Development of a Rapid Molecular Assay for Point-of-Care Testing of *Treponema pallidum* for Early Detection of Syphilis

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Abstract

INTRODUCTION: In 2023, syphilis cases in the U.S. surged to over 209,000, the highest case total since 1950. Current diagnosis of syphilis is limited to clinical recognition and serological testing, which is limited by a lack of sensitivity early in the disease. To improve early detection of primary syphilis, a real-time polymerase chain reaction (PCR) test was developed to directly detect *Treponema pallidum* (causative agent of syphilis) from relevant clinical samples. METHODS: The test targets two conserved regions within the *T. pallidum* genome, and also includes an internal control. Reaction conditions were optimized to permit room temperature storage and compatibility with extraction-free and pipette-free direct detection using the Collect-to-Test (C2T)® cartridge and the point-of-care-compatible T-COR 8® instrument. RESULTS: The real-time PCR test demonstrated a time-to-detection of under 45 minutes. The test was verified for specificity against 21 non-*T. pallidum* organisms, and found to have a limit of detection of 1.0 to 5.0 x10^3 treponemes/mL. Stability testing showed a shelf-life of up to one year at room temperature. CONCLUSIONS. Initial verification of the T. pallidum test demonstrated 100% specificity and acceptable limit of detection. This test could significantly improve access to early and rapid syphilis diagnosis at the point of care.

Introduction

Syphilis is a STI with rising incidence. There is a significant unmet need for its improved identification and diagnosis. Incorporation of IVD assays into the POC setting may provide an important means for diagnosing patients before they are lost to follow-up, particularly during the primary and secondary stages of infection when risk of transmission and disease progression is high. The *T. pallidum* (Syphilis) C2T Test is designed to be used with Tetracore's T-COR 8™ System.

- The **Sample Collection Device** provides a solution to allow direct testing from lesion exudate specimens and a variety of other relevant clinical specimens (eg, saliva). The buffer within the Sample Collection Device resuspends the sample, preparing it for direct PCR testing with no need for extraction.

- The **C2T cartridge** is a self-metering reaction device that has a sample reservoir configured to accept varying fluid amounts. A plunger is used to transfer sample fluid from the metering reservoir into the reaction chamber, which contains dried reagents. The C2T cartridge accepts a range of volumes and thus does not require a pipette to supply an exact volume for processing.

- The **T-COR 8** is a portable multi-sample instrument, capable of full functionality on internal battery power, and weighing less than 10 lbs. It is the size of a laptop and can easily fit in a lab space at a doctor's office or in a community STI clinic. It is CE-IVD(D). Its analytics are comparable to standard laboratory equipment, including a proprietary algorithm (SmartCt™) which analyzes and interprets results across each of the channels to provide a summary *Detected*/*Not Detected* call (Ct values and amplification curves visible under certain operator modes only).

Methods

Multiplexed Assay Design

Two different real-time PCR assays were designed, targeting separate and highly homologous regions within the *polA* gene. Both assays include a FAM-labeled probe (one assay included two probes designed to detect both strands of the resulting amplicon). These assays were combined with a third assay targeting a unique synthetic sequence used as the internal control (IC) and detected with a HEX-labeled probe.

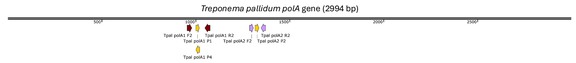

Figure 1. Design of the two assays targeting the *polA* gene of *T. pallidum*.

Test Production and Operation

Multiplexed mastermix was produced and lyophilized into individual C2T cartridges, which were pouched with desiccant and stored at room temperature until use. Each test was run on the T-COR 8 instrument using 100 µL of sample, mimicking the recommended addition of 5 drops from the swab device.

Results

Reduction of the *T. pallidum* (Syphilis) C2T Test Run Time

With a goal of achieving a time-to-answer of < 1 hour, the PCR cycling protocol was modified to decrease the overall run time. These modifications included:

- Decreasing the time of the initial denaturation step
- Combining each cycle's annealing/extension cycles
- Decreasing each cycle's time of annealing/extension
- Maximizing the annealing temperature to create a lower ramping requirement for heating and cooling
- Programing the protocol to use the active cooling capability of the T-COR 8 instrument

These modifications allowed for the total assay run time to be decreased to 43 min; with an approximate 2 min loading time, this **equates to a sample-to-answer time of 45 min**.

T. pallidum (Syphilis) C2T Test Sensitivity

Samples were created using live and heat-killed versions of 2 strains of *T. pallidum*, representative of the 2 main currently circulating strains. Cryopreserved cultures were diluted at decreasing concentrations and tested directly—no extraction was performed. **Both *T. pallidum* strains were detected with ≥90% sensitivity down to <100 treponemes/test.**

Table 1. Sensitivity for direct (no extraction) detection of 2 *T. pallidum* strains.

	T. pallidum SS14			*T. pallidum* Nichols Seattle		
	Rate	Tp/test	Tp/mL	Rate	Tp/test	Tp/mL
Live culture	95.8% (23/24)	1.9×10^1	5.0×10^3	95.5% (21/22)	3.7×10^1	1.0×10^3
Heat-killed culture	90.9% (20/22)	9.4×10^1	2.5×10^3	100% (24/24)	4.7×10^1	1.3×10^3

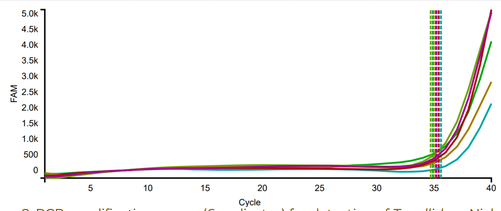

Figure 2. PCR amplification curves (6 replicates) for detection of *T. pallidum* Nichols Seattle at a concentration of 1.0×10^3 Treponemes/mL on the T-COR 8.

Results (cont.)

Specificity and Selectivity of the *T. pallidum* (Syphilis) C2T Test

A number of non-*T. pallidum* organisms were tested in triplicate at a high concentration; these organisms were selected due to their propensity of being present within and around the target sample types (i.e., skin wounds and lesion exudates). DNA was extracted to allow for the highest concentration of each organism to be tested. Samples were tested with or without *T. pallidum* DNA included at the LoD (5.0×10^1 gc/reaction). **No organism provided a false positive, and *T. pallidum* was detected at the LoD in the presence of all organisms.**

Table 2. Specificity and selectivity evaluation of the *T. pallidum* (Syphilis) C2T Test.

Organism	Concentration Tested (cfu/mL)	No *T. pallidum* Target	+ *T. pallidum* Target at LoD	
Staphylococcus aureus	1.8×10^8	0/3 detected	3/3 detected	33.4 (0.2)
Bacteroides fragilis	1.0×10^8	0/3 detected	3/3 detected	33.2 (0.9)
Staphylococcus saprophyticus	1.4×10^8	0/3 detected	3/3 detected	33.5 (0.4)
Mycoplasma hominis	4.8×10^8	0/3 detected	3/3 detected	32.2 (0.4)
Mycoplasma genitalium	5.9×10^8	0/3 detected	3/3 detected	33.3 (0.3)
Proteus vulgaris	2.9×10^8	0/3 detected	3/3 detected	33.6 (0.4)
Klebsiella pneumonia	1.9×10^8	0/3 detected	3/3 detected	33.4 (0.5)
Enterococcus faecium	1.0×10^8	0/3 detected	3/3 detected	33.0 (0.2)
Proteus mirabilis	2.7×10^8	0/3 detected	3/3 detected	33.2 (0.4)
Staphylococcus epidermidis	1.8×10^8	0/3 detected	3/3 detected	33.6 (0.1)
Clostridium perfringens	1.7×10^8	0/3 detected	3/3 detected	33.5 (1.1)
Escherichia coli	1.4×10^8	0/3 detected	3/3 detected	33.7 (0.3)
Enterobacter cloacae	2.7×10^8	0/3 detected	3/3 detected	33.7 (0.3)
Chlamydia trachomatis	4.2×10^8	0/3 detected	3/3 detected	33.8 (0.4)
Pseudomonas aeruginosa	5.2×10^8	0/3 detected	3/3 detected	33.0 (0.9)
Enterococcus faecalis	2.0×10^8	0/3 detected	3/3 detected	32.7 (0.2)
Neisseria gonorrhoeae	2.8×10^8	0/3 detected	3/3 detected	33.6 (0.2)

Potentially Interfering Agents

The impact of potentially interfering over-the-counter agents expected to be found in or near sampling areas were evaluated. **At the concentrations tested, no agent proved inhibitory to the *T. pallidum* (Syphilis) C2T Test, and *T. pallidum* was detected in all reactions.** These agents include:

- Abreva (10% of reaction)
- Lotrimen (10% of reaction)
- 5% KY vaginal moisturizer
- 0.25% Vagisil
- 5% Balneol
- 5% Preparation H
- 10% Summer's Eve Douche
- 1% Betadine

Repeatability and Across-Lot Variability

Repeatability was determined by calculating the standard deviation for the Cts achieved across 2 concentrations of *T. pallidum*, each tested repeatedly in the same production lot. The across-lot variability was determined by calculating the standard deviation for the Cts achieved across 2 concentrations of *T. pallidum* target, each tested repeatedly across 6 different production lots. **Both tests showed highly repeatable detection with tight standard deviations across multiple production lots.**

Test and *T. pallidum* Concentration	Reactions (N)	Mean Ct (SD)
Repeatability		
• 5×10^1 c/rxn	24	33.0 (0.6)
• 1×10^2 c/rxn	12	32.4 (0.8)
Across-lot variability		
• 1×10^2 c/rxn	13	32.5 (0.5)
• 1×10^4 c/rxn	11	27.5 (0.3)

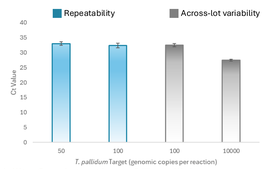

Figure 3. Repeatability and across-lot variability for detection of *T. pallidum*.

Stability

Produced tests were found to be able to **detect a low target level when held at an elevated temperature (37°C) for up to 6 months and room temperature (25°C) for up to 1 year.**

Conclusion

In this study, the *T. pallidum* (Syphilis) C2T Test:

- Allows for an interpreted point-of-care detection of *T. pallidum* to aid in the diagnosis of syphilis.
- Allows for a sample-to-answer time of 45 min.
- Robustly detects multiple *T. pallidum* strains with ≥90% sensitivity down to <100 treponemes/test.
- Is highly specific and not susceptible to inhibition with potentially interfering agents.
- Shows excellent repeatability and across-lot variability with minimal standard deviation.
- Is stable at room temperature for a minimum of 1 year.

Plan Forward

The *T. pallidum* (Syphilis) C2T Test is being developed by Tetracore according to ISO 13485 standards. The validation of the *T. pallidum* (Syphilis) C2T Test is planned to encompass the following:

Nonclinical validation studies—performed using laboratory-generated samples to ensure that the device meets our requirements of analytical sensitivity, precision, inclusivity, specificity, selectivity, interference, and stability. These results are reported in this abstract.

Preclinical validation studies—to include testing to determine the analytical sensitivity, precision, inclusivity, specificity, selectivity, interference, and stability in contrived clinical specimens using the *T. pallidum* (Syphilis) C2T Test to confirm the ability of the test to detect *T. pallidum* organism in relevant unextracted clinical specimens.

Clinical validation studies—to include evaluation of 30 syphilis-positive and 30 syphilis-negative clinical specimens using the *T. pallidum* (Syphilis) C2T Test. These specimens will also be tested using a CLIA laboratory test, to allow for a direct comparison.

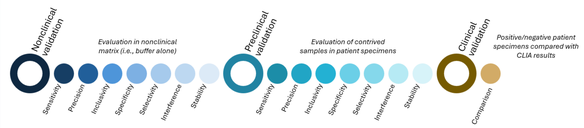

Figure 4. Development plan for the T. pallidum (Syphilis) C2T Test, with a final plan for a clinical study and 510(k) with CLIA waiver submission.

Acknowledgements

This work was supported by contract from the National Institutes of Health (NIH) National Institute of Allergy and Infectious Diseases (NIAID), Contract 75N93022C00025 and 75N93024C00073.

The authors wish to thank the following individuals for their contributions to this work: Daniel Normand, Mechanical Engineer; Ian Hicklin, laboratory technician. Additionally the authors thank our collaborator, Dr. Lorenzo Giacani, in the Department of Global Health at the University of Washington for supplying *Treponema* cultures.

Abbreviations

C2T, Collect-to-Test; cfu, colony forming units; gc, genomic copies; IC, internal control; IVD, in vitro diagnostic; PCR, polymerase chain reaction; POC, point-of-care; STI, sexually transmitted infection.