The Potential of Metatranscriptomics for Identifying Screening Targets for Bacterial Vaginosis

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Abstract

Background: The ribosomal RNA content of a sample collected from a woman with bacterial vaginosis (BV) was analysed to determine the active microbial community, and to identify potential targets for further screening.

Methodology/Principal Findings: The sample from the BV patient underwent total RNA extraction, followed by physical subtraction of human rRNA and whole transcriptome amplification. The metatranscriptome was sequenced using Roche 454 titanium chemistry. The bioinformatics pipeline MG-RAST and desktop DNA analysis platforms were utilised to analyse results. Bacteria of the genus Prevotella (predominately P. amnii) constituted 36% of the 16S rRNA reads, followed by Megaspheara (19%), Leptotrichia/Sneathia (8%) and Fusobacterium (8%). Comparison of the abundances of several bacteria to quantitative PCR (qPCR) screening of extracted DNA revealed comparable relative abundances. This suggests a correlation between what was present and transcriptionally active in this sample: however distinct differences were seen when compared to the microbiome determined by 16S rRNA gene amplicon sequencing. To assess the presence of P. amnii in a larger pool of samples, 90 sexually active women were screened using qPCR. This bacterium was found to be strongly associated with BV (P<0.001, OR 23.3 (95%CI:2.9–190.7)) among the 90 women.

Conclusions/Significance: This study highlighted the potential of metatranscriptomics as a tool for characterising metabolically active microbiota and identifying targets for further screening. Prevotella amnii was chosen as an example target, being the most metabolically active species present in the single patient with BV, and was found to be detected at a high concentration by qPCR in 31% of cohort with BV, with an association with both oral and penile-vaginal sex.

Introduction

The advent of molecular-based screening utilising massively parallel DNA sequencing has increased our capability to characterise the microbial ecology of human clinical samples, both rapidly and economically [1]. In addition it has allowed a better understanding of the normal endogenous microbiota. The vaginal microbiota is complex, varying at different stages of reproductive life, as well as during the menstrual cycle. Bacterial vaginosis (BV) is a condition affecting the vaginal microbiota where in the childbearing age woman the natural microbiota (typically Lactobacillus spp.) is depleted, and replaced by an overgrowth of mixed, primarily anaerobic bacteria [2]. This condition has significant associations with miscarriage, premature birth and pelvic infections, and can increase a woman’s risk of acquiring sexually transmitted infections and HIV [3]. No single aetiological agent has been identified yet for BV, and is now generally considered likely to be of polymicrobial aetiology.

BV is typically diagnosed using either the Nugent scoring method [4] that examines bacterial composition via a Gram smear or the Amsel criteria [5] that considers factors such as presence of discharge, amine production, presence of clue cells and a vaginal pH greater than 4.5. Through microbiome studies, the microbiology of BV has been better characterised. These studies have generally been carried out using PCR-derived 16S rRNA gene fragments, using “universal” bacterial 16S RNA gene primers [2]. While this approach is able to provide a comprehensive understanding of the bacterial community membership, it is not able to determine which members are transcriptionally active.

Metatranscriptomics is the analysis of the RNA transcripts being expressed by a community at a given point of time. For bacteria, the predominant RNA classes are ribosomal (primarily 16S rRNA and 23S rRNA), which are able to be used as taxonomic identifiers. The metatranscriptomic screening of clinical samples has been described for gut and dental microbiomes [6,7], with only one study to date applying this methodology to BV [8].
important difficulty faced with the application of this technique to clinical samples is the low levels of RNA present in many samples and the overabundance of host RNA. This requires reduction of host RNA and subsequent linear amplification of RNA for transcriptomic analysis [9,10].

This study describes a method to analyse microbial rRNA from vaginal samples that is applicable to variety of clinical sample types. It demonstrates how the data gained can provide valuable information for larger qPCR based screening studies.

Materials and Methods

Sample collection

A 26 year old woman presenting with abnormal vaginal discharge, odour, 4/4 Amsel criteria and a Nugent score of 10 was recruited with written consent from Melbourne Sexual Health Centre (MSHC), Victoria, Australia. This patient reported no recent antibiotic use, had no recent male sexual partner, and reported only having one female partner in the prior 3 months and three in the past 12 months. Vaginal discharge was collected using ten flocked swabs and immediately rotated and pooled in 4 ml RNAlater (Life Technologies, Grand Island, USA). The RNAlater solution was then stored at −80°C until processing.

For the second component of this study, extracted DNA (stored at −30°C) was utilised from a randomly selected subset of 90 samples obtained from a cross sectional study of sexually active women attending MSHC in 2003/4 [11]. This set of samples comprised 34 women with normal microbiota (Nugent 0–3), 20 with intermediate microbiota (Nugent 4–6) and 36 with BV (Nugent 7–10). Written consent for all de-identified participants and ethical approval for this study was obtained previously from The Alfred Hospital Human Research Ethics Committee.

Nucleic acid preparation

Figure 1 provides an overview of the workflow for this study. Initially, total RNA was extracted from 2 ml RNAlater solution [12]. Total RNA extraction was performed using Trizol (Life Technologies) with use of 1-Bromo-3-chloropropane in place of chloroform followed by RNA purification using a RNeasy column (Qiagen, Hilden, Germany) [13]. Any remaining DNA was digested using the Turbo DNA-free enzyme (Life Technologies).

Human ribosomal content was subtracted using the MICROBEnrich Kit (Life Technologies) as per manufacturer instructions followed by removal of small RNAs with the MEGAscreen kit (Life Technologies). RNA concentration was determined by Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA). To increase the RNA content needed for sequencing, whole transcriptome amplification (WTA) and reverse-transcription using the TransPlex Kit (Sigma-Aldrich Corporation, St. Louis, USA) was utilized. This resulted in a cDNA concentration of 30 ng/µl (from original RNA content of 3.5 ng/µl) with 2 µg submitted to AGRF (Australian Genome Research Facility Ltd., Brisbane, Australia) for 454 sequencing (FLX titanium chemistry; Roche/454 Life Sciences, Branford, USA).

Genomic DNA was precipitated from the remaining Trizol buffer using 0.3 ml of 100% ethanol per 1 ml of Trizol buffer followed by two washes of the DNA pellet with 0.1 M trisodium citrate in 10% ethanol [14]. An amplicon-based metagenomic library was generated from the extracted DNA using the universal bacterial PCR primers 27F and 338R that target the V1–V2 hypervariable regions of the 16S rRNA gene as previously described [15]. Both cDNA and 16S rRNA gene amplicon libraries were subsequently bar-coded using MID tags and sequenced together utilising a quarter region of a 454 sequencing plate.

Sequence analysis

Subsequent to sequencing, all raw sequencing data was de-multiplexed according to their MID tags and the data obtained from each sample was then imported into Genomics Workbench version 4.5.1 (CLC Bio, Aarhus, Denmark), for removal of WTA primers and random primer sequences from each applicable read and data was filtered (low quality score limit of 0.05; maximum 2 ambiguous nucleotides allowed; minimum sequence length of 100 nt). Each dataset was then screened for chimeric sequences using UCHIME [16], with the trimmed and filtered data submitted to the MG-RAST (http://metagenomics.anl.gov) bioinformatics pipeline for analysis (cDNA library MG-RAST ID = 4461586.3; DNA amplicon MG-RAST ID = 4461792.3) [17]. A 90% cut-off was used for database searches within MG-RAST as an arbitrary cut-off for genus identities using the RDP database, and a 98% cut-off was used for species level identification [18]. MG-RAST generates abundance counts based on the number of unique hits a particular sequence has against a particular database. It is therefore highly likely that a single read may have multiple abundance counts assigned to it if there is an equal relatedness. The identities for each read were sorted using Excel 2007 (Microsoft Corporation, Redmond, USA) to eliminate multiple identical hits for individual reads, with manual analysis being carried out using the BLAST algorithm for discrepant samples. Graphical representation of bacterial abundances was achieved using Krona charts [19]. Functional genes from the cDNA library were characterised by SEED analysis within MG-RAST.

Reads derived from the cDNA library assigned to each major genus (comprising >10% of total population) using MG-RAST were also imported into Lasergene 8 (DNASTAR Inc., Madison, USA) for manual sequence alignment was carried out using a 98% sequence match cut-off. The consensus sequences of alignments were assigned an identity using the BLAST algorithm with a ≥98% identity required to assign a species name.

Quantitative PCR (qPCR) screening

All assays were performed on the LC480 real-time instrument (Roche Diagnostics, Mannheim, Germany) using 5 µl DNA in a 20 µl reaction. The bacteria targeted were Atoohobium vaginae, Leptotrichia/ Sneathia spp., Gardnerella vaginalis [21], and the genera Prevotella [22] and Lactobacillus [23]. A primer set for Prevotella amnii was developed using the forward and reverse primers 27F and 338R [24] with the incorporation of a TaqMan probe 5′-[6FAM] GCG CAC ACT GGG TGG TGG GCG ATC TC-3′, specific to P. amnii, based on 454 sequencing data from this study and all available relevant sequences on the Genbank database. The total bacterial content of each sample was determined by the modification of an assay by Nikkari et al. [2002] [25] with the incorporation of a TaqMan probe, 516F (5′-6FAM) TGC CAG CAG CCG TAA [BHQ1]-3′ to calculate relative bacterial abundances and to determine sample adequacy for stored DNA samples.

Statistical analyses

Relative bacterial abundances were compared using a paired t test or chi square test. The association of P. amnii with BV, defined as a Nugent score of 7–10, was made using a chi square test, reported with odds ratios (OR) with 95% confidence intervals (CI). All analyses were carried out using Stata version 12 (StataCorp LP, College Station, USA) [26].
Results

Nugent 10 patients’ cDNA library

In total, 89,969 raw sequencing reads were obtained with an ultimate post data filtration of 78,285 reads (average length $= 268 \pm 91$ bp; GC content $= 49 \pm 3\%$). Overall, this library consisted of 72,826 (93\%) bacterial reads, 3,865 (4.9\%) human reads and 34 (0.04\%) either plastid, fungal or viral reads (Table 1). Of the bacterial reads, 31,857 (43.7\%) were of the 16S rRNA gene and 4,349 (6.0\%) were non-ribosomal RNA (e.g. mRNA) with the remaining matching other ribosomal regions such as the 23S rRNA gene and intergenic regions. Of the 16S rRNA gene reads, the most dominant genus identified was *Prevotella* (36\% of the reads), followed by the genera *Megasphaera* (19\%), *Leptotrichia/Sneathia* (8\%), *Fusobacterium* (8\%), and 9\% of the reads matching uncultured members of the Fusobacteriales (Figure 2). Of the *Prevotella* reads, 78\% formed contigs of $\geq 1000$ bp in length that matched *P. amnii*. Analysis of assembly free reads of the *Prevotella* spp. reads at the species level (11,725 reads with $\geq 98\%$ match to the RDP database) showed similar results, however distinction between *P. amnii* and *P. bivia* was not possible for the majority of these sequences (data not shown). The major *Fusobacterium* was *F. nucleatum*, and the majority of *Megasphaera* reads were $\geq 98\%$ similar to the currently uncharacterised *Veillonellaceae* bacterium S3PF24 (Genbank accession JX104009). The bioinformatics pipeline of MG-RAST was able to annotate 2,053/4,349 of the non-ribosomal RNA reads, which SEED analysis revealed to consist of genes involved primarily in protein metabolism (20.6\%), carbohydrate/lipid utilisation (16.3\%) and cluster based subsystems (13\%). The most abundant functional gene read identities were primarily represented by genes expressed by the genus *Prevotella* (Table 2).

Comparison of cDNA to DNA from Nugent 10 patient

All of the predominant bacteria with $>1\%$ total abundance identified in the cDNA library were also detected using the 16S rRNA gene amplicon-based approach on extracted DNA. There
was a significant difference in the relative abundances given between both libraries for the most dominant taxa, such as the genus *Lactobacillus* (primarily *L. iners*) that comprised 1.4% of the cDNA reads and 23% of the DNA amplicon library (*p* <0.0001). Also of particular note were reads pertaining to the genus *Gardnerella* that were present in 7% of the cDNA library, but were nearly non-existent in the DNA amplicon library with only seven reads detected (0.006%). When comparing the relative abundances of the seven taxa screened for using qPCR to the abundances given for the cDNA library, there was no significant difference (*p* = 0.9093). We found that the proportion of *Lactobacillus* spp. determined by qPCR to be 1.9% (cDNA = 1.4%; DNA amplicon = 23.1%), and the proportion of *Prevotella* spp. was 48% (cDNA = 35%; DNA amplicon = 11%) (Table 3).

**qPCR screening of sexually active women**

*P. amnii* was detected in 11/36 (30.6%) women possessing a Nugent score of 7–10, whereby nine had a Nugent score of 7 and two with a Nugent score of 10. This bacterium was also detected in a single case possessing intermediate microbiota with a Nugent score of 8.

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**Figure 2. Bacterial diversity of cDNA library.** Graphical representation of the bacterial genera identified through 16S rRNA gene matches for the Nugent = 10 cDNA using the RDP database search tool in MG-RAST.

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score of 6, and in none of 34 cases of normal microbiota (Nugent 0–3) (p < 0.001; Table 4). The bacterial load of *P. amnii* in these 12 positive cases was high, averaging $1.41 \times 10^9$ copies per swab (range = $7.74 \times 10^6$ to $4.66 \times 10^9$). This bacterium was found to be strongly associated with BV (p < 0.001, OR 23.3 (95%CI:2.9–190.7)) among the 90 women. When the 90 women were stratified by different sexual exposures the association between BV and *P.

Table 1. Summary of Nugent 10 cDNA 454 sequencing reads analysed by MG-RAST.

| Summary of MG-RAST reads | n     | %   |
|--------------------------|-------|-----|
| Bacterial                | 72826 | 93.0|
| 16S rRNA gene            | 31857 | 40.7|
| Other ribosomal content* | 36621 | 46.8|
| Functional genes         | 4348  | 5.6 |
| Viral†                   | 1     | -   |
| Fungal‡                  | 4     | -   |
| Plastid                  | 29    | 0.4 |
| Human                    | 3865  | 4.9 |
| Poor quality†            | 1560  | 2.0 |
| Total                    | 78285 | -   |

*23S rRNA gene and intergenic regions; † human coronavirus; ‡ two reads of *Schizothyrium commune* cytochrome oxidase subunit 1 (cox1) gene, single reads of *Botryotinia fuckeliana* glycosyl hydrolase gene and *Ascomycota* hypothetical protein; †† unable to be assigned to any taxonomy doi:10.1371/journal.pone.0076892.t001

Table 2. Top 20 Bacterial non-16S/23S gene Nugent 10 cDNA reads using 80% identity with a minimum alignment of 50 amino acid residues (n = 1361).

| Function                                      | # reads | % reads | % of reads |
|-----------------------------------------------|---------|---------|------------|
| TonB-dependent receptor plug domain protein   | 103     | 7.57    | 100        |
| putative lipoprotein                          | 76      | 5.58    | 100        |
| glyceraldehyde-3-phosphate dehydrogenase, type I | 57    | 4.19    | 87.7       |
| MotA/TolQ/ExbB proton channel family protein  | 40      | 2.94    | 100        |
| translation elongation factor Tu              | 39      | 2.87    | 46.2       |
| phosphoenolpyruvate carboxykinase (ATP)       | 39      | 2.87    | 100        |
| 50S ribosomal protein L27                     | 30      | 2.20    | 100        |
| translation elongation factor G               | 32      | 2.35    | 59.4       |
| TonB-dependent receptor                       | 24      | 1.76    | 100        |
| ExbD/TolR family protein                     | 32      | 2.35    | 100        |
| phosphoenolpyruvate carboxykinase             | 19      | 1.40    | 94.7       |
| ribosomal protein L28                         | 21      | 1.54    | 100        |
| ribosomal protein S12                         | 18      | 1.32    | 100        |
| M16 family peptidase                          | 16      | 1.18    | 100        |
| D-phosphoglycerate dehydrogenase              | 15      | 1.10    | 100        |
| glyceraldehyde 3-phosphate dehydrogenase      | 26      | 1.91    | 96.2       |
| ribosomal protein L6                          | 14      | 1.03    | 42.9       |
| 305 ribosomal protein S13                     | 14      | 1.03    | 28.6       |
| 305 ribosomal protein S9                      | 13      | 0.96    | 100        |
| chaperone protein DnaK                        | 13      | 0.96    | 0          |

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Table 3. Comparison between Nugent 10 RNA and DNA bacterial abundances.

| Taxa                            | RNA library | 16S rRNA (454) | qPCR |
|---------------------------------|-------------|----------------|------|
| Prevotella                      | 35.0%       | 11.0%          | 47.7%|
| Megasphaera                     | 18.8%       | 8.7%           |      |
| Uncultured Fusobacteriales      | 8.3%        | -              |      |
| Leptotrichia/Sneathia           | 7.5%        | 1.8%           | 6.1% |
| Fusobacterium                   | 7.4%        | 2.0%           |      |
| Gardnerella spp.                | 6.9%        | <1%            | 6.53%|
| Clostridiales                   | 4.8%        | 1.3%           |      |
| Atopobium spp.                  | 2.4%        | 19.0%          | 2.0% |
| Lactobacillus spp.              | 1.4%        | 23.1%          | 1.9% |
| Bacteroides                     | 1.2%        | 2.6%           |      |
| Mobiluncus                      | 0.8%        | 20.4%          |      |
| TM7 group                       | 0.5%        | 5.4%           |      |
| Eubacterium                     | 0.4%        | -              |      |
| Butyribrio/Uncultured Lachnospiraceae | 0.3% | 2.8%           |      |
| *P. amnii*                      | 28.06%      | 15.8%          |      |
| Megasphaera Type I              | 0.13%       | 0.02%          |      |

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amnii persisted for different sexual exposures in men. While the associations between BV and P. amnii were not significant among women reporting receptive oral sex with a woman or sex with a woman in the last 3 months, these analyses involved only small numbers (Table 4).

Discussion

This study explored the active microbial diversity of a vaginal sample taken from a lesbian woman with symptomatic BV to demonstrate the potential of metatranscriptomics for identifying targets for screening studies. Prevotella amnii was found to be the most active species present in this sample and was also detected at a high concentration by qPCR in 30.6% of BV cases in sexually active women. Although we identified P. amnii as the predominant Prevotella species in this sample (78% of Prevotella reads; 28.1% of total bacterial population), there may be a possible overestimation in the cDNA abundance given, as conserved regions shared by multiple Prevotella spp. may be involved through chimeric assembly. This must be kept in mind when comparing to qPCR data (33.1% of Prevotella reads; 15.8% of total bacterial population) that would otherwise suggest that this species is more active than others in this sample. This bacteria has only recently been shown to form a symbiotic relationship with G. vaginalis [28]. These two Prevotella spp. share many phenotypic traits and it has been noted that culture-based methodologies may not differentiate between these two species, with full length 16S rRNA gene sequencing recommended for species differentiation [29].

The other predominant bacteria in this case were identified through 16S rRNA gene as the currently uncharacterised isolate Veillonellaceae bacterium S3PF24 (phylogenetically belonging to the genus Megaphaga) that was originally isolated from a vaginal environment, and the bacterium Fusicobacterium nucleatum. F. nucleatum has been described in cases of BV previously [30]. Interestingly it has been implicated in amniotic infection and pre-term birth in cases where the bacterium was also detected in the patients’ oral cavity [31,32]. F. nucleatum, as well as both Prevotella and Megaphaga spp. are regularly associated with periodontal disease [33,34,35], and it is of interest to note that the single Nugent score 10 participant analysed in this study had a history of recent female-female oral sex. These three bacteria were more abundant than the usual predominant G. vaginalis and A. vaginae in the cDNA library, which collectively only comprised 10% [2].

Further work is warranted to determine the role of these bacteria, in particular P. amnii, in the pathogenesis of BV. Of particular interest is whether their association with oral sex represents a possible route of transmission.

Although not a focus of this study, mRNA reads were also identified in this study that were primarily associated with protein metabolism and carbohydrate/lipid utilisation pathways of the genus Prevotella. The low abundance of mRNA reads (5.6%) was not surprising, given the low general relative abundance of mRNA in relation to total RNA in bacterial cells [36]. To focus more upon this class of RNA transcripts, an additional enrichment step, such as the removal of bacterial rRNA can be employed [37], in the same manner as human rRNA was reduced in this study.

A comparison was made between the cDNA and DNA amplicon libraries to determine what proportion of the microbial community was active, and differences were observed between these libraries. This may suggest that there was a significant difference between what is present and what is transcriptionally active, although these findings are limited by the fact that this is a single sampling point. The broad range amplification of bacterial DNA using the 16S rRNA gene may have potential bias in the community structure, as has been found in numerous studies [38,39,40,41]. Our data suggests that G. vaginalis was underrepresented in the DNA amplicon library when compared to the qPCR data from the same DNA sample. Despite being one of the most utilized primers for microbiome analysis, there has been suggestion in the literature that the forward PCR primer 27F may not reliably amplify this species [42], and our analysis has demonstrated this similarly through qPCR for G. vaginalis. Also, given that L. iners was found to be underrepresented in the cDNA library when compared to the DNA amplicon library, we applied a genus-specific Lactobacillus qPCR assay to the DNA sample, as well as one that targeted the genus Prevotella that was present at a much higher abundance in the cDNA library. We found that there was no statistical difference in the abundances given between qPCR and the cDNA library for these genera. Based on this limited data, we can suggest misrepresentation of several taxa in the DNA amplicon library occurred, and that in this case, the vast majority of the bacteria physically present in this sample were also transcriptionally active. This further highlights the advantage of transcriptomics for such analysis as our data suggest that this analysis gives better representation of bacterial levels across the board.

MG-RAST for the analysis of metatranscriptomic work can be carried out using a standard desktop or laptop computer. However, this method may lead to misclassification of certain

Table 4. Prevalence of Prevotella amnii in cohort of sexually active women (n = 90).

| Nugent category | 0–3 | 4–6 | 7–10 | Total | p value* | OR (Nugent 7–10) |
|-----------------|-----|-----|------|-------|---------|------------------|
| Male Sexual Partner (3 months) | 0/25 (ND) | 1/19 (5.3%) | 11/30 (36.7%) | 12/74 (16.2%) | <0.001 | 24.89 (95% CI:3.00–206.81) |
| Vaginal Sex (3 months) | 0/24 (ND) | 1/19 (5.3%) | 11/30 (36.7%) | 12/73 (16.4%) | <0.001 | 24.32 (95% CI:2.93–202.10) |
| Receptive Oral Sex (3 months) | 0/25 (ND) | 1/16 (6.3%) | 11/31 (35.5%) | 12/72 (16.7%) | 0.001 | 22.00 (95% CI:2.65–182.61) |
| Receptive Male Oral Sex (3 months) | 0/20 (ND) | 1/15 (6.7%) | 11/30 (36.7%) | 12/65 (18.5%) | 0.002 | 19.68 (95% CI:2.36–164.44) |
| Female Sexual Partner (3 months) | 0/11 (ND) | 0/2 (ND) | 2/16 (12.5%) | 2/29 (6.9%) | 0.418 | 4.66 (95% CI:10.20–106.01) |
| Receptive Female Oral | 0/10 (ND) | 0/2 (ND) | 2/16 (12.7%) | 2/24 (8.3%) | 0.336 | 5.95 (95% CI:0.26–138.25) |
| Total | 0/34 (ND) | 1/20 (5%) | 11/32 (36.6%) | 12/90 (13.3%) | <0.001 | 23.320 (95% CI:2.85–190.74) |

*Chi square for proportion with Prevotella amnii in the three Nugent categories; †Haldane’s estimation; OR = Odds Ratio; ND = Not Detected.

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reads. For example, in this study it was found that many Gardnerella reads were being classified as the genus Bifidobacterium, Megabacera reads as the genera Veillonella and Dalilster, and those of the genera Liptotrichia/Sneathia as Strepobacillus. Therefore care must be taken in the interpretation of results, and manual checking of representative reads is required to ensure an accurate identification of sequencing reads to the genus level.

In summary, this study has shown how metatranscriptomics can be utilised as a useful tool to carry out an in-depth examination of the active microbial ecology of an environment, and is capable of generating more taxonomic and potential functional data than an amplicon sequencing-based approach alone, and avoid potential misrepresentation issues caused by PCR primer selection. We have demonstrated that this kind of metatranscriptomic approach can be used to identify targets for screening in larger studies. As this study focussed on a single individual, generalisations based on the data are not possible and transcriptomic analyses on a large cohort is warranted. This being said, our limited findings reinforce that P. amnii may be an important BV-associated bacterium, and should be included in future studies investigating the pathogenesis of BV. Its previously known association with periodontal disease, and the association with oral sex in this study, make it interesting to speculate about transmission of pathogenic bacteria between the oral and vaginal cavities, and their role in the development of BV.

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Author Contributions

Conceived and designed the experiments: JT CB SG CF KT. Performed the experiments: JT. Analyzed the data: JT CB SG CF KT. Contributed reagents/materials/analysis tools: CB SG CF KT. Wrote the paper: JT CB SG CF KT. Sample collection: KT.

References

1. Nakamura S, Nakaya T, Iida T (2011) Metagenomic analysis of bacterial infection by means of high-throughput DNA sequencing. Exp Biol Med (Maywood) 236: 960–971.
2. Lamont RF, Sobel JD, Akins RA, Hassan SS, Chaiworapongsa T, et al. (2011) The vaginal microbiome: new information about genital tract flora using molecular-based techniques. BJOG 118: 535–549.
3. Turovsky Y, Sutazyk N, Chikudina ML (2011) The artiology of bacterial vaginosis. J Appl Microbiol 110: 1115–1128.
4. Nugent RP, Krohn MA, Hillier SL. (1991) Reliability of diagnosing bacterial vaginosis is improved by a standardized method of gram stain interpretation. J Clin Microbiol 29: 297–301.
5. Ansari R, Toten PA, Spiegel CA, Chen KC, Eschenbach D, et al. (1983) Nonspecific vaginitis. Diagnostic criteria and microbial and epidemiological associations. Am J Med 74: 14–22.
6. Frias-Lopez J, Durazo-Aroche A (2012) Effect of periodontal pathogens on the metatranscriptome of a healthy multispecies biofilm model. J Bacteriol 194: 2082–2089.
7. Gouadec MJ, Durban A, Pignatelli M, Allalouf JJ, Jimenez-Hernandez N, et al. (2011) Metatranscriptomic approach to analyze the functional human gut microbiota. PLoS One 6: e17447.
8. Shipitsyna E, Roos A, Datcu R, Hallen A, Fredlund H, et al. (2013) Composition of the vaginal microbiota in women of reproductive age - Sensitive and Specific Molecular Diagnosis of Bacterial Vaginosis Is Possible? PLoS One 8: e62803.
9. Iscove NN, Barbara M, Gu M, Gibson M, Moti C, et al. (2002) Representation is faithfully preserved in global cDNA amplified exponentially from sublibrary of human mRNAs by cDNA microarrays. Genome Res 16: 527–335.
10. Bradshaw CS, Morton AN, Gaizlend SM, Morris BR, Moss LM, et al. (2005) Higher-risk behavioral practices associated with vaginal bacterial vaginosis compared with vaginal candidiasis. Obstet Gynecol 105: 105–114.
11. Trent DA, Whitehead A (2009) Simultaneous extraction of high-quality RNA and DNA from small tissue samples. Journal of Heredity 100: 246–250.
12. Chomczynski P, Mackey K (1993) Substitution of chloroform by bromo- chloroform in the single-step method of RNA isolation. Anal Biochem 225: 163–164.
13. Chomczynski P (1993) A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cells and tissue samples. Biotechniques 15: 532–534, 536–537.
14. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, et al. (2010) Vaginal microbiome of reproductive-age women. Proc Natl Acad Sci U S A 107 Suppl 1: 4680–4687.
15. Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27: 2194–2200.
16. Meyer F, Paarmann D, Souza M, Olson R, Glass EM, et al. (2008) The metagenome RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics 9: 366.
17. Lazarevic V, Whiteson K, Hernandez D, Francois P, Schrenzel J (2010) Study of inter- and intra-individual variations in the salivary microbiota. BMC Genomics 11: 523.
18. Ordway BD, Bergman NH, Phillippy AM (2011) Interactive metagenomic visualization in a Web browser. BMC Bioinformatics 12: 585.
19. Fredricks DN, Fiadler TL, Thomas KK, Mitchell CM, Marrazzo JM (2009) Changes in vaginal bacterial concentrations with intravaginal metronidazole therapy for bacterial vaginosis as assessed by quantitative PCR. J Clin Microbiol 47: 721–726.
20. Zariffard MR, Saifuddin M, Sha BE, Spear GT (2002) Detection of bacterial vaginosis-related organisms by real-time PCR for Lactobacillus, Gardnerella vaginalis and Mycoplasma hominis. FEMS Immunol Med Microbiol 34: 277–281.
21. Fuerst JP, Firmesse O, Gourmelon M, Brudonne C, Tap J, et al. (2009) Comparative assessment of human and farm animal faecal microbiota using real-time quantitative PCR. FEMS Microbiol Ecol 68: 351–362.
22. Leng Z, Liu X, Chen X, Zhu H, Nelson KE, et al. (2011) Diversity of cervicovaginal microbiota associated with female lower genital tract infections. Microb Ecol 61: 704–714.
23. Gupta K, Stapleton AE, Hooton TM, Roberts PL, Fennell CL, et al. (1998) Inverse association of H2O2-producing lactobacilli and vaginal Escherichia coli colonization in women with recurrent urinary tract infections. J Infect Dis 178: 446–450.
24. Niki S, Fozor AE, Lepp PW, Cisarik PR, Lasich Wilson S, et al. (2002) Broad-range bacterial detection and the analysis of unexplained death and critical illness. Emerg Infect Dis 8: 188–194.
25. StataCorp (2011) Stata Statistical Software: Release 12. College Station, TX: StataCorp LP.
26. Sinnaasap S, Hoffmann NG, Morgan MT, Mates FA, Feidler TL, et al. (2012) Bacterial communities in women with bacterial vaginosis: high resolution phylogenetic analyses reveal relationships of microbiota to clinical criteria. PLoS One 7: e37581.
27. Pybus V, Onderdonk AB (1997) Evidence for a commensal, symbiotic relationship between Gardnerella vaginalis and Prevotella bivia involving ammonia: potential significance for bacterial vaginosis. J Infect Dis 175: 406–413.
28. Lawson PA, Moore E, Fierens E (2008) Prevotella amnii sp. nov., isolated from human amniotic fluid. Int J Syst Evol Microbiol 58: 109–92.
29. Ling Z, Kong J, Liu F, Zhu H, Chen X, et al. (2010) Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. BMC Genomics 11: 488.
30. Gauthier S, Teun A, Himaya E, Morand M, Chandad F, et al. (2011) The origin of the Bacteroides vulgatus involved in intra-amnionic infection and preterm birth. J Matern Fetal Neonatal Med 24: 1329–1332.
31. Hay PE, Lamont RF, Taylor-Robinson D, Morgan DJ, Isaac C, et al. (1994) Abnormal bacterial colonisation of the genital tract and subsequent preterm delivery and late miscarriage. BJM 308: 295–298.
32. Nadkarni MA, Browne GV, Chihour KL, Byun R, Nguyen KA, et al. (2012) Pattern of distribution of Prevotella species/phylotypes associated with healthy gingiva and periodontal disease. Eur J Clin Microbiol Infect Dis 31: 2899–2909.
33. Kamar PS, Greifen AI, Moeschberger ML, Leys EJ (2005) Identification of candidate periodontal pathogens and beneficial species by quantitative 16S rDNA analysis. J Clin Microbiol 43: 3944–3947.
34. Gauthier S, Teun A, Himaya E, Morand M, Chandad F, et al. (2011) The origin of the Bacteroides vulgatus involved in intra-amnionic infection and preterm birth. J Matern Fetal Neonatal Med 24: 1329–1332.
35. Pybus V, Onderdonk AB (1997) Evidence for a commensal, symbiotic relationship between Gardnerella vaginalis and Prevotella bivia involving ammonia: potential significance for bacterial vaginosis. J Infect Dis 175: 406–413.
36. Lawson PA, Moore E, Fierens E (2008) Prevotella amnii sp. nov., isolated from human amniotic fluid. Int J Syst Evol Microbiol 58: 109–92.
37. Ling Z, Kong J, Liu F, Zhu H, Chen X, et al. (2010) Molecular analysis of the diversity of vaginal microbiota associated with bacterial vaginosis. BMC Genomics 11: 488.
38. Neidhardt FC (1996) Chemical composition of Escherichia coli. In: Emoto M, Yabuuchi E, editors. Escherichia coli and

Bacterial Vaginosis Metatranscriptome Screening
39. Winsley T, van Dorst JM, Brown MV, Ferrari BC (2012) Capturing Greater 16S rRNA Gene Sequence Diversity within the Domain Bacteria. Appl Environ Microbiol 78: 5938–5941.

40. Sim K, Cox MJ, Wopereis H, Martin R, Knol J, et al. (2012) Improved detection of bifidobacteria with optimised 16S rRNA-gene based pyrosequencing. PLoS One 7: e32543.

41. Soergel DAW, Dey N, Knight R, Brenner SE (2012) Selection of primers for optimal taxonomic classification of environmental 16S rRNA gene sequences. ISME Journal 6: 1440–1444.

42. Srinivasan S, Fredricks DN (2008) The human vaginal bacterial biota and bacterial vaginosis. Interdiscip Perspect Infect Dis 2008: 750479.
