Analytical validation of the CSI-Dx® Tick-Borne Assay and the RAPID-Dx Bioinformatic Pipeline in Urine

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INTRODUCTION

Culture-based methods have been regarded as the gold standard of diagnosis for infectious diseases (Laupland and Valiquette, 2013) and used as an essential tool in determining treatment regimens. However, these methods can take up to 96 hours to identify a pathogen and determine its susceptibility to antibiotics (Afshari et al., 2012). Some organisms, such as the causal agent of Lyme disease, *B. burgdorferi*, require special media and may take much longer to grow to detectable levels, if they grow at all (Schutzer et al., 2019). Factors that decrease the efficacy of culture-based methods include previous antibiotic treatment, growth media requirements that can be difficult or impossible to replicate, poor sample quality or preprocessing, low microbial load, and minor infection severity (Fenollar et al., 2006; Mancini et al., 2010; Afshari et al., 2012; Blauwkamp et al., 2019). Such methods fail to identify a pathogen as often as 50% of the time (Srinivasan et al., 2015). Situations where culture-based methods fail to identify pathogenic organisms in cases involving infection (culture-negative infections), have been shown to increase the risk of further complications due to uncertainties involving identification of pathogen(s) and associated resistances, which can delay the proper treatment required.

Certain infectious disease states (i.e., Lyme Disease) are not diagnosed via culturing methods due to the slow growth rate of the causative pathogen. Serological tests are therefore implemented to assess human immune responses to pathogens through detection of antibodies. However, these methods tend to be unreliable during the early stages of infection — known as the "window period" — as the immune system has not yet formed a robust enough response to be detected via these techniques (Moore et al., 2016). This window period results in high false-negative rates leading to missed diagnoses. In addition, some serologic tests, such as the Western Blot/Immunoblot, may be difficult to interpret and add in an element of unnecessary subjectivity to patient diagnosis (Branda et al., 2017).

PCR-based methods like real-time PCR (qPCR) have been shown to yield results faster with at least 3 times more sensitivity than culture-based assays (Boutaga et al., 2003; Guiducci et al., 2019). Notably, qPCR technology has also been used in situations to circumvent high false negative rates inherent to culture-based assays due to pathogenic lifestyles, sampling factors, and treatment (Gallo et al., 2008). However, PCR methods are limited by the number of targets that can be amplified per assay (Mancini et al., 2010). Though methods for maximizing this capability exist, PCR is not an ideal choice for the diagnosis of infectious diseases with multiple causal agents, or for identifying novel pathogens.

The advent of next-generation sequencing (NGS) presents a promising avenue for developing methods to detect infections that hold clear advantages over current gold standards. NGS enables clinicians to identify pathogenic microorganisms present in a sample by determining their genetic sequence without inherent biases associated with culturing microbes. There have been several cases in which NGS has identified pathogens when routine testing could not (Goldberg et al., 2015). While

metagenomic (MG) approaches, which sequence all DNA in a sample, remain a promising tool for characterizing the composition of microbial populations, there are caveats that can bias clinical diagnosis. Due to the stability of DNA, MG approaches are unable to distinguish between past and current infections as they may detect the DNA of dead pathogens (naked DNA) that are no longer impacting the host patient (Chiu and Miller, 2019). However, the emergence of metatranscriptomics (RNA sequencing) technology has provided the means to characterize active organisms and genes in a sample. Using metatranscriptomics (MT), several clinically-based studies were able to characterize relevant immune response pathways and microbial characteristics associated with the conditions ranging from pediatric asthma to COVID-19 (Pérez-Losada et al., 2015; Zhang et al., 2020).

In this study we present the analytical validation of the CSI-Dx® Tick-borne Assay, a MT assay for the broad detection of microbes in clinical human urine samples. Study design and analytical validation of this assay were based on guidelines from Clinical and Laboratory Standards Institute (CLSI). This study assessed the performance of CSI-Dx® using 6 internal validity metrics, including the limit of detection (LoD) of 7 representative microorganisms, limit of blank (LoB), reproducibility between sequencing runs and analysis (precision), analytical accuracy, ability to detect co-infections of closely related taxa (interference), and stability of samples as well as test system performance over time.

METHODS

Analytical Validation Sample Collection & Preservation

Urine specimens were collected from individuals without previous Tickborne associated disease diagnosis. Samples were collected in 50mL urine collection containers containing Urine Conditioning Buffer (Zymo Research). Study participants were instructed to conduct a lymphatic flush prior to specimen collection.

Limits of blank (LoB) and detection (LoD)

The LoB and LoD of 7 representative positive control microorganisms (Table 1) spanning the Eukaryota, Bacteria, and Virus domains were individually spiked into 50 mL aliquots of pooled human urine collected from clinically healthy individuals. Positive control microbes were intentionally selected to assess the CSI-Dx® test system's ability to identify both gram-positive and gram-negative bacteria, as well as microbes of varied GC content and genome size. Controlled microbial concentrations spanning seven 0.5 log-fold CFU dilutions (3 – 3000 cells/µL) were spiked into urine specimens in triplicate for each positive control microorganism. Additionally, triplicate "blank" urine specimens without any representative microbe spike in and up to 4 no-template control "NTC" specimens consisting of sterile nuclease-free water and Urine Conditioning Buffer were processed in union with the controlled spike-in samples. Each assay contained at most 28 samples (24 urine and up to 4 NTC) per positive control microorganism. Prepared samples underwent subsequent RNA extraction and library preparation as described in the Supplementary Information. 15 pg of a characterized synthetic RNA sequence (ERCC) was spiked into each RNA extract prior to library preparation to serve as an internal control. Prepared, multiplexed libraries were then subject to Illumina Sequencing on the NextSeq 550 platform 4 times per control microorganisms

Table 1: List of the seven representative microorganisms incorporated into the -CSI-Dx® LoD validation experimental design.

Organism	Gram-Status	%GC	Genome Size	ATCC #	Reference
Borrelia burgdorferi B31	Neither (diderm)	28.2	1.3 Mb	35210	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3028687/
Borrelia afzelii BO23	Neither (diderm)	27.8	1.3 Mb	51992	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6019248/
Staphylococcus aureus M10/0148 (MRSA+)	Gram Positive	32.8	2.8 Mb	BAA-2312	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6086073/
Pseudomonas aeruginosa 283 [7]	Gram Negative	66	6.9 Mb	17649	https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1068618/
Escherichia coli CFT073	Gram Negative	50	5.2 Mb	700928	https://aem.asm.org/content/77/10/3268#T1
Candida albicans CBS 562	Fungus	33.6	14.7 Mb	18804	https://www.atcc.org/products/18804
Zika Virus (Synthetic RNA)	RNA Virus	--	1.5 Kb	VR-3198SD	https://www.atcc.org/products/vr-3252sd

Generated sequence data was subject to systematic parallel bioinformatics processing using CSI's RAPID-Dx bioinformatic pipeline. Final taxa annotation counts across all 4 replicate sequencing runs of each control spike in were collated into a text file for downstream analysis and visualization.

Accuracy

The analytical accuracy of the CSI-Dx® assay was evaluated via the processing of 24 blank (non-spiked) urine samples and 20 urine samples spiked with select representative microorganisms at a concentration equivalent to 2-fold above the respective established LoD of each respective microbe. All 44 prepared urine specimens underwent CSI-Dx® processing and RAPID-Dx analysis as described previously. Resulting RAPID-Dx output was collated from all accuracy samples, any blank samples in which a representative microbe was annotated at a normalized count above threshold was counted as a false-positive (FP) whereas any sample that was subject to a microbial spike-in at 2X LoD that did not yield a normalized count above threshold was considered a false-negative (FN). Resulting analytical specificity (TN/[TN+FP]) and sensitivity (TP/[TP+FN]) values were subsequently calculated.

Interference

To evaluate the CSI-Dx® assay's ability to detect co-infections of closely related microbial taxa, an interference experiment was conducted. *B. burgdorferi* and *B. afzelli* were spiked into pooled urine specimens at ratios of

1:1, 4:1, and 1:4 where the "1" values were spiked at 2X established LoD. Triplicate samples were prepared and underwent CSI-Dx® processing and RAPID-Dx analysis for each ratio (n = 9).

Stability

To assess sample stability and test system performance over time, a concentration of *B. burgdorferi* equivalent to 1X LoD was spiked into urine specimens in triplicate at each time point and placed at RT for a period of 1, 3, 7, 14, and 30 days. The time point set ups were performed in reverse such that the 30-day time point was set up at 30 days from target completion and subsequent time points were spiked as the target completion date approached. This allowed all time points to be processed simultaneously to ensure equivalent assay conditions for all samples. Each time point was analyzed for the presence of *B. burgdorferi* over the course of time. Stability of NGS sequencing libraries post freeze-thaw was evaluated through the comparison of ERCC-normalized counts of each respective control representative microbe across replicate sequencing runs 2 – 4 to their respective LoDs.

Precision / Reproducibility

Precision of the CSI-Dx® assay was assessed by examining negative controls on all analytical validation runs (n=32). Negative controls were considered successful if they contained fewer than 2.5 million filtered, non-ERCC sequences and fewer than the respective LoD for each of the 7 taxa used for the LoB/LoD experiments. These criteria then allowed us to assess both cross contamination among samples on the run, as well as environmental contamination.

RESULTS

The CSI-Dx® Tick-Borne assay was developed to leverage NGS technology to detect trace concentrations (cells/mL) of tick associated pathogens in clinical urine specimens. To assess the analytical validity of the assay, *Limit of Detection, Accuracy, Stability, and Interference e*xperiments were conducted with contrived clinical urine specimens consisting of a controlled addition of one or multiple of a panel of 7 representative microbial taxa (Table 1). GC content varied from 28-66%, and genome size ranged from 1.5 Kb to 14.7 Mb. These taxa included five bacteria, one fungus, and one virus. Notably, two of the chosen bacteria are prominent tick-borne pathogenic taxa and are members of the same genus. When considering all validation experiments together, a total of 767 experimental metatranscriptomic samples were processed, yielding a total of 12.4 billion raw sequences, 11.2 billion of which were retained after quality filtration. Experimental samples that were found to yield over 2.5 million sequences and possess a fraction of ERCC reads greater than 0.001% of filtered sequence abundance were considered to pass QC and were incorporated into downstream validation analysis. Precision was also evaluated by analyzing QC pass/fail results from all 121 negative control samples tested over the course of the comprehensive analytical validation.

Limit of Blank (LoB) and Limit of Detection (LoD)

Limit of Blank (LoB) calculations were conducted to identify the observed baseline signal of each of the 7 representative taxa in pooled urine specimens from individuals with no prior tick-borne illness diagnoses. LoB calculations were conducted as described in the Clinical and Laboratory Standards Institute (CLSI) EP17-A2 document considering blank pooled urine specimens collected over the course of the 7 Limit of Detection (LoD) spike-in experiments (CLSI, 2012). A total of 84 pooled blank urine specimens underwent CSI-Dx® processing to assess the LoB of each representative taxon. Two pathogens, *E. coli* and *S. aureus*, were found to yield increased LoBs of 683.25 and 40,014.26 ERCC-normalized counts, respectively. *P. aeruginosa* was found to yield a LoB of 60.94 ERCC-normalized counts. The remaining representative taxa, *B. afzelii*, *B. burgdorferi*, *C. albicans*, and *Zika virus* were found to yield low LoB measures, with *B. afzelii* yielding a LoB of 3.13 ERCC normalized sequences, and the remaining taxa yielded a LoB of 0. The LoB of additional tick-associated pathogens that were not representative spike-in taxa within the scope of this analytical validation were also calculated,

all of which were found to yield a LoB of 0. In summary, the LoB results from representative taxa indicate that while certain pathogens such as *E. coli* and *S. aureus* are relatively prevalent in healthy urine specimens, pathogens associated with Tick-associated illnesses such as Borrelia burgdorferi and *B. afzelii*, along with the eukaryotic representative taxon *C. albicans* yield trace to absent pathogen signal (Table 2).

Table 2. Comprehensive LoB/LoD per representative taxa. ERCC sequences denote the value of the raw counts for each taxon divided by the number of ERCC sequences.

Representative Taxon	***Limit of Blank (LoB)***	***Limit of Detection (LoD)***
Borrelia burgdorferi	0 ERCC sequences	3 cells/µL
Borrelia afzelii	3.14 ERCC sequences	3 cells/µL
Candida albicans	0 ERCC sequences	3 cells/µL
Staphylococcus aureus	40,014.26 ERCC sequences	NA*
Zika Virus	0 ERCC sequences	1000 particles/µL
Escherichia coli	683.25 ERCC sequences	NA*
Pseudomonas aeruginosa	60.94 ERCC sequences	316 cells/µL

Nonparametric LoD calculations based on CLSI's EP17-A2 *Classical Method* were conducted to determine the lowest cell concentration at which the CSI-Dx® assay could consistently yield annotation counts above the predetermined LoB threshold for 100% of samples (Table 2, Figure 1). *B. afzelii*, *B. burgdorferi*, and *C. albicans* yielded all yielded an LOD of 3 cells/mL, respectively. *P. aeruginosa* and *Zika virus*, yielded LoD measures of 316 cells/mL and 1000 genome copies/mL, respectively. Together, the data serve as an indication of the CSI-Dx® assay's ability to detect trace concentrations of tick associated bacteria (*B. afzelii* and *B. burgdorferi*) as well as fungi (*C. albicans*) and viruses (*Zika virus*) in clinical urine specimens.

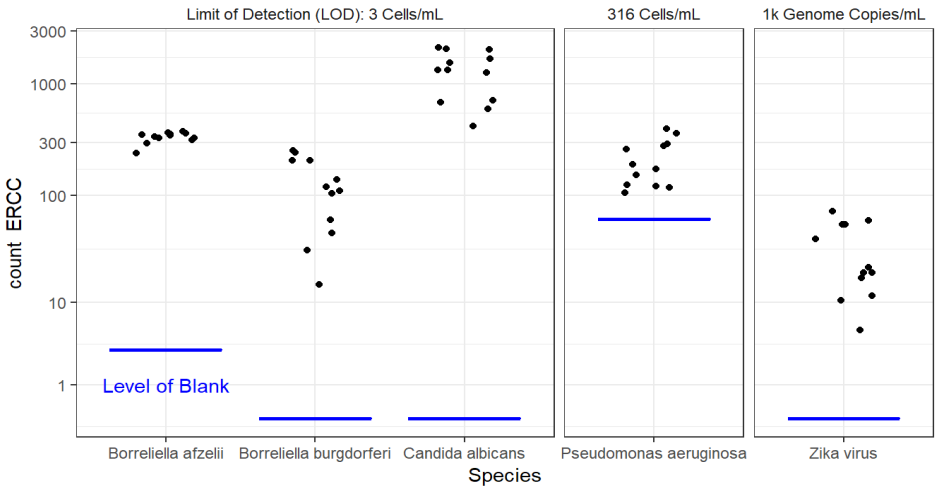

Figure 1. Limit of Detection (LoD) CLSI non-parametric classical calculation result dot-plots display ERCC normalized counts of the lowest spike-in concentration at which 100% of analytical samples (12/12) yielded counts above the Limit of Blank (LOB). Three taxa (*B. afzelii*, *B. burgdorferi*, and *C. albicans*) yielded an LoD of 3 cells per mL. P. aeruginosa and Zika virus yielded LoD results of 316 and 1000 cells/mL, respectively.

Accuracy Results

After the calculation of per-taxon LoDs, a follow up analytical accuracy experiment was conducted in which 24 contrived negative specimens and 20 contrived positive specimens (2X LoD) were subject to CSI-Dx® sample processing (Table 3). A total of 4 contrived negative samples, and 2 contrived positive samples were observed to fail assay QC due to insufficient sequencing depth or insufficient internal ERCC control sequence counts. All 20 remaining contrived negative specimens yielded a negative (below detection) result for all assayed tick associated pathogens (Table 3, Figure 2). All 18 contrived positive samples that passed assay QC yielded a positive result (above detection) for their respective spiked in taxon of interest (B. afzelii [5/5], B. burgdorferi [4/4], *C. albicans* [5/5], *Zika virus* [4/4]). Only one taxon, *P. aeruginosa*, was found to yield positive signal above threshold in multiple (n = 12) samples in which it was not the controlled lab spike-in.

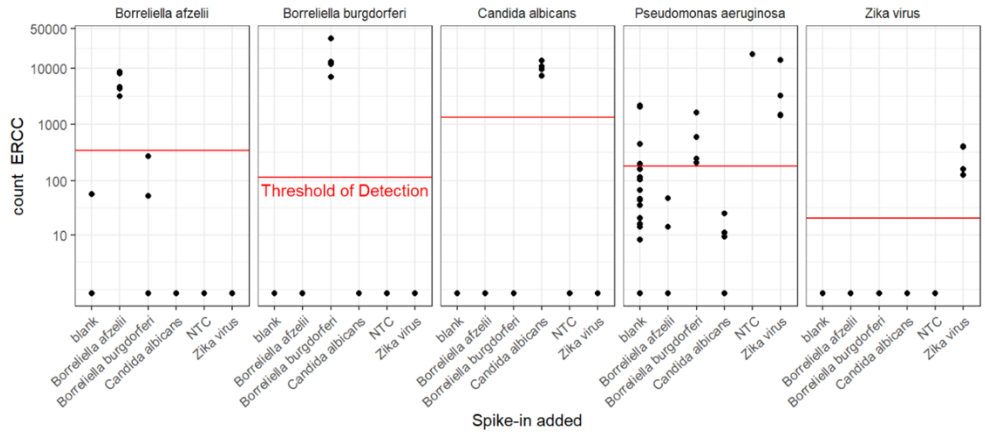

Figure 2. Analytical accuracy experimental results reveal 100% analytical sensitivity (18/18) and analytical specificity (20/20) of contrived clinical urine specimens. Per-taxon limit of detection bars calculated from the prior Limit of Detection are displayed on each dot-plot facet.

Table 3. Per taxa/cohort analytical sensitivity and specificity results.

True Sample cohort	***Number of Samples (n)***	***B. afzelii detected (n)***	**B. Burgdorferi detected (n)**	**C. albicans detected (n)**	**Zika virus detected (n)**	**Assay Failed QC**	**Not detected (n)**
Blank	24	0	0	0	0	4	20
Borrelia afzelii	5	5	0	0	0	0	0
Borrelia burgdorferi	5	0	4	0	0	1	0
Candida albicans	5	0	0	5	0	0	0
Zika virus	5	0	0	0	4	1	0
Total	44	5	4	5	4	5	20

Interference Results

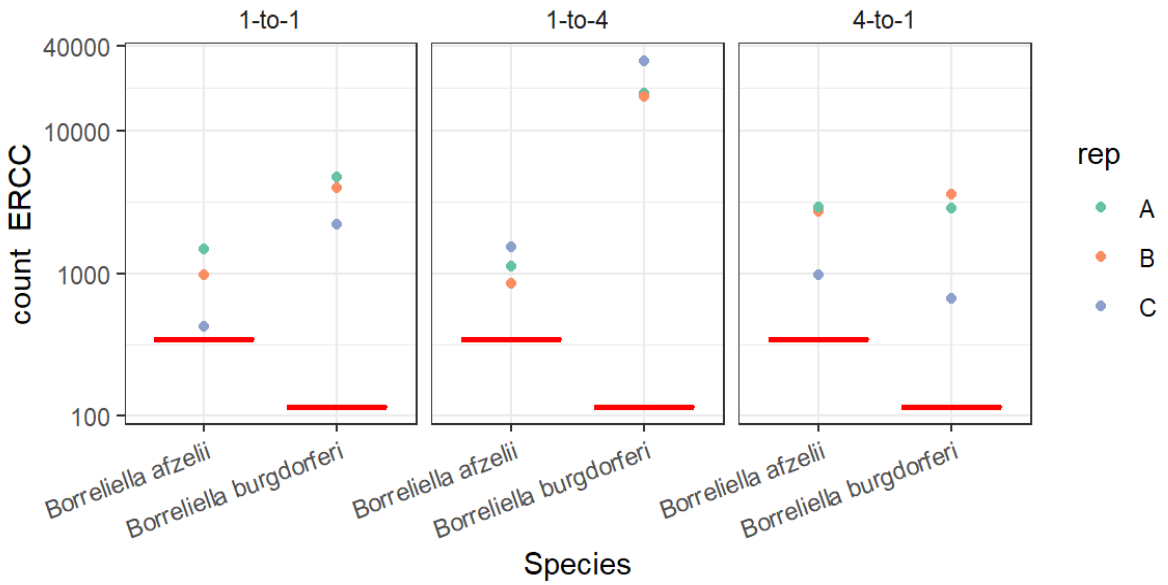

Figure 3. Box plots showcasing detection results of co-infectious Borrelia burgdorferi and Borrelia afzelii within each controlled ratio spike in (1:1,4:1,1:4).

The interference experiments, in which 4:1, 1:1, and 1:4 ratios of *B. afzelii* and *B. burgdorferi* were spiked into matched urine specimens, demonstrated that the CSI-Dx® assay can successfully detect multiple pathogens of closely related species in 100% (n = 6/6) of samples (Figure 3). Counts for *B. afzelii* and *B. burgdorferi* were above LoD in all tested samples. Therefore, both tick-borne pathogens were able to be qualitatively detected, showing a lack of interference based on the presence of a closely related taxa. This serves as an indication of the CSI-Dx® assay's ability to identify co-infections of closely related taxa in clinical urine specimens.

Stability & Precision Results

Stability was evaluated with respect to the passage of time at room temperature. *B. burgdorferi* counts remained above detection within 3 replicate urine specimens across Day 1, Day 3, Day 7, Day 15, through Day 30 in all samples (Figure 4). This shows the CSI-Dx® assay can reliably detect pathogens in preserved

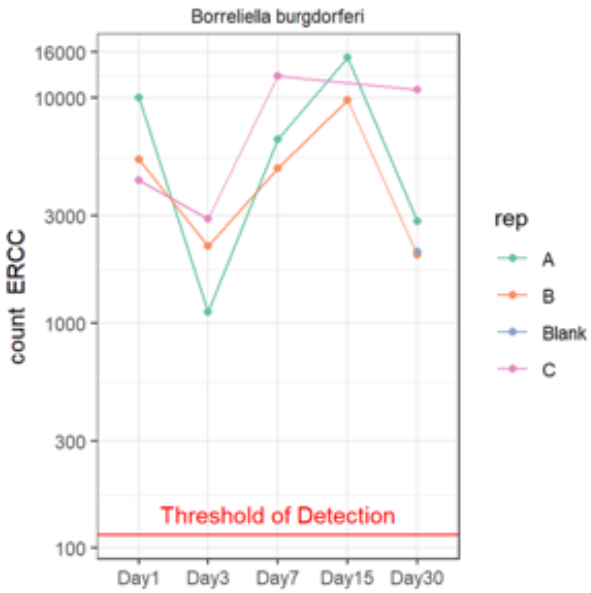

Figure 4. Dot plot of ERCC normalized Borrelia burgdorferi annotations observed over time (Day 1 – Day 30) within triplicate (n = 3) urine specimens.

samples up to a month after collection, removing any concern regarding the logistics of sample transportation. A total of 121 negative control (NTC) specimens underwent CSI-Dx® processing and analysis to assess the precision of the CSI-Dx® assay. Only 1 of the 121 samples

was observed to fail assay QC due to excess (> 2.5 million non-ERCC sequences) coverage, indicating a precision of over 99% for the CSI-Dx® assay.

DISCUSSION

Here, we present the development and analytical validation of the CSI-Dx® Tick-Borne Assay. This novel, metatranscriptomic (MT) based assay is intended for the broad detection of tick-associated pathogens in clinical urine samples. This analytical validation was designed and performed based on Clinical and Laboratory Standards Institute (CLSI) guidelines for laboratory developed tests. Recent studies which have also reported on similar analytically validated assays, have largely focused on metagenomic (MG), DNA-based methods (Blauwkamp et al., 2019; Miller et al., 2019). Considering the differences in NGS sequencing technology used between pre-existing assays and the CSI-Dx® Tick-Borne Assay, MT analysis can generate gene expression data and ultimately identify active pathways from a given sample, whereas MG is unable to differentiate between active and inactive genes. Existing studies of analytically validated MG-based assays have also discussed this limitation in depth (Blauwkamp et al., 2019; Miller et al., 2019). This critical difference between the two NGS methods highlights the novelty and potential for clinical utility of the CSI-Dx® Tick-Borne Assay compared to current analytically validated NGS assays.

In the context of detecting tickborne pathogens using NGS-based assay, the CSI-Dx® Tick-Borne Assay may be a more promising assay compared to previously analytically validated mNGS assays. Rather than using a single threshold to determine LoD for all organisms (Miller et al., 2019), our analytical validation study calculated LoB for individual organisms, which ultimately allowed for a more sensitive assay through a lower derived LoD. More specifically, the identification of tickborne pathogens is an ideal target for CSI-Dx® since initial LoB calculations for *B. burgdorferi* and *B. afzelii* were low, 0 and 3.14 ERCC sequences, respectively. With a low initial LoB, a more sensitive LoD for these tickborne pathogens of interest was ultimately calculated at 3 cells/uL. Calculated LoB and LoD for more common organisms found in the environment and human skin, like *S. aureus*, *E. coli*, and *P. aeruginosa* were larger from 60 to 40,014 ERCC sequences and 4-3000 cells/uL, respectively.

The overall accuracy for the CSI-Dx® Tick-Borne Assay resulted in 100% for both analytical specificity and analytical sensitivity (Table 1). While there are several existing types of Lyme disease diagnostic assays, reported measures of accuracy were found to be highly variable for sensitivity (9.6%-100%) and specificity (44%-100%). Sensitivity and specificity values were highly variable since individual studies assessed different diagnostic assays for various stages of Lyme disease among clinical patient samples, rather than analytical validation samples that contain known concentrations of organisms (Cook and Puri, 2016). More recently, analytically, and clinically validated mNGS-based assays reported sensitivity between 98-99% and specificity between 73%-93% (Blauwkamp et al., 2019; Miller et al., 2019). Additionally, Miller et al. (2019), noted that their reported sensitivity of 73% refers to clinical rather than analytical sensitivity and discussed reasons that could have contributed to a lower calculated clinical sensitivity. While our analytical accuracy results are comparable to existing studies, more work is required to assess whether the accuracy of the CSI-Dx® Tick-Borne Assay will alter with clinical samples.

Ticks are capable of co-transmitting human pathogens that can range from spirochetal bacteria, rickettsia bacteria, flaviviruses, and protozoan parasites (Diuk-Wasser et al., 2016a). Epidemiology studies have estimated around 19% of Lyme disease cases result in concurrent co-infections involving babesiosis (Diuk-Wasser et al., 2016b). Additionally, symptoms of co-infection with Lyme disease and babesiosis have been found to largely resemble symptoms from other conditions like anemia, thrombocytopenia, and influenza, compared to patients only diagnosed with Lyme disease (Krause et al., 1996). The wide range of clinical symptoms

indicative of Lyme disease and cases of associated co-infection, have long been a diagnostic challenge that contributes to the rate of missed/delayed diagnosis and prescription of ineffective antibiotic treatment (Aucott et al., 2009). In the current study, the CSI-Dx® Tick-Borne assay was able to identify multiple pathogens from the same urine sample. Specifically, both taxa (*B. afzelii* and *B. burgdorferi*) were qualitatively identified and suggest that the sensitivity and specificity of the CSI-Dx® Tick-Borne Assay is not impacted by the presence of multiple closely related taxa in the same urine sample. In a larger scope, not only can this technology be used to contribute to the growing literature of Lyme disease co-infection epidemiology, but it can also be utilized to aid in refining treatment regimens for patients suffering from co-infections with tickborne-pathogens.

The CSI-Dx® Tick-Borne assay analytical validation yields a low (3 cells/mL) clinically-relevant LoD for tick associated pathogens in urine specimens. These benefits of the CSI-Dx® assay include: 1) faster turn-around time compared to current tickborne-pathogens detection methods, 2) untargeted nature of CSI-Dx® allows for broad-spectrum tickborne-pathogen detection in a single urine sample, 3) detection of active pathogens associated with infection, 4) longer established stability (30 days) compared to similar analytically validated assays, and 5) sample matrices required for this assay are non-invasive (i.e., urine). While results from this analytical validation suggests CSI-Dx® to be a competitive assay for clinical application, future work is required to assess the performance of this assay on clinical urine samples.

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