Antimicrobial resistance genes and modelling of treatment failure in bacterial vaginosis: clinical study of 289 symptomatic women

David G. Bostwick,¹ John Woody,¹ Courtney Hunt² and William Budd¹

¹Granger Genetics, Richmond, VA, USA
²Desert Jewel Gynecology Center, Phoenix, AZ, USA

Clinical management of bacterial vaginosis (BV) is difficult owing to inaccurate diagnostic tests, limited drug choices, and a high rate of recurrence. To our knowledge, there has not been a previous study of antimicrobial resistance (AMR) genes in community practice using next-generation sequencing (NGS). A case–control study (1 : 1 age-matched with and without BV) was undertaken in a series of 326 nongravid women of reproductive age with symptoms of BV to determine the prevalence of AMR genes. NGS was used to describe the complete vaginal microbiota and identify bacterial genes associated with resistance to: macrolides and/or lincosamides – ermA, ermB, ermC, erM, ermTR and mefA; tetracyclines, β-lactams, streptomycin, gentamicin and/or tobramycin – acrA, acrB, mecA, tet, tetA, tolC and aac2; 5-nitroimidazoles – nim and nimB; and triazoles – cdr1 and mdr1. An evidence base was created to inform treatment decisions applicable to individual patients. AMR genes were identified in all drug classes: macrolides, 35.2 %; lincosamides, 35.6 %; tetracyclines, 21.8 %; aminoglycosides (streptomycin, gentamicin and tobramycin), 5.2 % each; 5-nitroimidazoles, 0.3 %; and triazoles, 18.7 %. There was more than a fourfold-higher frequency of AMR genes in pathogens from BV than from non-BV patients for macrolides (58.2 versus 12.3 %, respectively), lincosamides (58.9 versus 12.3 %) and tetracyclines (35.6 versus 8.0 %) (Fisher’s exact test; all p<0.001). For each patient with BV, the spectrum of resistance genes was matched to the pathogens present. AMR genes were present in the majority of vaginal microbiomes of patients with symptoms of BV.

INTRODUCTION

The most common vaginal infection of women during the reproductive years, bacterial vaginosis (BV), has a prevalence of 27–41 % (Klebanoff & Turner, 2014) in women in the USA. It is characterized by replacement of the healthy lactic acid-rich H2O2-producing planktonic Lactobacillus-dominant microbiome by a diverse polymicrobial biofilm of multiple taxa of commensal facultative anaerobes and other pathogenic bacteria. Symptoms include malodorous vaginal discharge, discomfort and elevated pH. It is associated with increased susceptibility to HIV and other sexually transmitted infections, adverse pregnancy outcome, and potentially greater risk of pelvic inflammatory disease (Taylor et al., 2013).

Recidivism is arguably the greatest clinical challenge with BV; it is reportedly very high [41 (Mitchell et al., 2012) to 67 % within months] despite standardized treatment with oral or intravaginal metronidazole or clindamycin, frustrating patients and clinicians (Parma et al., 2014; Swidsinski et al., 2014). A major cause of treatment failure, antibiotic resistance, results from the presence of antimicrobial resistance (AMR) genes, non-genetic determinants of resistance, and drug resistance of the characteristic polymicrobial BV-infected biofilm (Marrazzo et al., 2008).

A major goal in tackling antibiotic resistance is early and more accurate detection and diagnosis of infection and resistance for patient benefit, infection control, surveillance and prudent antibiotic stewardship (Tuite et al., 2014). Resistance data from cultures have been available for decades, but provide no information regarding numerous abundant BV-associated bacteria that are difficult or impossible to culture, including many of the most abundant found only recently with advanced molecular diagnostics, such as Atopobium vaginae, Atopobium parvulum, Lactobacillus iners and other Lactobacillus spp., and multiple Lachnospiraceae spp. (BVAB1, BVAB2 and BVAB3).
(Srinivasan & Fredricks, 2008). In addition to providing a comprehensive description of the vaginal microbiome, molecular diagnostic testing with next-generation sequencing (NGS; Srinivasan et al., 2012) offers promise as a rapid method for targeted genetic identification of AMR genes, providing more complete clinical assessment of multiple genes simultaneously.

The aim of this study was to describe and compare the spectrum of AMR genes in symptomatic women with and without BV. A precision medicine model was created to predict the probability of treatment success for an individual woman based on her prevalence of BV-associated bacteria and the accompanying AMR gene signature.

**METHODS**

This study was approved by AIBioTech’s intramural Institutional Review Board.

**Case selection.** We undertook a retrospective patient-based diagnostic case–control study with consecutive recruitment of all NGS swab specimens examined at AIBioTech between 1 July 2014 and 14 February 2015. Vaginal and cervical swabs were collected from community gynaecology practices in the USA. Records were retrieved from the files of the laboratory information system (Renaissance Software) and de-identified to maintain patient confidentiality after matching NGS results with clinical data. The diagnosis of BV was based on Amsel criteria (clinical criteria), as modified by Gutman et al. (2005). Recorded variables included patient age, date of collection, pregnancy status, menopausal status and clinical indication for vaginal swab specimen evaluation (e.g. vaginitis, vaginosis, leucorrhoea).

Of a total of 487 samples received, the following were excluded: women with vulvo-vaginal candidiasis, trichomona or sexually transmitted infections (n=7), those with incomplete sequencing data or insufficient samples for processing (n=36), pregnant women (n=19), peri-menopausal and post-menopausal women (n=60), and asymptomatic women (n=39). Of the remaining 326 samples, all from asymptomatic women, data were available for 289 for AMR genes. Cases of BV were age-matched 1:1 with controls without BV, with a single extra case in the non-BV group (144 in BV group).

**DNA isolation.** Cervical and vaginal swab specimens were collected and stored using the BD Universal Viral Transport System (Becton Dickinson) according to the manufacturer’s instructions. Extraction of DNA was performed with the RTP Bacteria DNA Mini kit (STRATEC Molecular). Briefly, 400 μl of each sample was pelleted and resuspended with buffer solution R, incubated sequentially in a thermo-mixer for 10 min at 65 °C and 10 min at 95 °C following transfer into extraction tubes containing a lyophilized enzyme mix, vortexed with 400 μl binding buffer B6, and then transferred to an RTA Spin Filter. The column was washed twice with buffer and then each sample was centrifuged for 1 min at 18 000 g to eliminate residual ethanol. Column drying was followed by elution with 150 μl of the provided buffer at 65 °C and brief centrifugation. Samples were stored at 4 °C for up to 1 month or at −20 °C for up to 6 months.

**Library preparation.** Fourteen universal primer pairs were designed to generate seven overlapping amplicons that span the variable regions of the 16S rRNA gene sequences. Accepted extracted DNA input for library generation ranged from 2 to 21 ng (mean 12 ng). Barcoded DNA libraries were generated using the Ion AmpliSeq Library kit 2.0 – 96LV (Thermo-Fisher Scientific). Two separate primer pools were utilized in the initial amplification reaction. The pools were combined before proceeding with the FuPa reaction. The Ion Xpress Barcode Adapters 1-16 kit and Barcode Adapters 17-32 kit were utilized for sample barcoding and adaptor ligation (ThermoFisher Scientific). AMPure Agencourt XP (Beckman Coulter) magnetic PCR cleanup beads were utilized as indicated in the Ion AmpliSeq Library Preparation User Guide. (https://tools.thermofisher.com/content/sfs/manuals/MAN0007247_AmpliSeq_LibPrep_with_LibEqualizer_QR.pdf).

**Ion torrent sequencing.** Sample libraries were normalized using the Ion Library Equalizer kit (Thermo-Fisher Scientific), yielding a final sample library of 100 μl at 100 pM concentration according to the manufacturer’s specifications. Emulsion PCR (emPCR) was performed on the Ion OneTouch 2 instrument (Thermo-Fisher Scientific) as indicated in the Ion PGM Template OT2 200 kit (https://tools.thermofisher.com/content/sfs/manuals/MAN0007220_Ion_PGM_Template_OT2_200_Kit_UG.pdf). Briefly, normalized 100 pM sample libraries were pooled and loaded with OT2 kit reagents with Ion Sphere Particle (ISP) beads. Samples were processed for enrichment on the Ion OneTouch enrichment system within 16 h of completion of emPCR. Enrichment of ISPs was achieved using the reagent kit included in the Ion PGM Template OT2 200 kit and DynaBeads MyOne streptavidin C1 beads (Thermo-Fisher Scientific). The Ion Torrent Ion 316 Chip kit v2 (Thermo-Fisher Scientific) was prepared and loaded according to the manufacturer’s recommendations (https://tools.thermofisher.com/content/sfs/manuals/MAN0007273_IonPGMSequenc_200Kit_v2_UG.pdf). Chips were manually loaded with enriched ISPs with primed sequencing polymerase (provided in kit) using Rainin pipette tips SR-L200F. The Ion Torrent PGM was run according to Ion Torrent 316 chip specifications, with 500 flows, and use of 18 MΩ water system (multistage system, including carbon tank, RO membrane, UV irradiation, post-filtering, deionization, and an Elga water polisher). Standard compressed nitrogen gas was supplied to the PGM system.

**Table 1. AMR genes associated with BV-associated antibiotics and other antibiotics**

<table>
<thead>
<tr>
<th>Macrolide</th>
<th>Lincosamide</th>
<th>β-Lactam</th>
<th>Tetracycline</th>
<th>Aminoglycoside</th>
<th>5-Nitroimidazoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>ermA</td>
<td>ermA</td>
<td>acrA</td>
<td>acrA</td>
<td>acrA</td>
<td>acrA</td>
</tr>
<tr>
<td>ermB</td>
<td>ermB</td>
<td>acrB</td>
<td>acrB</td>
<td>acrB</td>
<td>acrB</td>
</tr>
<tr>
<td>ermC</td>
<td>ermC</td>
<td>tolC</td>
<td>tet</td>
<td>tolC</td>
<td>tolC</td>
</tr>
<tr>
<td>erM</td>
<td>erM</td>
<td>aac2</td>
<td>tetA</td>
<td>mecA</td>
<td>mecA</td>
</tr>
<tr>
<td>ermTR</td>
<td>ermTR</td>
<td>mecA</td>
<td>tolC</td>
<td>mecA</td>
<td>mecA</td>
</tr>
<tr>
<td>mecA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>streptomycin</td>
<td>acrA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>gentamicin</td>
<td>acrB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>tobramycin</td>
<td>acrB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>nis</td>
<td></td>
<td></td>
<td></td>
<td>mecA</td>
<td>mecA</td>
</tr>
<tr>
<td>nimB</td>
<td></td>
<td></td>
<td></td>
<td>meca2</td>
<td>meca2</td>
</tr>
</tbody>
</table>
Analysis of NGS data. A reference 16S rDNA non-redundant database was compiled for organisms likely to be present in the vagina based on published and online data from the Vaginal Microbiome Consortium (Fettweis et al., 2012). Full 16S sequences were compiled for over 1300 bacteria and used for comparison of targeted sequencing data. Briefly, patient sample sequences were separated by the Torrent Suite Software (Thermo-Fisher Scientific), and a custom bioinformatics solution (American International Biotechnologies) was created using Perl.

Following sequencing, a binary version (BAM) of a tab-delimited text file containing sequence alignment data (SAM), for each patient sample was extracted into the SAM format, and sequences less than 75 nt in length were removed. Reads greater than 75 nt in length were used to align to a manually curated USEARCH database of the organisms described (Budd et al., 2015). In order to be classified as detected, the organism had to exceed a threshold of 100 reads across more than one variable region of the 16S rDNA molecule. Each detected organism was approximated by dividing the number of reads mapped to the organism by the total number of reads mapped to all bacteria; only those accounting for at least 1% of reads per patient sample were counted.

Review of literature on AMR genes in BV. We undertook a literature review of all drugs recommended for patients with BV and others that may be useful for the most abundant BV-associated pathogens. This non-systematic literature search used available databases at the National Center for Biotechnology Information (NCBI), including PubMed, as well as other internet research tools such as Google search functions and others available through the American Society for Microbiology journals online. These databases were queried using a variety of key words in numerous combinations, including: antibiotic resistance in bacteria; antibiotic resistance; microbial resistance; drug resistance; drug resistance genes; bacterial vaginosis; and the names and classes of the drugs listed in Table 1. From the search results, we compiled the data from at least three papers per combination of organism–drug–gene (when available), and extracted the consensus sensitivity, intermediate, or resistance for each according to the in vitro MIC findings.

Analysis of AMR genes in BV. Multiple AMR genes were identified for each major class of drugs (Table 1), and amplicons for each were designed at <200 bp and tested in patient samples. AMR genes were sequenced using the Ion AmpliSeq targeted sequencing system. Genes of interest were identified by literature search and confirmed using the Antibiotic Resistance Database (ARDB). Sequences of each gene were obtained from NCBI and used as an input into the Ion Torrent AmpliSeq Designer software. One to three amplicons were created to successfully identify each resistance gene. Following sequencing, assembled gene sequences were aligned to a reference database using USEARCH with 99% identity required and a minimum of 10 aligned reads. Results were reported qualitatively as present or absent.

Creation of model to predict patient outcome according to NGS data and AMR genes. For women with BV, we created a hierarchical tree diagram according to the rank order of BV-associated bacteria from highest prevalence to lowest for a total of three branches; this created eight combinations of bacteria. At each sequential step in the rank ordering, the proportion of each bacterium was determined, and the one with the greatest proportion was assigned to that branch-point in the hierarchical tree and also removed from consideration of subsequent proportions. Each organism in the combinations was then queried for the presence of AMR genes, and these data were combined with those from the literature review of drug susceptibility to predict the likelihood of success or failure of metronidazole or clindamycin to eradicate each of the three bacteria in each of the eight combinations. No effort was made to address non-AMR gene-based mechanisms of resistance.

Statistics. Fisher’s exact test was used to compare the presence or absence of resistance genes in samples from patients with BV and non-BV. Age differences and AMR gene abundance differences were studied with Pearson’s correlation coefficient. Differences in mean number of genes per sample were evaluated with Spearman’s rank correlation test. The significance level was set at $P<0.05$ for all tests.

RESULTS

Patients ranged in age from 14.6 to 54.9 years (mean 33.8 years). No difference was observed in age between those with and without BV (mean 33.4 versus 34.1 years, respectively; range 15.0–54.6 versus 14.6–54.9 years, respectively; $P=56$, Pearson’s correlation coefficient test).

![Fig. 1. Prevalence of AMR genes (n=289 samples).](http://jmm.microbiologyresearch.org)
Table 2. Literature review of in vitro drug sensitivity and resistance for normal commensal bacteria (top four by prevalence) and common BV-associated pathogens (top ten by prevalence; Bostwick et al., 2016)

Entries indicate in vitro antibiotic sensitivity (S) and resistance (R) for select drug classes according to the presence or absence of the listed organism; 'I' indicates that the drug may or may not be effective against the organism (more than 30% resistance in some studies; variation in MIC results). Dash (–) indicates that no studies have been performed by current recommended methods. The results are based on a preponderance of data, but are subject to variance owing to differences in method of measurement of susceptibility, number of strains studied, subjectivity of interpretation of culture results, changes in sensitivity over time, etc.

<table>
<thead>
<tr>
<th>Class of drug</th>
<th>Macrolide</th>
<th>Lincosamide</th>
<th>β-Lactam</th>
<th>Tetracycline</th>
<th>Aminoglycoside</th>
<th>5-Nitroimidazoles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Top 4 normal commensals</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>Anaerobic</td>
<td>Erthromycin, azithromycin</td>
<td>Clindamycin</td>
<td>Penicillin</td>
<td>Tetracycline, doxycycline</td>
<td>Streptomycin</td>
</tr>
<tr>
<td>crispatus</td>
<td>Gram-positive</td>
<td>S (Ferris et al., 2004; Delgado et al., 2005; Klare et al., 2007)</td>
<td>S (Ferris et al., 2004; De Backer et al., 2006; Polatti, 2012)</td>
<td>S (De Backer et al., 2006)</td>
<td>R (Gad et al., 2014)</td>
<td>R (Gad et al., 2014)</td>
</tr>
<tr>
<td>iners</td>
<td>Anaerobic</td>
<td>S (Ferris et al., 2004; Delgado et al., 2005)</td>
<td>S (Ferris et al., 2004; Polatti, 2012)</td>
<td>S (De Backer et al., 2006)</td>
<td>R (Gad et al., 2014)</td>
<td>R (Gad et al., 2014)</td>
</tr>
<tr>
<td>jensenii</td>
<td>Anaerobic</td>
<td>S (Ferris et al., 2004; Delgado et al., 2005)</td>
<td>S (Ferris et al., 2004; De Backer et al., 2006)</td>
<td>S (De Backer et al., 2006)</td>
<td>R (Gad et al., 2014)</td>
<td>R (Gad et al., 2014)</td>
</tr>
<tr>
<td>gasseri</td>
<td>Anaerobic</td>
<td>S (Ferris et al., 2004; Delgado et al., 2005; De Backer et al., 2006)</td>
<td>S (Ferris et al., 2004; Polatti, 2012)</td>
<td>S (De Backer et al., 2006)</td>
<td>R (Gad et al., 2014)</td>
<td>R (Gad et al., 2014)</td>
</tr>
</tbody>
</table>

**Top ten pathogens**

| Gardnerella vaginalis | Anaerobic | Gram-variable | S (Piot et al., 1980; Kharsany et al., 1993; Ferris et al., 2004)* | S (Piot et al., 1980; Tomusiak et al., 2011; Ferris et al., 2004; Polatti, 2012) | S (De Backer et al., 2006) | S (Shanker et al., 1982; Catlin, 1992) | S (Piot et al., 1980; Catlin, 1992) | R (Shanker et al., 1982; Catlin, 1992) | I (Piot et al., 1980; Delgado et al., 2005; Polatti, 2012)† |

| Atopobium vaginae | Anaerobic | Gram-positive | S (De Backer et al., 2006) | S (Ferris et al., 2004; De Backer et al., 2006) | S (Ferris et al., 2004; De Backer et al., 2006) | R (Ferris et al., 2004) | R (Ferris et al., 2004) | R (Ferris et al., 2004) | R (De Backer et al., 2006; Polatti, 2012) |

| BVAB2 | Not yet cultured | – | – | – | – | – | – | – | R (De Backer et al., 2010) |

| Atopobium parvulum | Anaerobic | Gram-positive | – | S (Copeland et al., 2009) | – | – | – | – | – |

| Dialister micraerophilus | Microaerophilic | Gram-negative | S (Morio et al., 2007) | S (Morio et al., 2007) | S (Morio et al., 2007) | – | – | – | S (Jumas-Bilak et al., 2005; Morio et al., 2007) |

| BVAB1 | Not yet cultured | – | – | – | – | – | – | – | – |

| Prevotella timonensis† | Anaerobic | Gram-negative | S (Brook et al., 2013) | S (Brook et al., 2013) | R (Boyanova et al., 2010) | R (Boyanova et al., 2010) | R (Finegold, 1996) | R (Finegold, 1996) | S (Boyanova et al., 2010) |
Prevalence of AMR genes

The prevalence of AMR genes in the 289 study group samples was significantly greater in patients with BV than in those without BV (74.3 versus 22.1 %, respectively; all \( p < 0.0001 \), Fisher’s exact test). Of the 14 resistance genes evaluated, only 8 were identified in any of the samples. The mean number was significantly greater in BV than non-BV patient samples (1.5 versus 0.3, respectively; range 0–6 versus 0–3; \( P < 0.001 \), Spearman’s rank correlation).

Genetic resistance for the most common treatments for BV showed disparate results (Fig. 1). The AMR gene (ermTR) for lincosamides, including clindamycin, was the most common gene present in both BV and non-BV samples, with a BV prevalence of 61.8 %. Conversely, the AMR genes for 5-nitroimidazoles (nim and nimB), including metronidazole, were found in only two and zero samples from BV patients, respectively. Two genes (ermTR and tet) accounted for the majority of AMR genes, and coexisted in 28.7 % of patient samples – significantly more in BV than non-BV (51.1 versus 5.5 %, respectively; \( p < 0.0001 \), Pearson’s correlation coefficient 0.52).

Literature review of drug sensitivity and resistance for BV-associated commensals and pathogens

Antibiotic susceptibilities according to culture-based in vitro MIC data are shown in Table 2.

Prediction of patient outcome according to NGS data, AMR genes, and literature review

Eight combinations of bacteria were created from the first three branches of the rank-ordered hierarchical tree diagram for women with BV (Fig. 2). Each of these combinations was queried for the presence of AMR genes together with expected antibiotic susceptibility according to the literature review. Treatment failure was predicted for at least 52.2 % of women treated with metronidazole and 44.0 % of those treated with clindamycin (Table 3). Absence of published data on susceptibility and AMR gene prevalence in Lachnospiraceae spp. (BVAB1, BVAB2 and BVAB3) precluded assessment of results in 28.9 % of samples.

DISCUSSION

We found that AMR genes were common in the vaginal microbiome. This was especially true in women with BV when compared with symptomatic women without BV; there was an increase in overall abundance (74.3 versus 22.1 %, respectively) and number of AMR genes per specimen (mean 1.5 versus 0.3, respectively). To our knowledge, this is the first report of the results of a commercially available clinical test for AMR genes in community practice using NGS.

According to AMR gene identification, there were varying levels of susceptibility for the two primary drugs used to
treat BV (clindamycin and metronidazole). The highest level of AMR gene identification (61.8 %) was found for clindamycin, one of the lincosamides. Among the four major classes of \textit{erm} genes (\textit{ermA}, \textit{ermB}, \textit{ermC} and \textit{ermF}) in different bacteria, \textit{ermA} appears to be the primary gene responsible for clindamycin-associated AMR in \textit{Gardnerella vaginalis}. Macrolides, lincosamides (clindamycin), and streptogramin B (MLSB) antibiotics have similar modes of action and resistance, the latter from production by the \textit{erm} genes of rRNA methylase, which results in a change in the structure of the antibiotic-binding site and inhibition of bacterial protein synthesis. MLSB phenotype can be constitutive (rRNA methylase is always produced) or inducible (methylase is produced only in the presence of an inducing agent).

Unlike clindamycin, there was a low level of AMR gene identification (1.4 %) for metronidazole. \textit{nim} genes encode a reductase that converts nitroimidazole to a non-toxic derivative. These genes are usually found on plasmids, but are also found on bacterial chromosomes, and are transferable by a conjugative process. There are seven types of \textit{nim} genes, each of which is highly predictive of metronidazole resistance (odds ratio 26) (Löfmark et al., 2010). However, \textit{nim}-negative bacteria may also occasionally have a high level of resistance, indicating that other mechanisms exist. Silent \textit{nim} genes are rare. The number of \textit{nim}-positive metronidazole-resistant bacteria appears to have been increasing rapidly in recent years (Nagy et al., 2001). Several anaerobic bacteria associated with BV, including \textit{G. vaginalis} and \textit{Mobiluncus curtisi}, have low \textit{in vitro} susceptibility to metronidazole, likely contributing to relapse (Fredricks et al., 2005).

There is a high recurrence rate for BV despite treatment, perhaps due at least in part to AMR genes, with reported relapses in about 30–50 % of women within 2–3 months and up to 80 % at 12 months (Sobel et al., 1993; Boris et al., 1997). A controlled clinical trial found that 14 day treatment with metronidazole resulted in treatment failure of 38 % within 1 week and 57 % within 3 weeks (Schwebke & Desmond, 2007). Our model, which combined abundance of BV-associated bacteria with AMR gene data and literature review of antibiotic susceptibility, predicted treatment failure of at least 44.0 % in women treated with clindamycin and 52.2 % in those treated with metronidazole, similar to the published recurrence rates.

Patient outcome predictions derived from targeted sequence data of AMR genes may be imperfect owing to the multifactorial nature of drug resistance (Livermore & Wain, 2013; Tuite et al., 2014). The sources of resistance may be unknown, causing unexpected treatment failure, as AMR gene analysis may create false-positive or false-negative results. In the former, a resistance gene is found, but it is silent or is not expressed at a clinically significant level to provide resistance (Enne et al., 2006), potentially resulting in withholding of optimal therapy; in the latter, the resistance gene analysis is negative, but unknown factors cause failure despite proper treatment. The combination of AMR genetic analysis and conventional culture-based resistance testing may obviate some of these problems. In addition, AMR genetic analysis in complex bacterial communities such as BV cannot identify the specific bacterial species responsible for a resistance gene; if the gene resides in normal commensal bacteria, then that could create another source of false-positive results.

**Fig. 2.** Rank-order hierarchical clustering of 163 symptomatic cases of BV. \textit{Strep.}, \textit{Streptococcus}. 

Table 3. Prediction of response to recommended drug treatments for BV based on most prevalent bacteria according to hierarchical clustering

Data on sensitivity and resistance are derived from Table 2. Assumptions in the model include the following: only the three most abundant bacteria account for all susceptibility; *A. Atopobium* response to drug is an ideal fit with literature review findings of susceptibility; AMR genes are fully expressed; no influence of the interactions between bacteria (commensals and pathogens) as well as the influence of the biofilm. The prevalent bacterial combinations are derived from Fig. 2.

<table>
<thead>
<tr>
<th>Prevalent bacterial combination</th>
<th>No. samples (% BV samples)</th>
<th>Metronidazole treatment</th>
<th>Clindamycin treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Response&lt;sup&gt;†&lt;/sup&gt;</td>
<td>Predicted failure (%)&lt;sup&gt;‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>G. vaginalis+, A. vaginae−, A. parvulum+</td>
<td>68 (41.7)</td>
<td>I, R, S</td>
<td>41.7</td>
</tr>
<tr>
<td>G. vaginalis+, A. vaginae−, A. parvulum−</td>
<td>12 (7.4)</td>
<td>I, R, −</td>
<td>7.4</td>
</tr>
<tr>
<td>G. vaginalis+, A. vaginae−, A. parvulum+</td>
<td>8 (4.9)</td>
<td>I, −, S</td>
<td>0.7</td>
</tr>
<tr>
<td>G. vaginalis−, BVAB2+, BVAB1+</td>
<td>28 (17.2)</td>
<td>I, −, −</td>
<td>2.4</td>
</tr>
<tr>
<td>G. vaginalis−, BVAB2−, BVAB1+</td>
<td>7 (4.3)</td>
<td>−, ?, ?</td>
<td>?</td>
</tr>
<tr>
<td>G. vaginalis−, BVAB2−, BVAB1−</td>
<td>8 (4.9)</td>
<td>−, ?, −</td>
<td>?</td>
</tr>
<tr>
<td>G. vaginalis−, BVAB1−</td>
<td>5 (3.1)</td>
<td>−, −, ?</td>
<td>?</td>
</tr>
<tr>
<td>G. vaginalis−, BVAB2−, BVAB1−</td>
<td>27 (16.6)</td>
<td>−, −, −</td>
<td>At least 52.2$|$</td>
</tr>
</tbody>
</table>

<sup>†</sup>Includes influence of AMR genes for metronidazole (1.4% nim gene prevalence – see Fig. 1). [Example 1: *G. vaginalis*+, *A. vaginae*+, *A. parvulum*+ constitutes 41.7% of samples; the literature review (Table 2) predicts that *A. vaginae* is resistant to metronidazole, and so all of these patients (41.7%) are predicted to experience treatment failure. Example 2: *G. vaginalis*+, *A. vaginae*−, *A. parvulum*+ constitutes 4.9% of samples; the literature review (Table 2) predicts that all of these are potentially susceptible to metronidazole; AMR gene data (Fig. 1) predict that 1.4% of these will experience treatment failure for metronidazole, yielding predicted failure of 0.07% (4.9% of 1.4%) of total BV samples.]<br><br>Note: Predicted failure is shown as a percentage of BV samples.<br><br>§Includes influence of AMR genes for clindamycin (61.8% ermTR gene prevalence – see Fig. 1). [Example: *G. vaginalis*+, *A. vaginae*−, *A. parvulum*+ constitutes 41.7% of samples; the literature review (Table 2) predicts that all of these are potentially susceptible to clindamycin; AMR gene data (Fig. 1) predict that 61.8% of these will experience treatment failure for clindamycin, yielding predicted failure of 25.8% (41.7% of 61.8%) of total BV samples.]

<sup>$\|$</sup>Absence of data on the responses of BVAB2 and BVAB1 to metronidazole and clindamycin limits predictive value.

Mutations are perhaps the greatest source of conflicting results between genotype and phenotype (Robicsek et al., 2006). In the case of infections with Gram-negative bacteria, the problem is daunting owing to the presence of hundreds of resistance genes and variants. Despite these potential obstacles, tests for AMR genes are in successful widespread clinical use today, with reported sensitivity and specificity of 72.3–100 and 55–100%, respectively (Tuïte et al., 2014). Olender (2013) reported ‘very high’ concordance 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. Similarly, the Clinical and Laboratory Standards Institute (CLSI, 2012) reported that ‘tests for meca... are the most accurate... for prediction of resistance to oxacillin’. A recent report showed that AMR gene analysis was superior to routine methods for determination of low-level drug resistance for *Mycobacterium tuberculosis* (Chakravorty et al., 2015). Conversely, there was no correlation between *nim* expression and metronidazole resistance (Leitsch et al., 2014). In addition to AMR genes, virulence factors such as sialidase may be prognostically useful, as they are known to enhance bacterial survival, invasion and/or tissue destruction. Analysis of these factors and resistance genes may aid in diagnosis and treatment planning in select cases (Briselden et al., 1992).
NGS testing appears to offer important advantages over current methods of diagnosis. NGS allowed rapid assessment without culturing of all bacteria (and all fungi, all parasites, and some viruses) present, supporting personalized clinical management of BV by: (1) separating vaginitis caused by candidiasis, trichomonas or sexually transmitted infections from BV; (2) revealing the complete spectrum of pathogens (and non-pathogens) present, allowing full drug coverage; (3) documenting multiple AMR genes at the same time, recognizing the polymicrobial nature of BV and other forms of vaginitis; (4) ensuring optimal antibiotic stewardship by avoiding AMR; and (5) suggesting alternative drugs unencumbered by the presence of resistance-determining genes and other mechanisms of drug resistance.

The most common method for diagnosis of infection and the presence of AMR genes, bacteriologic culturing, is slow and fails to grow the majority of pathogens now known to be present within microbiomes (Livermore & Wain, 2013). In a recent report, cancer patients with known bacteraemia had positive cultures in only 30–40% of cases (Guido et al., 2012). Unlike culturing, molecular methods offer improved diagnostic accuracy by identifying numerous commensals and pathogens that were previously unknown (Fredricks et al., 2005). Incomplete definition of the microbiome is of greatest concern with AMR genes owing to the inability to assess all genes that contribute to resistance, as well as inability to identify resistance arising from mutation. Livermore & Wain (2013) noted that only a comprehensive test can replace existing methods of detection and antibiotic resistance. NGS fulfills these demands by avoiding non-comprehensive testing and its associated ascertainment bias, providing AMR data, and delivering quantitative as well as qualitative data on relative bacterial loads.

Our study has multiple limitations, including: use of pre-selected AMR genes (targeted sequencing), which may not all be known, as there may be hundreds or even thousands of genes and gene variants that could be identified with a functional approach (Card et al., 2014), with new ones emerging over time through adaptation and mutation; potential selection and ascertainment biases owing to the non-randomized retrospective collection of cases, although the relatively large sample size may partially offset these concerns; and being restricted to asymptomatic women of reproductive age, which may preclude generalization of the results to other women. Rapid alterations occur in the microbiome, including mutations, yet our study is limited to single samples (Mayer et al., 2015). There are likely subsets of BV with different clinical outcomes, including primary and recurrent BV, but we did not make this distinction. Strain-specific differences were not addressed in this report, but probably play a significant role in pathogenesis, virulence, and response to therapy (Harwich et al., 2010). Also, our results are not directly linked to treatment outcomes; so the clinical utility remains to be determined prospectively. Further, we provide no data on bacterial hosts for the AMR genes, the genetic context, or whether the genes are inactivated by point mutations or frameshifts (Card et al., 2014). Finally, the predictive model contains numerous assumptions that may or may not be valid.

Relative strengths of our study include: the comprehensiveness provided by NGS; the use of seven amplicons to cover all of the variable regions of the 16S rRNA gene, providing considerably greater coverage and redundancy than use of only one or two amplicons; the use of 100-read minimum across more than one variable region of the 16S rRNA molecule, increasing sensitivity of detection [other studies have used a less sensitive threshold such as 750–1000-read minimum (Fredricks et al., 2009)]; extensive use of confirmatory genetic markers (data not shown); and acquisition of samples from multiple community-based gynaecologists from around the USA, ensuring a diversity of patients.

As patterns of gene resistance in BV emerge with additional NGS studies, these data may allow precise patient and drug targeting to achieve an optimum antibiotic effect and avoid recidivism.

REFERENCES


