Development of qPCR Assay for Genital Microbial Signatures

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Introduction

Recently it has been proposed that the genital microbiome could serve as an alternative test during the collection of sexual assault evidence based on Locard's Principal of Exchange[1,2]. This principle states that, in forensic science, a transfer of materials occurs whenever two objects come into contact, and both can be utilized as forensic evidence [1].

This project involves developing a real time PCR assay for specific genital microbial taxa that have the potential to be signature organisms for the female and male genital microbiome. The specific organisms were selected based on a comparative analysis of deep sequencing data obtained from vaginal and penile microbial samples and generated at the McCord Research Lab, Figure 1.[2]

A set of real time PCR primers and probes were developed and tested using genital samples collected from nineteen female and male volunteers. Samples were were extracted, quantified and amplified using the qPCR-based methodology. Four *Lactobacilli* species were investigated and tested for vaginal specificity : *L. iners, L. crispatus, L. gasseri,* and *L. jensenii.*

These four *Lactobacilli* species have been proposed to make up the bulk of a healthy vaginal microbiome; whereas *Gardnerella vaginalis* is present in females with Bacterial Vaginosis (BV).

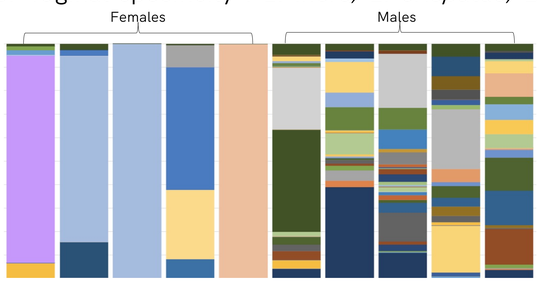


Figure 1: Initial results from a survey of 5 female and 5 male genital swabs indicate higher microbial diversity in the male samples. Each color indicates different bacterial species and their abundance in the sample [2].

Methods

Assay Design

MUSCLE Software
Reference 16S rRNA sequences for Lactobacillus, Gardnerella were extracted from the RDP database and aligned using MUSCLE software

IDT PrimerQuest tool
Primers and probes for the target of interest with a set of criteria where the Tm of the probe 6-9 °C

NCBI nucleotide Blast
Specificity of the design was examined using the BLAST search

IDT OligoAnalyzer tool
Hairpin and dimer formation was examined using the OligoAnalyzer tool.

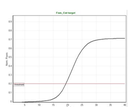
Methodology

Sample Collection
Nine vaginal samples and ten penile samples were collected using flocked and cotton sterile swabs, and MAWI iSWAB Microbiome Stabilizing Buffer.

Extraction
Extraction of samples was performed following the Zymo Research DNA MicroPrep Kit based on bead beating.

Quantification & Amplification
Samples were quantified using SYBRGreen qPCR paired with Universal Bacterial Primers [5]. Target amplification was performed using SYBRGreen & target-specific primers [3,4].

Data Analysis
Samples were analyzed using a comparative Ct model based on a fixed threshold.



Results

A community standard was utilized to test the designed primers *for Gardnerella* and *Lactobacillus*. Results showed that our initial design of *Lactobacillus* primers were insufficiently specific to be useful as a marker for vaginal samples, Figure 2. Results were positive for *Gardnerella* only in the vaginal sample.

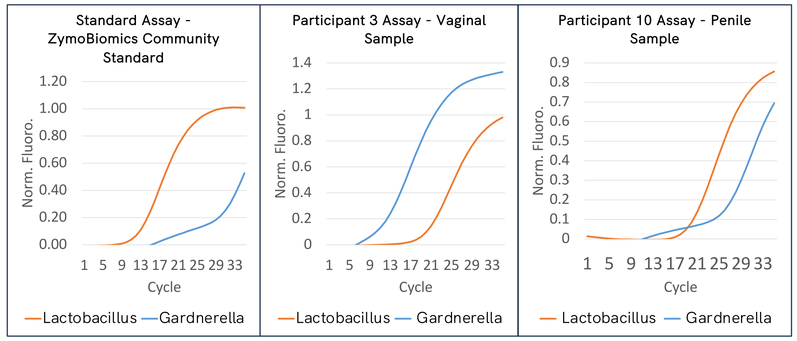


Figure 2: qPCR assays for detection of targeted bacteria. A. Amplification of *Lactobacillus* using community standard. B. Amplification in a vaginal sample using Gardnerella and Lactobacillus C. Amplification of penile sample using *Lactobacillus* & *Gardnerella* primers and probe design. (Response for *Gardnerella was deemed negative in the penile sample and it was not present in the control.)*

Therefore, a new set of primers for a subset of lactobacillus species were selected. These particular species had been previously reported in the literature to be specific to vaginal samples (Table 1) . Samples were then analyzed for *L. iners, L. gasseri, L. jensenii,* and *L. crispatus* (Figure 3, Table 2).

Table 1. Primers sequences for *L. iners, L. gasseri, L. jensenii,* and *L. crispatus*

Primer Name [3,4]	Sequence (5'-3')	Tm (°C)
L. iners F	TTGAAGATCGGAGTGCTTGC	63
L. iners R	TTATCCCGATCTCTTGGGCA	63
L. gasseri F	AGCGAGCTTGCCTAGATGAATTTG	60
L. gasseri R	TCTTTTAAACTCTAGACATGCGTC	60
L. crispatus F	AGCGAGCGGAACTAACAGATTTAC	63
L. crispatus R	AGCTGATCATGCGATCTGCTT	63
L. jensenii F	AAGTCGAGCGAGCTTGCCTATAGA	60
L. jensenii R	CTTCTTTCATGCGAAAGTAGC	60

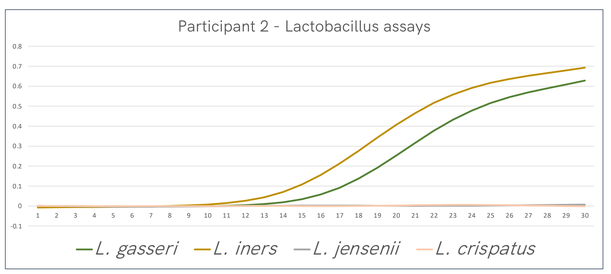


Figure 3. Participant 2 (vaginal) qPCR assays that detected primer sequences for *L. iners and L. gasseri.*

Results: *Lactobacillus* specificity

Sample #	Penile (P) Vaginal (V)	Quant (ng/uL)	*L. crispatus* [4]	*L. iners* [3]	*L. gasseri* [4]	*L. jensenii* [4]
1	V	1.3	-	16	-	20
2	V	0.3	-	17	19	-
3‡	V	6.6	-	30	-	-
4	V	0.05	-	-	-	16
5	V	2.3	-	15	-	20
6	V	2.1	-	-	-	17
7	V	0.2	20	-	24	-
8	V	4.8	-	11.75	-	-
9	V	1.1	19	-	18	-
10	P	0.1	-	-	-	-
11	P	0.003	-	-	-	-
12**	P	0.1	-	27	-	-
13	P	0.4	-	-	-	-
14	P	0.1	-	-	-	-
15	P	0.5	-	-	-	-
16**	P	5.6	-	-	-	18
17**	P	0.04	>30	30	-	-
18*	P	4.4	24	25	-	27
19	P	0.2	-	-	-	-

*Table 2. Ct values for four Lactobacillus species tested using male and female genital samples. All vaginal samples showed presence of at least one of the Lactobacilli species. 7 of 10 penile samples showed none of the four Lactobacilli species, The remaining positive male participants indicated previous sexual contact or the use of antibacterial soap/antibiotics in a questionnaire. ǂ Bacterial composition was predominantly Gardnerella vaginalis (Figure B). *Participant disclosed sexual contact. ** Participant was taking antibiotics and/or using antimicrobial soap at time of collection.*

Conclusions

A qPCR assay was developed to detect the presence of genital specific bacteria. Initial tests involved designing sets of primers and probes for vaginal specific bacteria [2]. The results indicated that two different bacterial genera had potential as indicator species: Gardnerella and Lactobacillus. Lactobacillus required more specificity and additional primers were identified [3,4]. Of the nine vaginal samples tested, eight showed specificity to *Lactobacilli.* The ninth sample was positive for *Gardnerella vaginalis.* 70% male/penile samples showed no presence of *Lactobacilli.* Those males who were positive either disclosed sexual contact or the use of antibiotics/antimicrobial soap in their questionnaire.

References

[1] Miller, M. T. (2014). Locard Exchange Principle. In M. T. Miller (Ed.) Academic Press.
[2] Ghemrawi, M., Torres, A. R., Duncan, G., Colwell, R., Dadlani, M., & McCord, B. (2020).
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[4] Tamrakar, R., Yamada, T., Furuta, I. et al. BMC Infect Dis 7, 128 (2007)
[5] Mao, D. P., Zhou, Q., Chen, C. Y., & Quan, Z. X. (2012). BMC Microbiol, 12, 66.

Acknowledgements

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