Isolation and Characterization of *Gardnerella vaginalis* Clinical Isolates

Laura W. Bush
INSTITUTE *for* WOMEN'S HEALTH
TEXAS TECH UNIVERSITY HEALTH SCIENCES CENTER

Betsaida Delgado[1], London Mena[2], Gary Ventolini[3], Jane Colmer-Hamood[1,4], and Abdul Hamood[1,2]

[1]Dept. of Immunology & Molecular Microbiology, TTUHSC, Lubbock; [2]Dept. of Surgery, TTUHSC, Lubbock; [3]School of Medicine at the Permian Basin, TTUHSC, Odessa; [4]Dept. of Medical Education, TTUHSC, Lubbock

TEXAS TECH UNIVERSITY
Honors College

ABSTRACT

Bacterial vaginosis (BV) is the most common vaginal infection in women of reproductive age. It is associated with an increased risk for pre-term birth, miscarriages, and pelvic inflammatory disease. Under healthy conditions, the vagina is predominated by lactobacilli. However, during BV, the lactobacilli are primarily replaced by facultative anaerobic bacteria, the most prominent being *Gardnerella vaginalis* (GV). Although GV is also found in healthy women, it exists in low concentrations, whereas, it is found in high concentrations in patients with BV. This poorly understood population shift may be due to the ability of GV to effectively compete with lactobacilli. GV may accomplish this competition through: efficient binding to the vaginal epithelial cells, efficient biofilm development, tolerance to the lactobacilli-produced lactic acid and hydrogen peroxide (H_2O_2), or the presence or absence of virulence-associated genes. In this study and using standard laboratory techniques, we analyzed 17 GV clinical isolates for the above described possibilities. We obtained 5 isolates from Biodefense Emerging Infections Resources and 12 from patients who presented at TTUHSC OB-GYN clinics. Using 16S-PCR, we confirmed that all the isolates are GV. The isolates did not vary in their attachment to human vaginal epithelial cells. Following their incubation with human vaginal epithelial cells for 6 h at 37ºC, the numbers of each isolate (colony forming units, CFU) attached to the epithelial cell varied between 10^4 to 10^7 CFU. Biofilm analysis, using the microtiter plate assay revealed that the isolates produced biofilms with variable masses. The crystal violet staining of the biofilm showed that the biofilm mass formed by the isolates varied from 0.15 to 0.98 OD_{600}. Lactobacilli produce 72 mg/mL D-lactic acid and 7 mg/mL L-lactic acid. Thus, we measured the resistance of the GV isolates to the combined concentrations. While three isolates were not affected, lactic acid treatment reduced the growth of other isolates by ninefold. The isolates varied in their resistance to H_2O_2. At 0.049 mM concentration, H_2O_2 reduced the growth of the isolates by 4 to 6 logs. At 0.195 mM and 0.39 mM concentrations, eight isolates survived and at 0.78 mM concentration, seven isolates survived. Finally, we tested the isolates for the presence of: sialidase, biofilm associated protein (BFAP), vaginolysin, siderophore uptake genes by specific primers and PCR. Results showed that 82% of the isolates carried the sialidase gene, 73% carried the BFAP gene, 91% carried the vaginolysin gene, and 18% carried the siderophore uptake gene, respectively. Our results suggested that: with the exception of the siderophore uptake gene, many virulence genes are conserved among GV clinical isolates; different GV isolates efficiently bind to the vaginal epithelial cells and develop biofilms; GV isolates are not resistant to *Lactobacillus*-levels of lactic acid or H_2O_2; and certain strains of GV resist high levels of H_2O_2 through a potential unique mechanism.

INTRODUCTION

Bacterial vaginosis (BV), which is responsible for more than 60% of vulvovaginal infections, is associated with complications of reproductive health including pelvic inflammatory disease, postpartum infections, enhanced susceptibility to HIV infections, preterm birth, and severe adverse outcomes in pregnancy. BV is characterized by a dramatic shift in the vaginal microflora. Lactobacilli, which predominate in the vaginal flora of healthy women, are significantly reduced and are replaced by strictly anaerobic or facultatively anaerobic bacteria. The predominant facultative species is *Gardnerella vaginalis* (GV). GV has been recovered from the vaginal fluid of almost all women diagnosed with BV. Analysis of vaginal biopsy specimens revealed that, during BV, the vaginal microflora exists as polymicrobial biofilms that adhere to the vaginal epithelial cells and consists mainly of GV. Compared with other BV-associated bacteria, only GV possesses the three main virulence-associated features necessary to cause infection and disease – adherence to epithelial cells, biofilm formation, and cytotoxicity.

The mechanism for the population shift during BV, as characterized by the decrease in the numbers of lactobacilli and the increase in the numbers of GV, is not clearly defined. GV is part of the vaginal microbiota of healthy women. However, during BV, GV becomes the predominant bacterium within the vaginal tract. One possible scenario to explain this population shift is that although GV is part of the normal vaginal flora, potential environmental changes select for certain "aggressive" strains of GV that are more efficient in adhering to the vaginal epithelium, forming biofilms, and displacing lactobacilli. Previous studies provided evidence that supports this scenario. Comparative analysis of the virulence of a GV strain obtained from a woman with BV and another strain obtained from a woman without BV suggested the presence of both commensal and pathogenic strains of GV. Compared with the BV-associated strain, the strain obtained from a woman without BV was impaired in its adherence. Similarly, comparative analysis of the genomic sequences of three GV strains obtained from women with symptomatic BV and another strain obtained from a healthy woman confirmed the presence of genetic differences between the two strains. Numerous genes were found only in the GV strains obtained from women with symptomatic BV. These genes code for proteins that likely increase the virulence of GV.

In this study, we conducted a comprehensive analysis of GV isolates obtained from women with BV. We obtained some of the isolates from Biodefense Emerging Infections (BEI) Resources and the others from patients with BV who presented at the OBGYN Clinic at TTUHSC. We analyzed the isolates for their attachment to vaginal epithelial cells and biofilm development. We also determined the resistance of the isolates to lactic acid at concentrations comparable to that produced by lactobacilli and variable concentrations of hydrogen peroxide (H_2O_2). Further, we screened the isolates for the presence of several GV virulence-related genes. Our study showed that although more isolates efficiently attached to the vaginal epithelial cells, they formed variable biofilms. Additionally, few isolates were resistant to high concentrations of H_2O_2. Further, among the different virulence related genes, most are conserved among the isolates. Our results suggest that attachment to vaginal epithelial cells as well as vaginolysin production are essential for the survival of GV during BV.

HYPOTHESIS

G. vaginalis **clinical isolates efficiently bind to vaginal epithelial cells and produce key virulence genes**

METHODS

Growing GV: GV strains were grown in NYC III medium for 48 h at 37°C with 5% CO_2 and the OD_{600} was then adjusted to ~1.0 with NYC III medium.

GV attachment: We examined the attachment of different strains to primary human vaginal epithelial cells (HVEC). HVEC were cultured and maintained in ReproLife Complete Reproductive Medium. 10^8 CFU/mL of culture was centrifuged down and re-suspended in 1 mL of ReproLife Medium (appropriate for HVEC growth). This culture was added to approximately 95% confluent HVEC to incubate for 6 hours in a 5% CO_2 with 37°C environment. Following this, the supernatant was extracted and the cells were washed twice with PBS. 1 mL of NYC III medium was added to the cells and cells were scraped to determine CFU attached.

Crystal violet assay: 10^8 CFU/mL cultures were centrifuged and re-suspended in fresh NYC III medium and added to 13-mm plastic disks. Following incubation for 48 h at 5% CO_2 at 37°C, the disks were rinsed with water and the biofilm biomass was determined by the crystal violet assay. The OD_{595} value was recorded; the values for the control disks were averaged and subtracted from the sample values.

Lactic acid tolerance: 1 mL NYC III medium plus D-lactic acid (72 mg/mL) and L-lactic acid (7 mg/mL) was added to 10^6 CFU/mL culture, incubated 24 h at 37°C in 5% CO_2. at 37°C. CFU's were determined.

Hydrogen peroxide tolerance: GV strains were grown for 48 h to an OD_{600} ~1. 10^6 CFU were added to 1 mL NYC III medium plus H_2O_2 in varied concentrations and incubated another 24 h at 37°C in 5% CO_2 after which CFU were determined.

RESULTS

Key for Figs. 1-3. Negative Control; ATCC 14019; JCP 7659; JCP 8070; JCP 7719; JCP 315-A; PT830; PT01; PT03; PT05; PT15; PT16-1; PT16-2; PT17; PT18; PT20; PT26; PT58; PT59

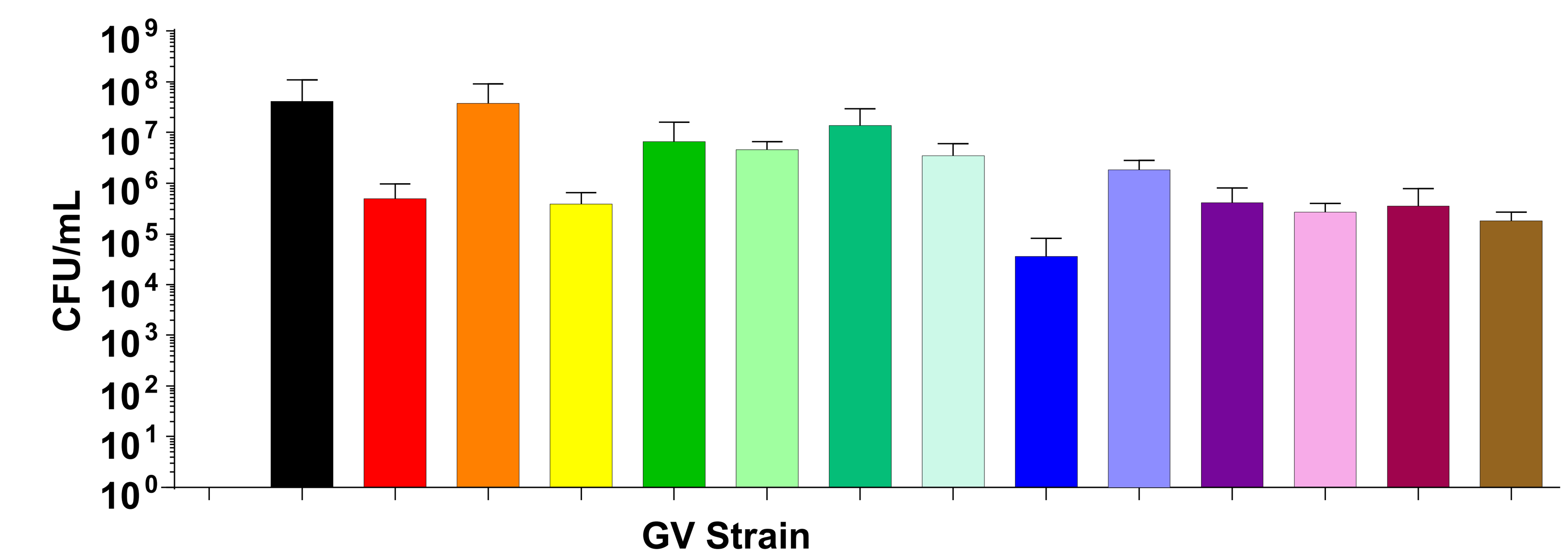

Fig. 1. GV attachment to HVEC: All isolates except PT16-1 and the negative control strain showed increased attachment to HVEC.

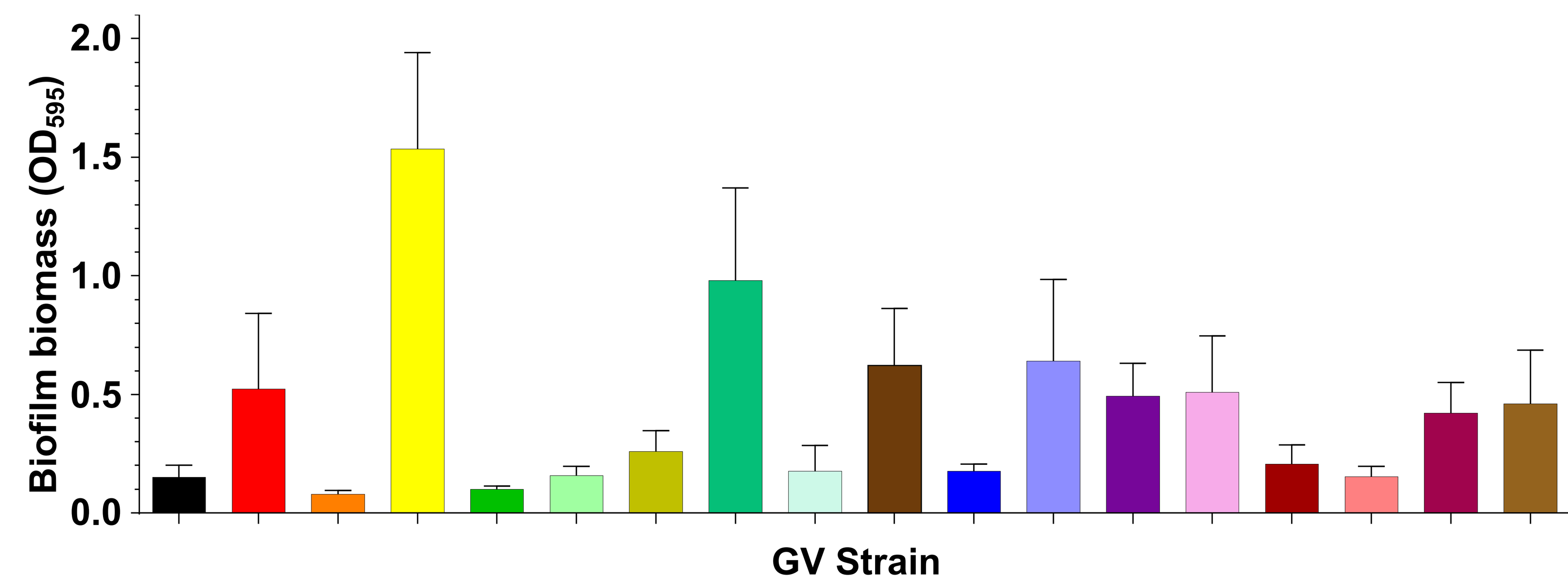

Fig. 2. Quantification of GV biofilm formation: Several clinical isolates showed increased biofilm biomass.

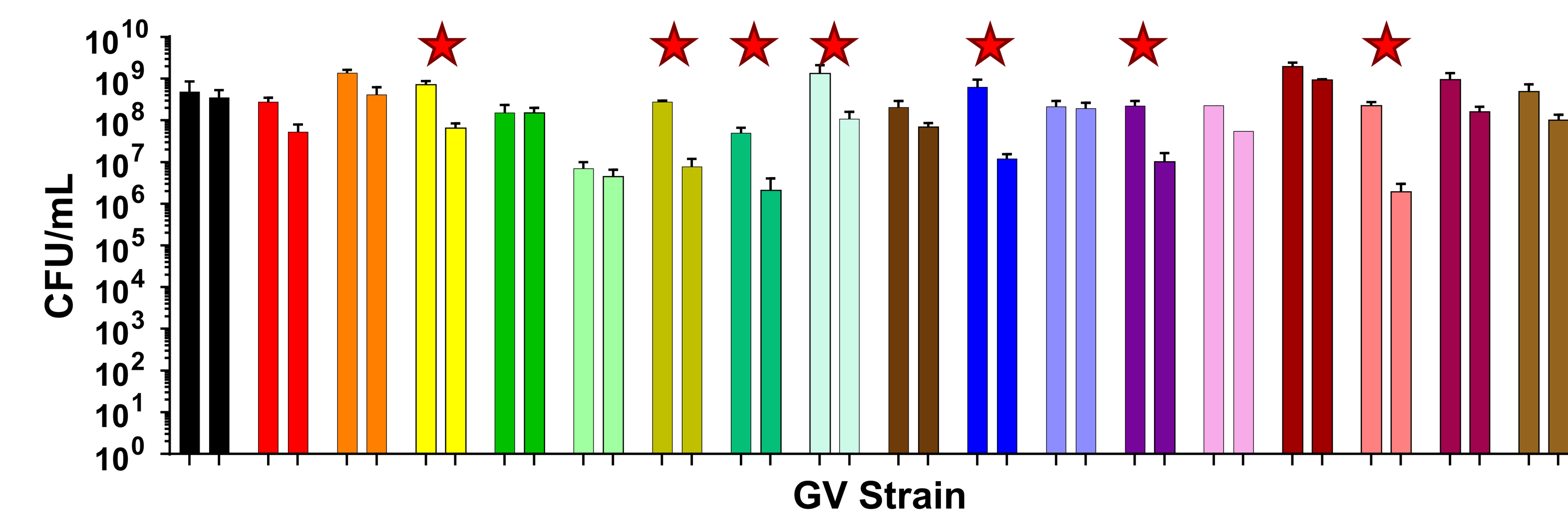

Fig. 3. Lactic acid tolerance test: First column represents the control and the second the lactic acid treatment. Strains marked with star showed a 1-log or greater reduction in CFU compared to control.

Table 1. Hydrogen peroxide tolerance test

H_2O_2 Concentration (mM)	0.78	0.39	0.19	0.098	0.049	0.024	0.012
Percent Resistant	39%	44%	44%	44%	78%	89%	100%

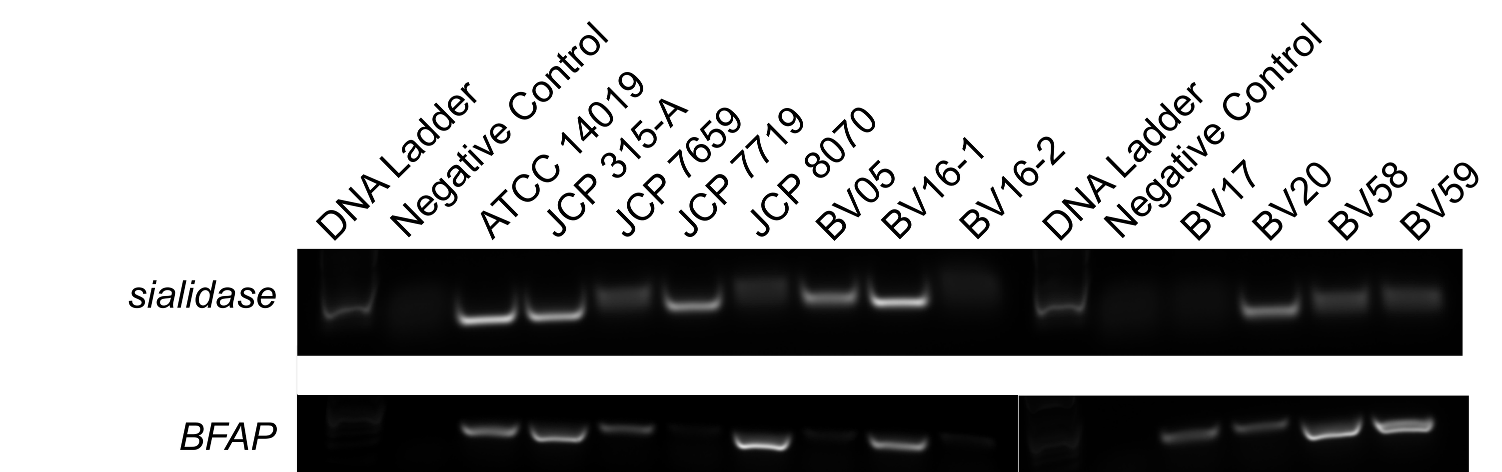

Fig. 4. Representative PCR agarose gels: PCR products for the virulence genes encoding sialidase and the biofilm-associated protein (BFAP).

Table 2. Summary of PCR for GV Virulence Genes

Strain	Genes encoding virulence factors				
	Sialadase	BFAP*	Vaginolysin	Siderophore biosynthesis	Siderophore uptake
ATCC 14019	+	+	+	+	+
JCP 7659	+	+	+	−	−
JCP 8070	+	+	+	−	−
JCP 7719	+	−	+	−	−
JCP 315-A	+	+	+	+	+
BV05	+	−	+	−	−
BV16-1	+	+	+	−	+
BV16-2	−	−	+	+	−
BV17	−	+	−	−	−
BV20	+	+	+	+	−
BV58	+	+	+	−	−
BV59	+	+	+	−	−

*BFAP, biofilm-associated protein

CONCLUSION

Our results suggest that:

- ❖ **Many virulence genes are conserved among GV clinical isolates, with the exception of the siderophore uptake gene;**
- ❖ **The majority of GV isolates from BV patients efficiently bind to vaginal epithelial cells and develop biofilms;**
- ❖ **GV isolates are resistant to levels of lactic acid produced by lactobacilli;**
- ❖ **Some strains of GV are resistant to high levels of H_2O_2 through a potentially unique mechanism.**

ACKNOWLEDGEMENTS

This study was supported by a grant from Laura W. Bush Institute for Women's Health, the Honors College Undergraduate Research Scholars Program supported by The CH and Helen Jones Foundations, and the Center for the Integration of STEM Education and Research.