Journal of Women's Health Care

Bostwick et al., J Women's Health Care 2016, 5:4
DOI: 10.4172/2167-0420.1000322

Research Article Open Access

Utility of Next-generation sequencing in Managing Bacterial Vaginosis: Examples from Clinical Practice

Bostwick GD[1]*, Hunt CA[2], Parker LR[3], Daniel Mcdyer[4], Woody J[1] and Budd W[1]

[1]*Granger Diagnostics, LLC, North Chesterfield, Richmond, Virginia, USA*

[2]*Desert Jewel Obstetrics and Gynecology Center, Phoenix, Arizona, USA*

[3]*Department of Obstetrics and Gynecology, Wake Forest University School of Medicine and Lyndhurst Gynecologic Associates, Winston-Salem, North Carolina, USA*

[4]*Florida Woman Care of Jacksonville, Jacksonville, Florida, USA*

***Corresponding author:** David G Bostwick, Chief Medical Officer, Granger Diagnostics, 601 Biotech Drive, North Chesterfield, VA, USA, Tel: 804-305-7648; E-mail: dbostwick@grangerdiagnostics.com

Rec date: June 27, 2016; **Acc date:** July 26, 2016; **Pub date:** Aug 10, 2016

Abstract

There is an unacceptably high recurrence rate for bacterial vaginosis and vaginitis owing to multiple causes, including misdiagnosis and empirical under-treatment and over-treatment. A diagnostic test that provided complete knowledge of the vaginal microbiome in patients with bacterial vaginosis (BV) would fill an important void. In initial evaluations of Next-generation Sequencing (NGS), this new test offers great promise. As a clinical diagnostic for primary or recurrent bacterial vaginosis, NGS by simple vaginal swab offers heightened sensitivity and specificity compared to culture or limited three-organism genetic tests. NGS holds valuable potential in multiple BV quandary situations, including: (a) Recurrent BV in a patient that failed initial therapy, (b) Symptoms of BV in a patient with false-positive Amsel criteria, (c) *Gardnerella vaginalis*-negative BV with mixed infection. Multiplex next-generation sequencing (NGS) provides a complete and accurate description of the composition of the complex polymicrobial vaginal microbiome and associated antimicrobial resistance-determining genes, facilitating personalized diagnosis and therapy for patients with bacterial vaginosis, STIs and vaginitis.

Keywords: Bacterial vaginosis; Drug resistance determinants; Vaginal microbiome; Metagenomics; Next-generation sequencing

Introduction

Bacterial vaginosis is the most common cause of vaginitis and vaginal discharge, with a prevalence of up to 29% of women [1]. This infection is associated with multiple serious health outcomes, including human immunodeficiency virus and other STI acquisition, human papillomavirus infection [2], post-abortion pelvic inflammatory disease, post-cesarean endometritis, low birth weight, preterm labor, and preterm delivery [3,4]. A high rate of recurrence is among the greatest clinical challenges with BV, reportedly very high despite standardized treatment with oral or intravaginal metronidazole or clindamycin, frustrating patients and clinicians [5,6]. Treatment failure results from diagnostic error, re-infection from sexual partners, the presence of antimicrobial resistance genes, non-genetic determinants of resistance, and drug resistance of the BV-infected biofilm [7]. A complete and accurate description of the composition of the complex polymicrobial vaginal microbiome can only be obtained by multiplex next-generation sequencing (NGS) testing, thereby assisting with the clinical challenge of BV [8]. These tests have recently shown that BV consists of a spectrum of multiple bacterial community clusters [8-10] dominated by *Gardnerella vaginalis, Lactobacillus iners*, or anaerobic combinations with or without *Lactobacillus spp.* in varying proportions [10]. Also, multiple significant BV-associated bacteria had previously escaped notice with all other detection methods such as cultures; undetected were *Atopobium vaginae* and *A. parvulum, Lactobacillus iners* and other *Lactobacillus spp.,* and multiple *Lachnospiraceae spp* (BVA1, BVA2, and BVA3) [11].

In addition to identifying all incident bacterial species, 16S ribosomal RNA gene sequencing (NGS) allows numerous other improvements compared with existing methods of detection [12]. Advantages include measurement of the relative proportion and absolute number of bacteria present (both pathogens and normal commensals), as well as identification of antimicrobial resistance-determining genes for the individual patient (precision medicine). In the following case studies, drawn from clinical experience with more than 2000 cases diagnosed with NGS, the utility of NGS testing in management of individual patient quandaries in BV was demonstrated. In each case, NGS provided novel information on the spectrum of infection and drug resistance. This study was approved by the Institutional Review Board of American International Biotechnology LLC, Richmond, Virginia.

Case 1: Recurrent BV that Failed Therapy

An otherwise-healthy 39 year-old with a prior history of recurrent BV treated empirically with metronidazole presented with a two-week history of irritation and thin watery grey-white vaginal discharge. Amsel criteria as modified by Gutman et al. [13] were positive for BV. Vaginal swab for NGS testing was obtained, but she was empirically placed immediately on metronidazole. NGS results subsequently showed recurrent BV, with 56% mixed anaerobes (*Atopobium parvulum, Dialister micraerophilus, Enterococcus faecalis, Megasphaera micronuciformis, Prevotella disiens, Prevotella timonensis, Sneathia sanguinegens*) 22% *Gardnerella vaginalis*, 20% Lactobacillus iners, 3% *Atopobium vaginae*, and antimicrobial drug resistance for clindamycin and macrolides (ermTR). Metronidazole was continued for a total of ten days.

She returned 3 months later with recurrent BV symptoms, with positive modified Amsel criteria. NGS testing revealed recurrent BV: 40% *Gardnerella vaginalis*, 46.5% mixed anaerobes (*Enterococcus faecalis, sulfureus*, and *villorum*; and *Streptococcus oralis, mitis*, and *pseudopneumoniae*); 12% *Lactobacillus* "other" (non-*crispatus* and non-*iners*); and 2.5% *Mycoplasma hominis*. Antimicrobial drug resistance remained positive for clindamycin and macrolides (ermTR). Metronidazole treatment was given again for a total of ten days.

Discussion and conclusion of case 1

Empirical treatment of BV with metronidazole and/or clindamycin has one of the lowest success rates for patient management in all of microbiology, with 50%- 80% recurrence within 12 months [14-16]. In a controlled clinical trial of 14-days of metronidazole for symptomatic BV, Schwebe and Desmond reported 38% failure within one week and 57% within three weeks [17]. These findings caused Eschenbach to conclude that "…treatment of BV… is in a sorry state…" [18].

He noted that antibiotic cure with metronidazole was greater than 90% when it was first introduced, but subsequent decades have seen this drop to 50-80%, with most contemporary reports at the low end of this range: "How can cure rates steadily decrease over 30 years?" Similarly, Austin et al. reported an increase in baseline clindamycin resistance over two decades [19].

Carr et al. argued that incomplete diagnosis of BV was clinically and economically inefficient, resulting in both empirical under-treatment and over-treatment, costly outcomes that cause unnecessary side effects, delayed diagnosis, heightened risk of AMR (antimicrobial resistance), and increased likelihood of recurrence [20] (Figure 1).

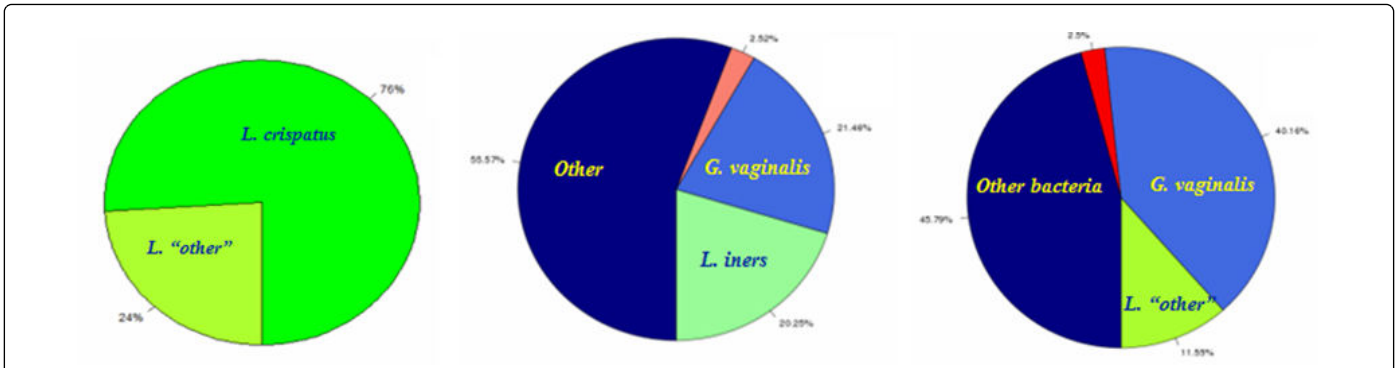

Figure 1: Vaginal microbiome: normal reference standard in non-gravid women during reproductive years (left); at presentation with BV (center); and with subsequent recurrent BV (right) in Case 1. Normal reference standard consists of *lactobacilli* with a predominance of *L. crispatus*. At presentation, BV was characterized by predominantly *G. vaginalis* and mixed anaerobes, with a minority of *L. iners*. After treatment that failed to account for drug resistant genes and the entire spectrum of pathogens present, recurrentBV was characterized predominantly by *G. vaginalis* (increased number) and mixed anaerobes, with loss of *L. iners*.

Molecular diagnostic testing of 16s ribosomal RNA with next-generation sequencing (NGS) provides a comprehensive description of the vaginal bacterial microbiome and offers promise as a rapid method for targeted genetic identification of multiple AMR genes simultaneously [21]. NGS testing allowed evaluation of resistance genes in non-culturable bacteria, facilitating study of many of the most abundant BV-associated bacteria found only recently with advanced molecular diagnostics, including *Atopobium vaginae, Atopobium parvulum, Lactobacillus iners* and other *Lactobacillus* spp., and multiple *Lachnospiraceae* spp (BVAB1, BVAB2, and BVAB3) [22].

Van de Wijgert et al. suggest that NGS will eventually replace Nugent scoring, the current diagnostic gold standard for BV [10]. It is also possible that NGS testing will change the treatment of BV, driving the use of narrow spectrum agents likely to cause less harmful disruption of the vaginal flora; intermittent therapy; or targeted combination therapy.

Case 2: Symptoms of BV with False-positive Amsel Criteria

A 23 year-old otherwise-healthy woman presented with a one-week history of symptoms of bacterial vaginosis (irritation, discomfort, thin discharge); Amsel criteria as modified by Gutman et al. [13] were positive for BV. Vaginal swab for NGS testing was obtained, but she was empirically placed immediately on metronidazole (5-nitroimidazole). NGS results subsequently showed no evidence of BV, with the microbiome dominated by *L. crispatus* and *L. iners*; AMR gene determination was negative. Metronidazole was discontinued (Table 1). She returned 3 months later with recurrent BV symptoms, with positive modified Amsel criteria. NGS testing revealed mixed infection with BV and *Candida albicans*. AMR genes that were previously negative had converted to positive (resistance) to 5-nitroimadazoles for BV treatment and triazoles (e.g., fluconazole) for *Candida* treatment (Table 1).

Antimicrobial Class	Antimicrobial Class Members	Antimicrobial Resistance-Determining Genes	Drugs at Initial Presentation	At Recurrence (3 months later)
Penicillins (Beta Lactams)	Penicillin, Amoxicillin, Nafcillin, Oxacillin	ACRA, AcrB, TolC, Aac2	Sensitive	Sensitive

Aminoglycosides	Gentamycin	ACRA, AcrB, TolC, Aac2	Sensitive	Sensitive
	Tobramycin			
	Amikacin			
Macrolides	Erythromycin	ermA, ermB, ermC, ermM, ermTR, mefA	Sensitive	Sensitive
	Azithromycin			
	Clarithromycin			
Tetracyclines	Tetracycline	ACRA, AcrB, tet, tetA, TolC	Sensitive	Sensitive
	Doxycycline			
	Minocycline			
Lincosamides	Clindamycin	ermA, ermB, ermC, ermM, ermTR	Sensitive	Sensitive
	Lincomycin			
5-nitroimidazoles	Metronidazole	Nim, NimB	Sensitive	Resistant
Triazoles	Fluconazole	MDR1	Sensitive	Resistant

Table 1: Results of Antimicrobial Resistance-Determining Genes at Presentation of Bacterial Vaginosis and at Recurrence: Case 2.

Discussion and conclusion of case 2

In the initial visit, the patient received metronidazole for only a few days until the NGS test was reported as negative, but it is possible or even likely that this medication caused profound alterations in the vaginal flora and the emergence of AMR that was observed in the follow-up visit. Mayer et al. recently reported that "…under antibiotic pressure, the vaginal microbiome undergoes rapid shifts in a matter of hours…" [23].

They noted that anaerobes cleared more rapidly than *Gardnerella vaginalis*, but BV-associated bacteria quickly re-emerged after cessation of therapy, and suggested the possibility of intermittent prophylactic treatment (Figure 2).

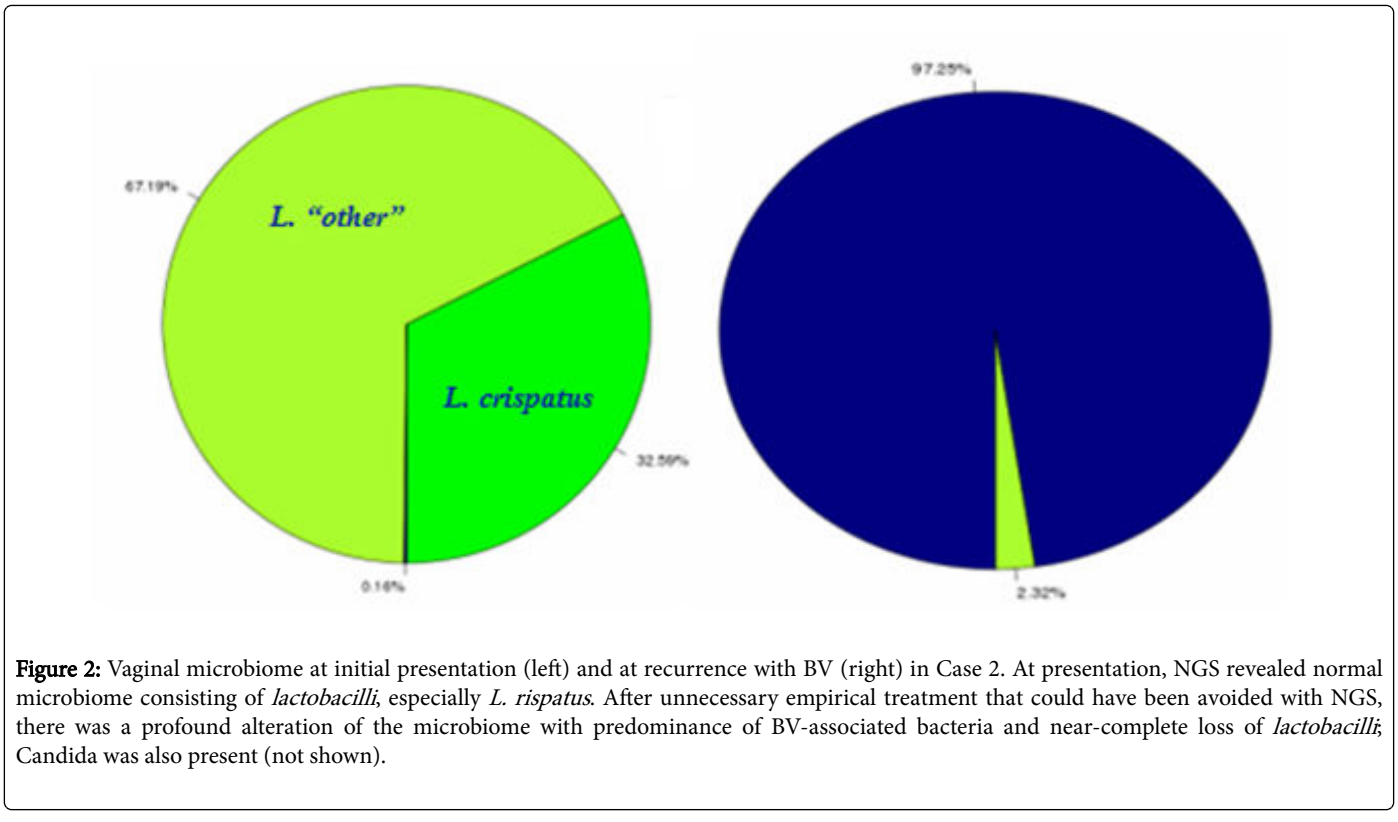

Figure 2: Vaginal microbiome at initial presentation (left) and at recurrence with BV (right) in Case 2. At presentation, NGS revealed normal microbiome consisting of *lactobacilli*, especially *L. rispatus*. After unnecessary empirical treatment that could have been avoided with NGS, there was a profound alteration of the microbiome with predominance of BV-associated bacteria and near-complete loss of *lactobacilli*; Candida was also present (not shown).

A major goal in tackling antimicrobial resistance (AMR) is rapid and targeted detection and diagnosis of infection and resistance for patient benefit, infection control, surveillance, and prudent antibiotic stewardship, including identification of alternative drugs unencumbered by the presence of AMR genes [24]. Major initiatives were recently launched for controlling AMR: the U.S. National Action Plan for Combating Antiobiotic Resistant Bacteria (March, 2015) and the World Health Organization to Tackle Antimicrobial Resistance (May 2015).

According to Mullaney, "…antibiotic resistance is a major threat to human health and well-being." He noted that the only way to effectively combat AMR is to understand the complete range of different resistance genes that allow bacteria to resist antibiotics; to do this, the entire microbiome needs to be investigated, and NGS is the only method that provides for such an investigation [25].

We recently reported that antimicrobial resistance (AMR) genes are common in the vaginal microbiome, especially in women with BV when compared with symptomatic women without BV; there was an increase in overall abundance (74.3% vs. 22.1%, respectively) and number of AMR genes (mean, 1.5 vs. 0.3, respectively) [21]. There were varying levels of susceptibility for the two primary drugs used to treat BV, with the highest level of AMR genes identified (61.8%) with clindamycin, one of the lincosamides.

AMR gene analysis is in widespread clinical use in management of other infectious diseases, with reported sensitivity and specificity of 72.3-100% and 55-100%, respectively [24]. Concordance was high between the phenotypic method (MIC determination) and the presence of the *ermX* gene for *Corynebacterium* isolated from the nasal mucosa, noting that this was the most common mechanism of resistance [26]. According to the Clinical and Laboratory Standards Institute, "…tests for *mecA*… are the most accurate… for prediction of resistance to oxacillin [27]." AMR gene analysis was superior to routine methods for determination of low-level drug resistance for *Mycobacterium tuberculosis* [28].

Case 3: *G. Vaginalis*-negative Bacterial Vaginosis with Mixed Infection

An otherwise-healthy 34 year-old woman presented with a one-week history of watery vaginal discharge and irritation. Amsel criteria as modified by Gutman et al. [13]. were positive for BV. Vaginal swab for NGS testing was obtained, but she was empirically placed immediately on metronidazole. NGS results subsequently showed BV, with 54% BVAB1, 41% Lactobacillus iners, 4% mixed bacteria (*Enterococcus avium, hermanniensis, hirae*, and *villorum*), and 1% *Lactobacilli* "other" (non-*crispatus*, non-*iners*). In addition, significant co-infection with *Candida albicans* was identified (fungal and parasitic DNA sequence reads are not included in the percentages of bacterial reads). Antimicrobial drug resistance (MDR1 gene) was positive for triazoles (fluconazoles). Metronidazole treatment was continued for a total of ten days.

Discussion and conclusion of case 3

This case illustrates two important issues with BV: (1) *G. vaginalis* is neither necessary nor sufficient for diagnosis; and (2) Mixed infections are a frequent and confounding management problem with BV. Prior to the discovery of BVAB1 in 2005 [29], this patient may have been classified as having pure yeast vaginitis, with high probability of incomplete treatment, persistence of BV, and subsequent recurrence.

How often is G. vaginalis missing in BV? Srinivasan et al. found a prevalence of 3% non- *G. vaginalis* BV in symptomatic women in an STI clinic in Seattle [9], similar to the 5% described among women in a pregnancy follow-up clinic [30]. If one relies on current methods of BV identification that rely on only *G. vaginalis* for diagnosis (e.g., BD Affirm VPIII) it would have given a false-negative result in this case.

How often *G. vaginalis* is present in women without BV? The prevalence in virginal women was 28% [31] to 45% [32], but these studies were criticized for relying on self-reporting of sexual status [33]. Schwebke et al. found *G. vaginalis* in 38.5% of asymptomatic women with a "healthy" vaginal microbiome (predominance of *Lactobacilli* and a Nugent score of 0-3) [34]. After pregnancy, *G. vaginalis* was observed in 47.3% of women [30]. In symptomatic women without BV, the prevalence of *G. vaginalis* was 20% (STI clinic in London, with bacterial assessment by culture) [35]. Vaginal inoculation by pure culture of *G. vaginalis* produced symptoms of BV in 8% [36] and 41% of women, compared with 73% inoculated with BV-infected secretions [36] (Figure 3).

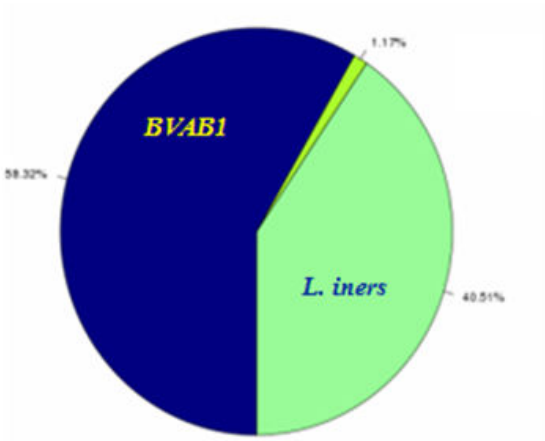

Figure 3: Vaginal microbiome at initial presentation with BV in Case 3. *Candida albicans* coinfection was also identified (not shown). See Figure 1, left, for normal reference standard. Recurrence reveals an otherwise-typical case of BV with mixed infection, but lacking *G. vaginalis.*

The frequent presence of *G. vaginalis* in asymptomatic women is an accepted finding, but its significance is uncertain. Does this represent incipient infection, resolving infection in transition phase, a low-virulence strain that usually acts as a normal commensal and only manifests virulence with loss of *Lactobacilli* and/or when combined with another factor such as the biofilm, or a normal-virulence strain held in check by the microbiome and other environmental factors? Importantly, *G. vaginalis* elicits a modest immune response similar in magnitude to *Lactobacilli*, in contrast with the robust response from *Atopobium vaginae* and other pathogens [37].

It is critical to consider the possibility of mixed infections in patients with presumptive BV that may include STI such as *Chlamydia* to avoid the serious threat of pelvic inflammatory disease or other complications. The prevalence of vaginal colonization with *Candida* spp. is higher in women without BV than in those with BV, but the opposite is true for *T. vaginalis* [10]. Using NGS, we were unable to

confirm these findings, but differences in results may be owing to different patient populations or insufficient number of patients to identify significant variance [21]. We also found that almost one-fourth of symptomatic women had co-existent *Candida* spp. (after excluding clinical cases of pure vulvovaginal candidiasis), usually *Candida albicans*, with no difference between BV and non-BV samples ($p = 0.8$). *Trichmonas vaginalis* and STIs were rare, precluding comparison of prevalence.

Conclusion

Next-generation sequencing (NGS) provides comprehensive description of the complex polymicrobial vaginal microbiome and associated antimicrobial resistance-determining genes, facilitating personalized diagnosis and therapy for patients with complicated bacterial vaginosis, STIs, and vaginitis; it will likely replace the now-inadequate vaginal culture in the near future.

References

1. Koumans EH, Sternberg M, Bruce C, McQuillan G, Kendrick J, et al. (2007) The prevalence of bacterial vaginosis in the United States, 2001-2004; associations with symptoms, sexual behaviors, and reproductive health. Sexually transmitted diseases 34: 864-869.
2. Peres AL, Camarotti JR, Cartaxo M, Alencar N, Stocco RC, et al. (2015) Molecular analysis and conventional cytology: association between HPV and bacterial vaginosis in the cervical abnormalities of a Brazilian population. Genetics and molecular research, GMR 14: 9497-9505.
3. Hillier S, Krohn MA, Watts DH, Wolner-Hanssen P, Eschenbach D (1990) Microbiologic efficacy of intravaginal clindamycin cream for the treatment of bacterial vaginosis. Obstetrics and gynecology 76: 407-413.
4. Watts DH, Krohn MA, Hillier SL, Eschenbach DA (1990) Bacterial vaginosis as a risk factor for post-cesarean endometritis. Obstetrics and gynecology. 75: 52-58. PubMed PMID: 2296423.
5. Swidsinski A, Baucke LV, Swidsinski S, Verstraelen H (2015) Polymicrobial Gardnerella biofilm resists repeated intravaginal antiseptic treatment in a subset of women with bacterial vaginosis: a preliminary report. Arch Gynecol Obstet 291: 605-609.
6. Parma M, Vanni SV, Bertini M, Candiani M (2014) Probiotics in the prevention of recurrences of bacterial vaginosis. Alternative therapies in health and medicine 20: 52-57.
7. Marrazzo JM, Thomas KK, Fiedler TL, Ringwood K, Fredricks DN (2008) Relationship of specific vaginal bacteria and bacterial vaginosis treatment failure in women who have sex with women. Annals of internal medicine 149: 20-28.
8. Budd WT, Harwich M, Bostwick DG, Meyers G, Dilts J, et al. (2015) Metagenomics Analysis Using Next Generation Sequencing of Vaginal Samples from Community Practices in the U.S. Cell Sci Res p: 2.
9. Srinivasan S, Hoffman NG, Morgan MT, Matsen FA, Fiedler 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: e37818.
10. Van de Wijgert JH, Borgdorff H, Verhelst R, Crucitti T, Francis S, et al. (2014) The vaginal microbiota: what have we learned after a decade of molecular characterization? PloS one 9: e105998.
11. Fredricks DN (2011) Molecular methods to describe the spectrum and dynamics of the vaginal microbiota. Anaerobe 17: 191-195.
12. Ravel J, Gajer P, Abdo Z, Schneider GM, Koenig SS, et al. (2011) Vaginal microbiome of reproductive-age women. Proceedings of the National Academy of Sciences of the United States of America 108: 4680-4687.
13. Gutman RE, Peipert JF, Weitzen S, Blume J (2005) Evaluation of clinical methods for diagnosing bacterial vaginosis. Obstetrics and gynecology 105: 551-556.
14. Bradshaw CS, Tabrizi SN, Fairley CK, Morton AN, Rudland E, et al. (2006) The association of Atopobium vaginae and *Gardnerella vaginalis* with bacterial vaginosis and recurrence after oral metronidazole therapy. The Journal of infectious diseases 194: 828-836.
15. Sobel JD, Schmitt C, Meriwether C (1993) Long-term follow-up of patients with bacterial vaginosis treated with oral metronidazole and topical clindamycin. The Journal of infectious diseases 167: 783-784.
16. Boris J, Pahlson C, Larsson PG (1997) Six years observation after successful treatment of bacterial vaginosis. Infectious diseases in obstetrics and gynecology 5: 297-302.
17. Schwebke JR, Desmond RA (2007) A randomized trial of the duration of therapy with metronidazole plus or minus azithromycin for treatment of symptomatic bacterial vaginosis. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 44: 213-219.
18. Eschenbach DA (2007) Bacterial vaginosis: resistance, recurrence, and/or reinfection? Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 44: 220-221.
19. Austin MN, Beigi RH, Meyn LA, Hillier SL (2005) Microbiologic response to treatment of bacterial vaginosis with topical clindamycin or metronidazole. Journal of clinical microbiology 43: 4492-4497.
20. Carr PL, Rothberg MB, Friedman RH, Felsenstein D, Pliskin JS (2005) "Shotgun" versus sequential testing. Cost-effectiveness of diagnostic strategies for vaginitis. Journal of general internal medicine 20: 793-799.
21. Bostwick DG, Woody J, Hunt C, Budd WT (2016) Antimicrobial Resistance Genes and Modeling of Treatment Failure in Bacterial Vaginosis: Clinical Study of 289 Symptomatic Women. Journal of medical microbiology.
22. Srinivasan S, Fredricks DN (2008) The human vaginal bacterial biota and bacterial vaginosis. Interdisciplinary perspectives on infectious diseases pp: 750479.
23. Mayer BT, Srinivasan S, Fiedler TL, Marrazzo JM, Fredricks DN, et al. (2015) Rapid and Profound Shifts in the Vaginal Microbiota Following Antibiotic Treatment for Bacterial Vaginosis. The Journal of infectious diseases. 212: 793-802.
24. Tuite N, Reddington K, Barry T, Zumla A, Enne V (2014) Rapid nucleic acid diagnostics for the detection of antimicrobial resistance in Gram-negative bacteria: is it time for a paradigm shift? The Journal of antimicrobial chemotherapy 69: 1729-1733.
25. Mullany P (2014) Functional metagenomics for the investigation of antibiotic resistance. Virulence 5: 443-447.
26. Olender A (2013) Antibiotic resistance and detection of the most common mechanism of resistance (MLSB) of opportunistic *Corynebacterium*. Chemotherapy 59: 294-306.
27. Institute CaLS (2012) Performance standards for antimicrobial disk susceptibility tests; Approved Standard-Eleventh Edition. Organisms requiring special consideration. Wayne, PA: Clinical and Laboratory Standards Institute.
28. Chakravorty S, Lee JS, Cho EJ, Roh SS, Smith LE, et al. (2015) Genotypic susceptibility testing of *Mycobacterium tuberculosis* isolates for amikacin and kanamycin resistance by use of a rapid sloppy molecular beacon-based assay identifies more cases of low-level drug resistance than phenotypic Lowenstein-Jensen testing. Journal of clinical microbiology 53: 43-51.
29. Fredricks DN, Fiedler TL, Marrazzo JM (2005) Molecular identification of bacteria associated with bacterial vaginosis. The New England journal of medicine 353: 1899-1911.
30. Menard JP, Fenollar F, Henry M, Bretelle F, Raoult D (2008) Molecular quantification of *Gardnerella vaginalis* and Atopobium vaginae loads to predict bacterial vaginosis. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 47: 33-43.
31. Fethers K, Twin J, Fairley CK, Fowkes FJ, Garland SM, et al. (2012) Bacterial vaginosis (BV) candidate bacteria: associations with BV and behavioural practices in sexually-experienced and inexperienced women. PloS one 7: e30633.

32. Tabrizi SN, Fairley CK, Bradshaw CS, Garland SM (2006) Prevalence of *Gardnerella vaginalis* and Atopobium vaginae in virginal women. Sexually transmitted diseases 33: 663-665.

33. Schwebke JR, Muzny CA, Josey WE (2014) Reply to Hickey and Forney. The Journal of infectious diseases 210: 1683-1684.

34. Schwebke JR, Flynn MS, Rivers CA (2014) Prevalence of *Gardnerella vaginalis* among women with lactobacillus-predominant vaginal flora. Sexually transmitted infections 90: 61-63.

35. Ison CA, Taylor RF, Link C, Buckett P, Harris JR, et al. (1987) Local treatment for bacterial vaginosis. British medical journal 295: 886.

36. Gardner HL, Dukes CD (1955) Haemophilus *vaginalis* vaginitis: a newly defined specific infection previously classified non-specific vaginitis. American Journal of Obstetrics and Gynecology 69: 962-976.

37. Eade CR, Diaz C, Wood MP, Anastos K, Patterson BK, et al. (2012) Identification and characterization of bacterial vaginosis-associated pathogens using a comprehensive cervical-vaginal epithelial coculture assay. PloS one 7: e50106.