outbreaks, including the most recent in Michigan in 2018, continue to fuel the development of rapid LGV diagnostics to ensure timely responses to future outbreaks (Convery and Kent, 2019; de Voux et al., 2016; Pathela et al., 2007; Smit et al., 2020). While commercially-available tests for LGV have come to market internationally, these assays have yet to receive FDA approval in the United States (Bernal-Martinez et al., 2020; Grange et al., 2021; Touati et al., 2021). In the U.S., laboratory detection of LGV still relies on methods that are costly, time-consuming, labor-intensive, and/or require specialized equipment and highly trained staff (Kersh et al., 2017). Although testing capacity is limited, some laboratories have developed in-house polymerase chain reaction (PCR)-based genotyping tests that, unlike commercially-available nucleic acid amplification tests (NAATs) for CT, are capable of differentiating LGV from non-LGV strains, while others have modified conventional procedures (Halse et al., 2006) similar to those described in previous reports (Bom et al., 2013; Christerson et al., 2012; Halse et al., 2006; Manning et al., 2021; Smit et al., 2020).

In 2005, Morre and colleagues published their sentinel study describing the first real-time PCR (RT-PCR) for LGV (Morre et al., 2005). In 2008, Chen et al. described a quadriplex real-time assay that not only targets the polymorphic membrane protein H (*pmpH*) gene, a CT gene that contains a 36bp deletion region often used to differentiate LGV from non-LGV strains, but also includes 2 additional targets, the CT cryptic plasmid to confirm CT status and the human ribonuclease P (*RNP*) gene [an internal control for human DNA (hDNA)] (Chen et al., 2008). Here we used Sanger sequencing of the outer membrane protein A (*ompA*) gene in a comparative analysis to evaluate the performance of the aforementioned quadriplex assay when run as 2 separate duplex assays (CT/hDNA and non-LGV/LGV) for the detection of LGV in rectal swab specimens.

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2. Methods

2.1. Bacterial strains and clinical specimens

A panel of genomic DNA from 41 bacterial and viral isolates was used to determine analytic specificity of the CT/hDNA and non-LGV/LGV assays (Table 1). DNA was purchased commercially from the American Type Culture Collection (ATCC; Manassas, VA) or extracted from isolates in the Laboratory Reference and Research Branch (LRRB) at the Centers for Disease Control and Prevention (CDC). Additionally, DNA was extracted from non-LGV isolates representing 12 CT serovars (A, B, Ba, C, D, E, F, G, H, I, J, K) (ATCC).

Rectal swabs (n=174) positive for CT by the Aptima Combo 2® assay (Hologic, Inc; Marlborough, MA) were obtained from 6 public health jurisdictions in Michigan (MI), Virginia (VA), New York (NY), Tennessee (TN), California (CA), and Indiana (IN) (collected between 2018 and 2019). Specimens were stored in Aptima transport media and shipped to CDC on dry ice. Work with remnant clinical specimens was determined not to be human subjects research through review by the National Center for HIV, Viral Hepatitis, STD, and TB Prevention.

2.2. Genomic DNA extraction

DNA extractions were automated using the iPrep/PureLink gDNA Blood kit (Invitrogen, Inc; Carlsbad, CA) or the QIAsymphony/DSP DNA Mini kit (Qiagen, Inc; Hilden, Germany). Controls for the LGV/non-LGV assay included DNA from non-LGV (VR-885D) and LGV (VR-902BD) strains (ATCC). VR-885D was also used as a positive control for CT cryptic plasmid DNA in the CT/hDNA duplex along with human DNA purchased through Thermo Fisher Scientific (Waltham, MA).

2.3. *Sanger sequencing of* ompA

CT positive rectal specimens (n = 174) were genotyped by amplifying the *ompA* gene as previously described (Batteiger et al., 2014; Dean et al., 2009; Lan et al., 1994). Cycle sequencing was performed with up to 4 primers (Table 2) using BigDye Terminator v3.1 (Applied Biosystems; Waltham, MA) followed by purification using the BigDye XTerminator kit (Applied Biosystems) and sequencing on the 3500XL Genetic Analyzer (Applied Biosystems) instrument.

2.3.1. Data analysis—Geneious Prime 2019.1.1 software (Biomatters Ltd; Auckland, New Zealand) was used for sequencing analysis. Consensus sequences generated from at least 1 forward and 1 reverse primer were aligned with CT reference sequences (serovars A-K, L1–L3) using MUSCLE v3.8.425. MEGA v10.0.5 was used to create a maximum-likelihood tree, which was annotated using the R package *ggtree* v2.0.4.

2.4. RT-PCR assay design

For the CT/hDNA duplex, target-specific PCR primer set and TaqMan probes were used to amplify 87bp and 73bp regions of the CT cryptic plasmid and human RNP gene (hDNA), respectively (Table 2).

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For the non-LGV/LGV assay, a 168bp fragment of the *pmpH* gene was amplified. Serovar-specific TaqMan probes were designed to span the 36bp deletion region to distinguish between the 2 serovar types.

All real-time reactions were run on a Rotor-Gene 6000 or Q machine (Qiagen, Inc) in either 25 μL (CT/hDNA assay) or 50 μL (non-LGV/LGV assay) volumes using PerfeCTa Multiplex qPCR Supermix (Quantabio; Beverly, MA) with the following cycling conditions: an initial hold at 95°C for 4 minutes, followed by 40 cycles at 95°C for 20 seconds then acquiring using the respective channel for the probe's fluorophore (orange and red for the CT/hDNA assay or green and yellow for the non-LGV/LGV assay) at 60°C for 60 seconds. While these duplex assays can be performed concomitantly as a quadriplex assay, as demonstrated in a previous study, they were performed as 2 separate assays in this study to alleviate signal bleed-through and minimize the primer-target competition often observed in multiplex PCR and to improve overall assay sensitivity (Chen et al., 2008).

2.5. Testing algorithm

Results from the real-time PCRs were interpreted as follows (Fig. 1): specimens with a signal in the human DNA (hDNA; ribonuclease P gene, RNP) channel in the CT/hDNA duplex were considered for non-LGV/LGV testing regardless of the CT result. Specimens with a signal in the non-LGV or LGV channel were classified as "non-LGV DNA detected" or "LGV DNA detected," respectively. Specimens without a signal in the non-LGV or LGV channels were reflexed to the CT result. If these specimens had a signal in the CT channel, they were classified as "CT DNA detected; strain type indeterminant"; however, if no signal was evident, these specimens were classified as "CT DNA not detected." Specimens lacking a signal for hDNA were classified as "Invalid" and excluded from the analysis.

2.6. Performance metrics

Limit of detection was determined by serially-diluting positive control DNA (1–10,000 copies per reaction for non-LGV/LGV and 1–1000 copies for CT/hDNA) in duplicate across 4 separate runs and determining the lowest copy number that consistently yielded a cycle threshold (Ct) signal. Reproducibility was determined by testing 4 replicates of 2 serial dilutions of positive control DNA for each target by the same operator in 3 separate runs at least 1 day apart. Assay sensitivity was determined by the number of specimens correctly classified as positive for each target compared with the known serovar as determined by Sanger sequencing, while the specificity was determined through confirmation of those correctly classified as negative for each target. Finally, accuracy was determined as the ability of the test to measure the "true" value for each target.

3. Results

3.1. Serovar determination via Sanger sequencing

Performance metric calculations were dependent upon reference method data; thus, we first genotyped the clinical specimens by Sanger sequencing the highly variable *ompA* gene on both strands (Fig. 2). Of the 174 CT+ clinical specimens, we were able to genotype 103 (59.2%); for the remaining 71 specimens, we were unable to amplify or sequence *ompA*.

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Sequencing was performed on 50 of the remaining 71 clinical specimens, but coverage was not sufficient (less than 2X) to determine the serotype and it was not attempted for 21 specimens due to low sample volume and/or low DNA yield. Although only genotyped specimens were used to measure the performance characteristics, all specimens were tested in both real-time PCR assays.

Of those genotyped, 92% were confirmed non-LGV (n = 95; including serovars B, D, Da, E, F, G, H, and J) as compared to 96% characterized as non-LGV by real-time PCR alone. Of the 8 LGV-positive specimens, 6/8 were serovar L2c and 2/8 L2g; only 7 were characterized as LGV+ by the real-time assays. All serovars were distributed across year and site.

3.2. Performance evaluation

Using Sanger sequencing as the reference method, we calculated the following performance characteristics: qualitative accuracy, diagnostic/analytic sensitivity, limit of detection, diagnostic/analytic specificity, and precision (Table 3).

The qualitative accuracy, or the alignment of RT-PCR results with those from the reference assay, was: 93.2% (96/103) for the CT/hDNA duplex and 99% (102/103) for the non-LGV/LGV duplex. Both the diagnostic and analytic sensitivity, or the ability of the RT-PCR assay to correctly identify LGV in an infected individual and control specimen, respectively, were: 93.2% (96/103) for CT and 100% (103/103) for hDNA; 100% (95/95) for non-LGV and 87.5% (7/8) for LGV. Based on data for the 103 CT+, the diagnostic specificity, or the ability of the RT-PCR assay to correctly identify true negatives was 100% for non-LGV/LGV and inclusion of the isolate panel provided analytical specificity of 100% (40/40) for CT and hDNA (Tables 1 and 3). These data reveal a positive predictive value (PPV) and negative predictive value (NPV) of 100% and 99% for the LGV/non-LGV duplex and 100% and 84% for the CT/hDNA duplex, respectively.

The limit of detection, based on serial dilutions of genomic DNA, was ≤100 copies/reaction. Additionally, the reproducibility of each assay, based on the ability to detect known concentrations of DNA on separate runs, measured at 100% (48/48) for all targets (CT, hDNA, non-LGV, and LGV).

4. Discussion

Laboratory diagnosis of LGV has long depended on time-consuming, labor-intensive methods such as culture, serology, and Sanger sequencing (Morre et al., 2008). Although molecular-based LGV testing is not novel (Chen et al., 2007; Chen et al., 2008; Chi et al., 2021; Kersh et al., 2017; Morre et al., 2005; Morre et al., 2005), few state public health laboratories have case volumes high enough to justify the time and resources required to build capacity for this approach (Halse et al., 2006; Pathela et al., 2007).

Based on the prototype from Morre et al., 2005, multiplex real-time PCR assays have been developed for LGV detection (Chen et al., 2007; Chen et al., 2008; Chi et al., 2021; Morre et al., 2005), including commercially-available options that are available internationally (Bernal-Martinez et al., 2020; Grange et al., 2021; Touati et al., 2021). While the quadriplex

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assay is useful in a variety of settings to simultaneously detect LGV, non-LGV, and mixed infections, the inclusion of multiple targets demonstrated reduced analytic sensitivity compared to the previous simplex approach (Chen et al., 2007). In this study, we describe an updated assay design that runs all 4 targets from the quadriplex assay in 2 duplex RT-PCRs to reduce impact on sensitivity.

The specificity was 100% for all targets analyzed. Likewise, the sensitivity was 93.2% to 100% for 3 of the 4 targets (CT, hDNA, and non-LGV) with lower sensitivity (87.5%) for the LGV target, leading to the detection failure of 1 case, likely due to the low number of true positives for LGV in this specimen panel. Despite its lower sensitivity, the probability that a person testing positive for LGV using this assay has LGV is high (PPV = 100%); and, equally important, the probability that a person testing negative for LGV does not have LGV is also high (NPV = 99%). For the CT/hDNA assay, the PPV and NPV was 100% and 84%, respectively. Together, these results suggest that the real-time assay yields similar results and thus, is comparable to conventional genotyping methods, like Sanger sequencing, for the detection of LGV in clinical specimens.

The low NPV for the CT/hDNA assay reflects the assay's inability to detect CT in 34% (60/174) of the NAAT CT+ rectal swabs. This result, despite the presence of the 7.5kb cryptic plasmid in nearly all isolates (Pickett et al., 2005), points to the difference in sensitivity achieved when amplifying targets directly from RNA (as with commercial NAATs) versus DNA (as in the real-time assays described). Thus, to conduct this assay independent of Sanger sequencing, we suggest that it only be used for clinical specimens that are confirmed CT+ by commercial NAAT, which is consistent with the 2019 European guidelines for the management of LGV, thus indicating that inclusion of the CT/hDNA assay may not be necessary prior to testing (de Vries et al., 2019).

In coupling the CT/hDNA duplex with the LGV/non-LGV assay, which uses a CT-specific gene for differentiating strain type, we detect CT in specimens either (1) positive for CT cryptic plasmid or (2) indirectly in specimens positive for LGV or non-LGV serovars. This is reflected in the real-time PCR testing algorithm (Fig. 1), which only reflexes to the CT results when the strain type is indeterminant.

In conclusion, these data suggest that the performance of this adapted assay is comparable to reference methods, offering similar diagnostic quality, yet is more cost-effective and yields results more quickly, increasing our capacity to detect LGV for routine surveillance and outbreak response efforts. Finally, further adaptations to this assay are underway (i.e., inclusion of other specimen types such as pharyngeal and oral) to reflect changes in the epidemiology of and better understand the burden of LGV in the United States.

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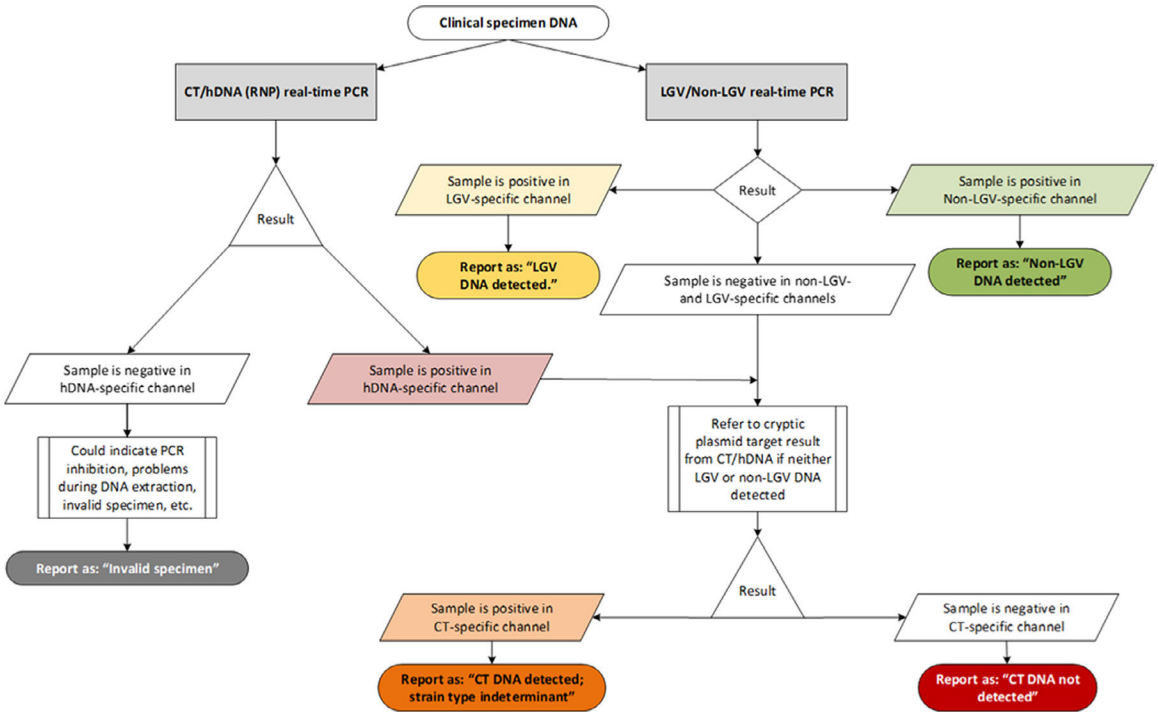

Fig. 1.

Testing Algorithm for the real-time PCR-based assay for LGV detection in clinical specimens. Specimens were previously tested for CT by the Aptima Combo 2 assay. CT+ specimens were selected and tested in both duplex assays (n = 174). Of the 174 CT+ rectal swabs, specimens positive for hDNA in the CT/hDNA assay were considered for further analysis in the non-LGV/LGV assay. Depending on the signal in the non-LGV and LGV channels, specimens were classified as non-LGV, LGV, or reflexed to CT results when the signal was not detected in the non-LGV or LGV channel.

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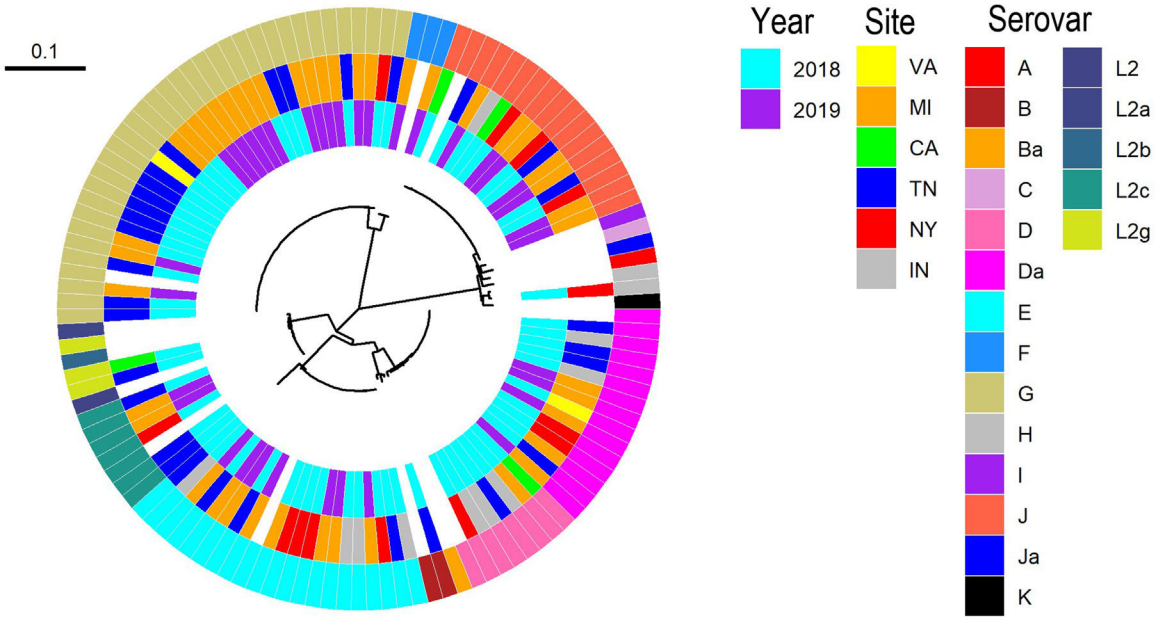

Fig. 2.

Phylogenetic analysis of *ompA* sequences from CT+ rectal swabs.

The *ompA* gene was amplified and sequenced from rectal swabs (n = 103) previously testing positive for CT (left). Metadata included: year of collection (inner ring), site (middle ring), and serovar (outer ring). Rectangles map to individual sequences and metadata is depicted by color (right). Sequences with associated metadata represent human rectal specimens (n = 103) and those without year and site represent reference isolates (n = 20).

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Diagn Microbiol Infect Dis. Author manuscript; available in PMC 2022 December 01.

Table 1

Bacterial and viral isolates used to determine analytic specificity.

Organism	Source	Catalog #	Strain designation
Acinetobacter baumanii	ATCC	17978DQ	5377
Bacteroides fragilis	ATCC	25285DQ	VPI 2553
Borrelia burgdorferi	ATCC	35210DQ	B31
Candida albicans	ATCC	10231DQ	3147
Candida glabrata	ATCC	2001D-5	CBS 138
Chlamydia pneumoniae	ATCC	53592	AR-39
Citrobacter freundii	ATCC	8090DQ	8090 / ATCC 13316
Escherichia coli	ATCC	25922DQ	Seattle 1946
Gardnerella vaginalis	ATCC	14019D-5	317
Haemophilus ducreyi	LRRB	*n/a*	CIP 542
Herpes simplex virus 1	ATCC	VR-539DQ	MacIntyre
Herpes simplex virus 2	ATCC	VR-540DQ	MS
Human papilloma virus type 16	ATCC	VR-3240SD	type 16
Human papilloma virus type 31	ATCC	VR-3256SD	type 31
Klebsiella pneumoniae subsp. *pneumoniae*	ATCC	700721DQ	MGH78578
Lactobacillus acidophilus	ATCC	4356	Scav
Legionella pneumophila subsp. *pneumophila*	ATCC	33152DQ	Philadelphia-1
Leptospira interrogans serovar Copenhageni	ATCC	BAA-1198D-5	Fiocruz L1-130
Mobiluncus mulieris	ATCC	35243DQ	SV 17J
Morganella morganii subsp. *morganii*	ATCC	35200D-5	AM-15
Mycoplasma hominis	ATCC	23114DQ	PG21
Mycoplasma genitalium	ATCC	33530	G37
Mycoplasma pneumoniae	ATCC	29342D	M129-B7
Neisseria gonorrhoeae	ATCC	700825DQ	FA1090
Neisseria meningitidis	ATTC	700532DQ	FAM18
Proteus mirabilis	ATCC	12453DQ	D1
Proteus vulgaris	ATCC	29905DQ	CDC PR1
Providencia stuartii	ATCC	33672D	495

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Organism	Source	Catalog #	Strain designation
Pseudomonas aeruginosa	ATCC	27853DQ	Boston 41501
Staphylococcus aureus subsp. *aureus*	ATCC	25923DQ	Seattle 1945
Treponema denticola	ATCC	35405D-5	a
Treponema pallidum subsp. *endemicum*	LRRB	*n/a*	Bosnia A
Treponema pallidum subsp. *pallidum*	LRRB	*n/a*	Nichols
Treponema pallidum subsp. *pallidum*	LRRB	*n/a*	St14
Treponema pallidum subsp. *pertenue*	LRRB	*n/a*	Gauthier
Treponema phagedenis	ATCC	27087	Kazan 8
Treponema phagedenis	LRRB	*n/a*	Reiter
Treponema refringens	LRRB	*n/a*	Noguchi
Trichomonas vaginalis	ATCC	30001D	C-1:NIH
Ureaplasma parvum	ATCC	27815	27
Ureaplasma urealyticum	ATCC	27618	T-strain 960

n/a = not applicable.

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Table 2

Primers and probes used for real-time PCR amplification and target detection in the CT/hDNA and non-LGV/LGV duplexes (top) and *ompA* PCR and sequencing primers (bottom).

Real-time PCR	***DNA sequence***	***Final concentration (nM)***
CT/hDNA duplex		
Primers [a]		
CT Forward	5’ – GGA TTG ACT CCG ACA ACG TAT TC-3’	50
CT Reverse	5’ – ATC ATT GCC ATT AGA AAG GGC ATT-3’	100
hDNA Forward	5’ – CCA AGT GTG AGG GCT GAA AAG-3’	80
hDNA Reverse	5’ – TGT TGT GGC TGA TGA ACT ATA AAA GG-3’	80
Probes		
CT	5’ – CalRed610 TTA CGT GTA GGC GGT TTA GAA AGC GG BHQ2 - 3’	100
hDNA	5’ – Quasar670 CCC CAG TCT CTG TCA GCA CTC CCT TC BHQ3 - 3’	80
non-LGV/LGV duplex		
Primers [a]		
non-LGV/LGV Forward	5’ – GGA TAA CTC TGT GGG GTA TTC TCC T-3’	600
non-GV/LGV Reverse	5’ – AGA CCC TTT CCG AGC ATC ACT-3’	600
Probes		
non-LGV	5’ – FAM GCT TGA AGC AGC AGG AGC TGG TG BHQ1 - 3’	200
non-LGV	5’ – VIC CCT GCT CCA ACA GT MGB NFQ - 3’	200
ompA *PCR/sequencing*	*DNA Sequence*	*Final Concentration (nM)*
PCR *Primers*		
ompA Forward	5’ – ATG AAA AAA CTC TTG AAA TCG-3’	200
ompA Reverse	5’ – CTC AAC TGT AAC TGC GTA TTT-3’	200
Sequencing *Primers*		
ompA Outer Forward	5’ – ATG AAA AAA CTC TTG AAA TCG-3’	400
ompA Outer Reverse	5’ – CTC AAC TGT AAC TGC GTA TTT-3’	400
ompA Nested Forward	5’ – TCC TTG CAA GCT CTG CCT GTG GGG AAT CCT-3’	400
ompA Nested Reverse	5’ – TGC AAG GAA ACG ATT TGC AT-3’	400

[a]All primers target the polymorphic membrane protein H (*pmpH*) gene.

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Table 3

Performance characteristics for real-time PCR duplexes.

Performance characteristic	CT/hDNA duplex	non-LGV/LGV duplex
Analytic sensitivity	93%/100%	100%/88%
Diagnostic sensitivity	93%/100%	100%/88%
Analytic specificity	100%/100%	100%/100%
Diagnostic specificity	*n/a*[a] / *n/a*[a]	100%/100%
Precision/Reproducibility	100%	100%
Qualitative accuracy	93%	99%
Limit of detection (LOD)	≤100 copies/reaction	≤100 copies/reaction
Positive predictive value (PPV)	100%	100%
Negative predictive value (NPV)	84%	99%

[a] *n/a*, not applicable.