

The Pathogenesis Of *Streptococcus anginosus* In Aerobic Vaginitis

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Background: Aerobic vaginitis (AV) is a newly defined type of bacterial vaginitis, but its pathogenesis is not yet clear. *Streptococcus anginosus* appears as an emerging pathogen in recent case reports, and colonizes in vagina of patients with AV. In this study, we investigate the pathogenesis of *S. anginosus* in AV.

Materials and methods: (1) We collected 41 vaginal specimens from 21 healthy, fertile women with normal vaginal flora (NM), 10 with bacterial vaginosis (BV) and 10 with AV; their microbiome structure was analysed by 16S rRNA gene sequencing. (2) *S. anginosus* and vaginal epithelial cells were cocultured in vitro, and cytotoxicity was tested by an LDH kit. (3) The *S. anginosus* virulence gene *sag* was knocked out, and the cytotoxicity of the mutant in vaginal epithelial cells was tested.

Results: (1) The microbiome structure of AV was dramatically different from that of BV and NM. The predominant genera of the three groups were *Streptococcus spp.*, *Gardnerella spp.* and *Lactobacillus spp.* *Streptococcus spp.* were significantly more abundant in AV than in BV (95% CI [0.1391, 0.8676], $P < 0.01$) and NM (95% CI [0.1391, 0.8676], $P < 0.01$). (2) *S. anginosus* was the dominant species in AV (95% CI [0.04672, 0.1097], $P < 0.01$). (3) The mean cytotoxicity of *S. anginosus* in vaginal epithelial cells was 58.34% for the wild type (WT) and 16.43% for the mutant; this difference was significant (95% CI [-60.55, -23.28], $P < 0.01$).

Conclusion: *S. anginosus* was the predominant microorganism in patients with AV in our study. *S. anginosus* caused vaginal epithelial cell lysis, indicating that *S. anginosus* is an AV pathogen. The *S. anginosus* virulence gene *sag* is vital for vaginal epithelial cell lysis.

Keywords: *Streptococcus anginosus*, aerobic vaginitis, vaginal microbiome, gene knockout, next-generation sequencing technology, NGS

Plain Language Summary

Aerobic vaginitis (AV), which is an infection caused by aerobic bacteria, was newly defined by Donders in 2002 and diagnosed according to Donders' score. However, not all AV cases are symptomatic or can be cured by antibiotics targeting aerobic bacteria. Therefore, study on the pathogen and pathogenesis of AV is very important. However, the data are limited. In this study, we found that *S. anginosus* was dominant and pathogenic in a subset of AV patients. All the results in our study are novel and unpublished.

Introduction

Aerobic vaginitis (AV) was first defined by Donders in 2002 as a type of vaginal infection caused by aerobic bacteria, and AV is diagnosed according to Donders' score criteria.¹ Patients with AV present with vaginal itching, purulent discharge with or without a rotten odour, and/or red vaginal wall. AV is not a new entity, but the second

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description of a type of bacterial vaginitis, which was first reported by Scheffey in 1956² and named desquamative inflammatory vaginitis (DIV) by Gardner in 1968;^{3,4} Both AV and DIV is distinct from bacterial vaginosis (BV). There is no difference between severe AV and DIV.⁵ Previous studies have shown that AV is associated with miscarriage,⁶ increased risk of amnionitis,⁷ placental histological inflammation,⁸ premature rupture of membranes,⁹ preterm delivery,^{6,10} foetal funisitis,⁸ and major cervical cytological abnormalities.^{11,12} The prevalence of AV is reported to be 5–27.6% in various areas.^{6,9,12–17} Thus, it is vital to identify AV so that standardized treatments can be developed to avoid complications.

Like BV, AV is considered an endogenous infection,¹⁸ but the causal pathogens of AV have not been studied as thoroughly as those of BV. *Escherichia coli*, *Streptococcus spp.*, *Staphylococcus aureus*, *coagulase-negative staphylococci*, *Staphylococcus epidermidis*, and *Enterococcus faecalis* have been found to colonize in significant numbers in AV patients.^{1,14,15} However, the pathogenesis of aerobic bacteria in AV is not yet clear. Further data are limited. Facilitated by the progression of sequencing technology and bioinformatics analysis, studies on the vaginal microbiome have increased in recent years. The work of the National Institutes of Health (NIH) has illustrated new insights into correlations between the vaginal microbiome and the general health of women.¹⁹ To date, no study on the pathogen of AV by next-generation sequencing (NGS) has been performed.

Except for *E. coli*, all of the colonizing bacteria in AV stated above are cocci. In this study, we concentrated on purely symptomatic AV caused by cocci. Our research focused on identifying pathogens by high-throughput 16S rRNA gene sequencing with newly designed primers.

Samples And Methodology

Subject Selection

Forty-one nonpregnant fertile women were enrolled in our study, in which there were 10 women with symptomatic AV diagnosed by Donders' score,¹ 10 women with BV diagnosed by Nugent score²⁰ (≥ 7) and 21 women with normal vaginal microbiota (NM). All of the participants were informed about the study, signed an informed consent form and were then examined by an appointed attending doctor in Beijing Tsinghua Changgung Hospital from July 2017 to September 2018. This study was reviewed and approved by the Ethics Committee Board at the

hospital. All participants were aged from 18 to 50 years and matched the following criteria: (1) did not use antibiotics or immunosuppressants during the last 30 days; (2) had no vaginal intercourse or vaginal procedures in the preceding week; (3) were free of systemic diseases such as infectious diseases, diabetes, autoimmune diseases, and cancer; (4) had at least one of the following clinical characteristics: itching, purulent discharge, dyspareunia, rotten odour, vaginal wall reddening (for AV only); (5) presence of cocci or chained cocci with or without parabasal epithelial cells in vaginal smears (for AV only).

Sample Collection And Preparation

The vaginal discharge of each participant was collected with two sterile cotton swabs of the lateral upper vaginal wall. One swab was examined with Donders' score and Gram stain, and the other swab was diluted in 1 mL of sterile PBS for total DNA extraction, kept in an icebox, and then stored at -80°C within one hour.

Total DNA Extraction, PCR Amplification And 16S rRNA Gene Sequencing

Total DNA from the vaginal discharge was extracted according to the manufacturer's protocol (TIANamp Bacteria DNA Kit, TIANGEN BIOTECH, Beijing, China). The extracted DNA concentration of each sample varied from 47.7–283.6 ng/mL as tested by a NANODROP ONE (Thermo Scientific), and the purity of the DNA was consistent.

The V1-V2 hypervariable regions of the bacterial 16S rRNA gene were amplified by BIO-RAD in a 50 μL reaction system including 30 cycles. Each reaction contained 20 pmol of each of the primers (27F': 5'-AGRGTTYGATYCTGG CTCAG-3'; 338R': 5'-GCTGCCTCCCGTAGGAGT-3'), 25 μL of 2Xtaq PCR Master Mix (TIANGEN), 2 μL of DNA template and 19 μL of double-distilled water (ddwater; TIANGEN). Each sample had a pair of specific barcodes flanking primers 27F' and 338R'. Thermocycling was initiated by a 3 min incubation at 94°C , followed by cycling parameters of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s and terminated by a 5 min incubation at 72°C . All PCR samples were tested by 1% agarose gel electrophoresis (400–500 bp). After generating sequence libraries with a TruSeq[®] DNA PCR-Free Sample Preparation kit (Illumina, USA), the concentration and purity were quantified with an Agilent 2100 Qubit 2.0 bioanalyzer and a BIO-RAD CFX96. Finally, the libraries were sequenced on the Illumina HiSeq 2500 platform using the 250 paired-end protocol.

Processing Of Sequencing Data

After sequencing, the raw data (Figures S1 and S2) were filtered according to the QIIME (V1.7.0) quality control process. Then, the paired reads of the clean data were spliced, and the sequences were merged with FLASH software (V1.2.7). Demultiplexing was performed with in-house R scripts, according to the sample-specific barcode. A 16S sequence reference index was built using the command “bowtie2-build”, with default parameters. All reads were aligned against the prebuilt index using bowtie2, with a parameter of “bowtie2-local”. Alignments were associated with taxa by a sequence-id-to-taxonomy map, provided by the reference database, using a custom Perl script. Unique reads were counted for each taxon, and the abundance was calculated for all taxa. Species with an abundance of less than 1% or read number less than 5 were excluded. For reference databases, we used the SILVA database (SSU 128 Ref NR 99 version from <https://www.arb-silva.de>). All taxonomic data are summarized at the species level. The data of all samples was available from <https://bigd.big.ac.cn/gsa/> (CRA002031).

Construction Of Δ sag ATCC33397

S. anginosus (ATCC33397=NCTC10713) was purchased from ATCC (01077) and cultured in brain heart infusion (BHI; BD) broth and agar. The primers used in this paper are listed in Table 1, and the working concentration for all primers was 10 pmol/ μ L. The erythromycin resistance gene fragment was amplified from plasmid pJDC9 (provided by Jing-Ren Zhang, Tsinghua University) with primers erm-F2 and erm-R2. After preparation of ATCC33397 genomic DNA, up- and downstream regions of sag gene clusters were amplified with primers Up-F1, Up-R1, Down-F3, and Down-R3. Each PCR contained 10 μ L of buffer, 4 μ L of dNTPs, 0.5 μ L of STAR, 40 ng of genomic DNA template and ddwater to produce a final volume of 50 μ L. PCRs were performed under the following conditions: 98°C for 10 min, [98°C for 30 s, 55°C for 1 min/kb, 72°C 3 min] 33 cycles, and 72°C for 5 min (PrimeSTAR DNA polymerase was purchased from Takara). All PCR fragments were purified according to the Magen Pure DNA Mini kit manufacturer’s protocol. Next, fusion PCR of the up/downstream regions of sag gene clusters and the erythromycin resistance gene fragment was performed. The first round in the fusion PCR process included 12 cycles, and the PCR volume was 50 μ L comprising 10 μ L of buffer, 4 μ L of dNTPs, 0.5 μ L of STAR, DNA template (upstream:erm:

Table 1 Primers Used In This Study

Name	Sequence (5'-3')	Target	Use
27F'	AGRGTTYGATYCTGGCTCAG	16S rRNA VI-V2	Sequencing
338R'	GCTGCCTCCCGTAGGAGT	16S rRNA VI-V2	Sequencing
Up-F1	AGAACTTTTCTGAGCCAATTGCTAGG	Upstream fragment of sag	Construction of mutant
Up-R1	ATCTCCATTATCTAGTTTGTCCCTTATAAAATGATGTGTAATATACAA	Upstream fragment of sag	Construction of mutant
erm-F2	ATAAGGAGGACAAACTAGATATAATGGAGATAAGACGGTTCGTGTT	erm	Construction of mutant
erm-R2	AACTAGAAAATACAAATCGCACTCATAGAAATATTTCCCTCCCGT	erm	Construction of mutant
Down-F3	TAATCTATGAGTCGCATTGATTTTCTAGTTGGATGTTAAACCCATTCC	Downstream fragment of sag	Construction of mutant
Down-R3	AGTCTGATAATATTTGTGTCGAATCTTTCC	Downstream fragment of sag	Construction of mutant
sag-F-primer	ATTACACATCAATTTATAAGGAGGACAAACTATGTTAA	sag fragment	Verification of sag cluster
sag-R-primer	TTCTCCTCATCTATCGTTTTCATTTTCAGAC	sag fragment	Verification of sag cluster

downstream=1:1:1>800 ng) and ddwater. The PCR conditions were the same as those described above except for the 12 cycles. The second round of the fusion PCR process included 25 cycles. The reaction contained 10 μ L of buffer, 4 μ L of dNTPs, 1 μ L of Up-F1 primer, 1 μ L of Down-R3 primer, 0.5 μ L of STAR, 10 μ L of purified up+erm+down DNA template and 23.5 μ L of ddwater. The PCR conditions were the same as described above except for the 25 cycles. Finally, the fusion PCR product was purified and stored at -20°C . All DNA fragments were verified by 1% agarose gel electrophoresis (Figure 1A–I).

Transformation of ATCC33397 was conducted using a competence-stimulated peptide (CSP; synthesized by ChinaPeptides), as previously reported.²¹ First, 100 $\mu\text{g}/\text{mL}$ of CSP and fusion PCR product was added to 200 μL of a diluted overnight culture of ATCC33397 (in BHI broth), which was then incubated at 37°C in 5% CO_2 for 3 h. Then, the incubation was continued at room temperature for 2 h. Finally, the transformation mixture was plated on BHI agar plates containing 100 $\mu\text{g}/\text{mL}$ erythromycin to select transformants (Figure 1J and K).

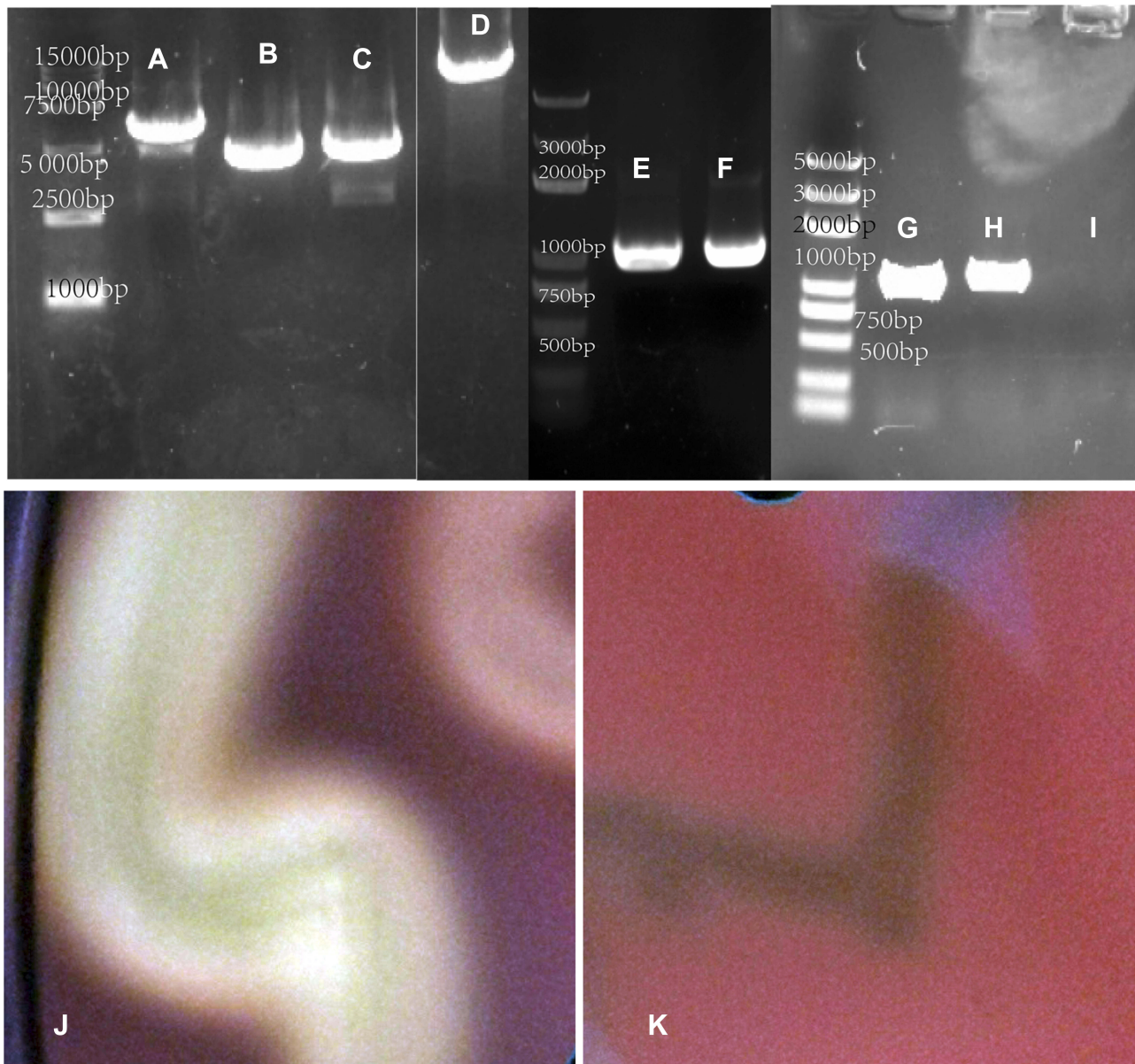


Figure 1 The PCR fragments for the knockout of the sag gene and the *S. anginosus* (ATCC33397/NCTC10713) growing on Blood Agar Plate (BAP). (A) The up+erm+down fragment after fusion PCR; (B) is the up+erm fragment and (C) is the erm+down fragment. (D) The sag fragment of ATCC33397 (WT); (E and F) are erm fragments; (G) is upstream and (H) is downstream of the sag coding area. (I) The sag fragment of the mutant amplified from Δ sag ATCC33397 with primers sag-F-primer and sag-R-primer shown in Table 1. (J) The wild type, with haemolytic activity, and (K) is the mutant with no sag gene and no haemolytic activity.

Table 2 The Symptoms Of Each Patient With AV. “1” Represents Yes, Meaning The Patient Had The Characteristic, And “0” Represents No

ID	Predominance	Purulent Discharge	Itching	Vaginal Wall Reddening	Dyspareunia	Rotten Odour
AV1	<i>S. anginosus</i>	1	1	0	0	1
AV2	<i>S. anginosus</i>	1	1	1	0	0
AV3	<i>S. anginosus</i>	1	1	1	0	0
AV4	<i>L. inners</i>	0	1	0	0	0
AV5	<i>Atopobium. vaginae</i>	1	0	0	0	1
AV6	<i>S. anginosus</i>	1	0	0	0	0
AV7	<i>S. anginosus</i>	1	1	1	1	0
AV8	<i>Aerococcus. christensenii</i>	1	1	1	1	0
AV9	<i>S. anginosus</i>	1	1	0	0	0
AV10	<i>S. anginosus</i>	1	0	0	0	0

Coculture Of *S. Anginosus* And Vaginal Epithelial Cells

VK2/E6E7 cell line was purchased from ATCC (CRL-2616) and cultured in Keratinocyte-serum free medium (K-SFM, Gibco) with 0.1 ng/mL human recombinant EGF (PeproTech), 0.05 mg/mL bovine pituitary extract (Science cell) and 44.1 mg/L Calcium chloride at 37°C

and 5% CO₂. The epithelial cell(VK2/E6E7) cytotoxicity produced by *S. anginosus* (ATCC33397) was determined with an LDH assay kit. VK2/E6E7 cells were cultured with wild-type ATCC33397 and the mutant Δsag ATCC33397 at a concentration of 10⁶ colony forming units (cfu)/mL for 1 h at 37°C and 5% CO₂ (initial MOI: 1).

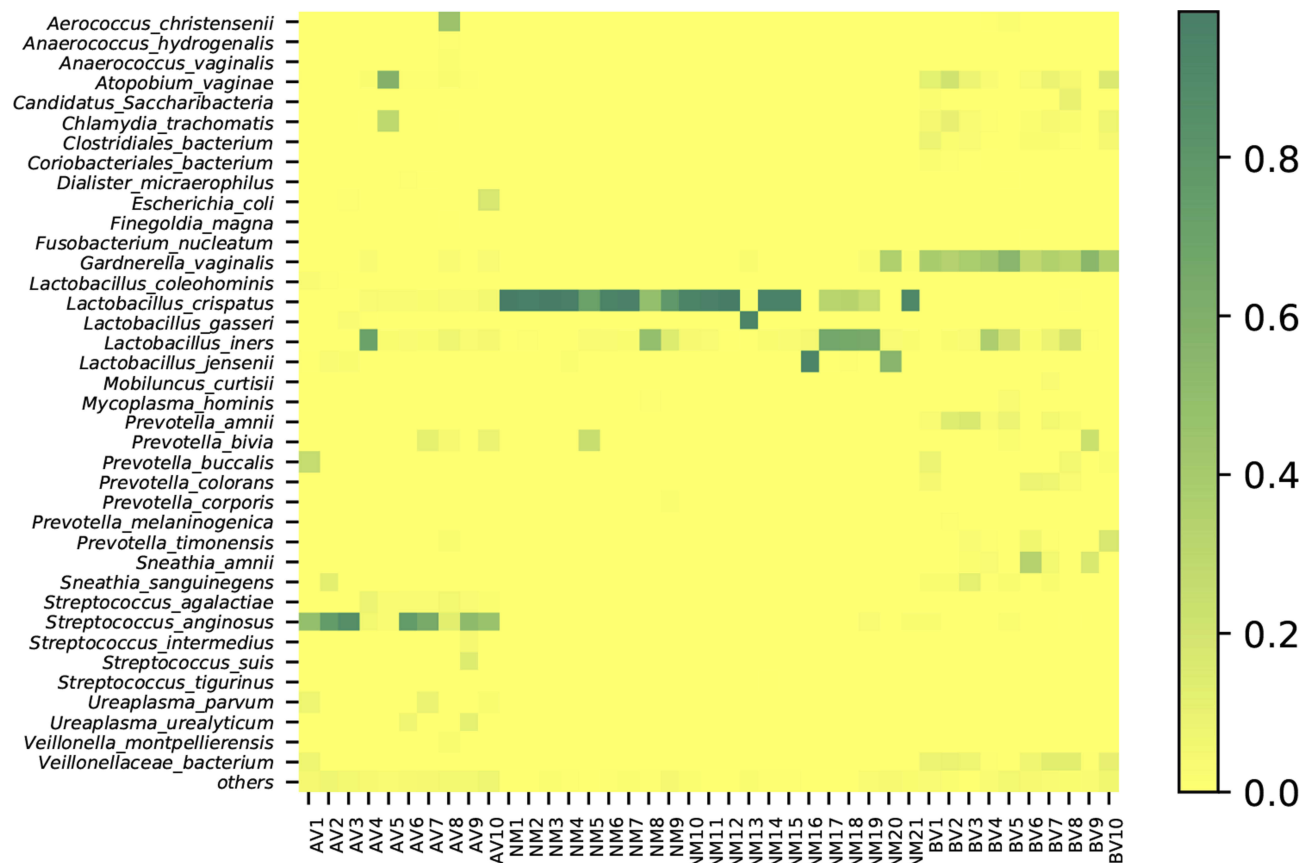


Figure 2 The clustering analysis of AV, BV and NM. The relevance of species to each sample is shown. The deeper the colour is, the more relevant the sample is to the species.

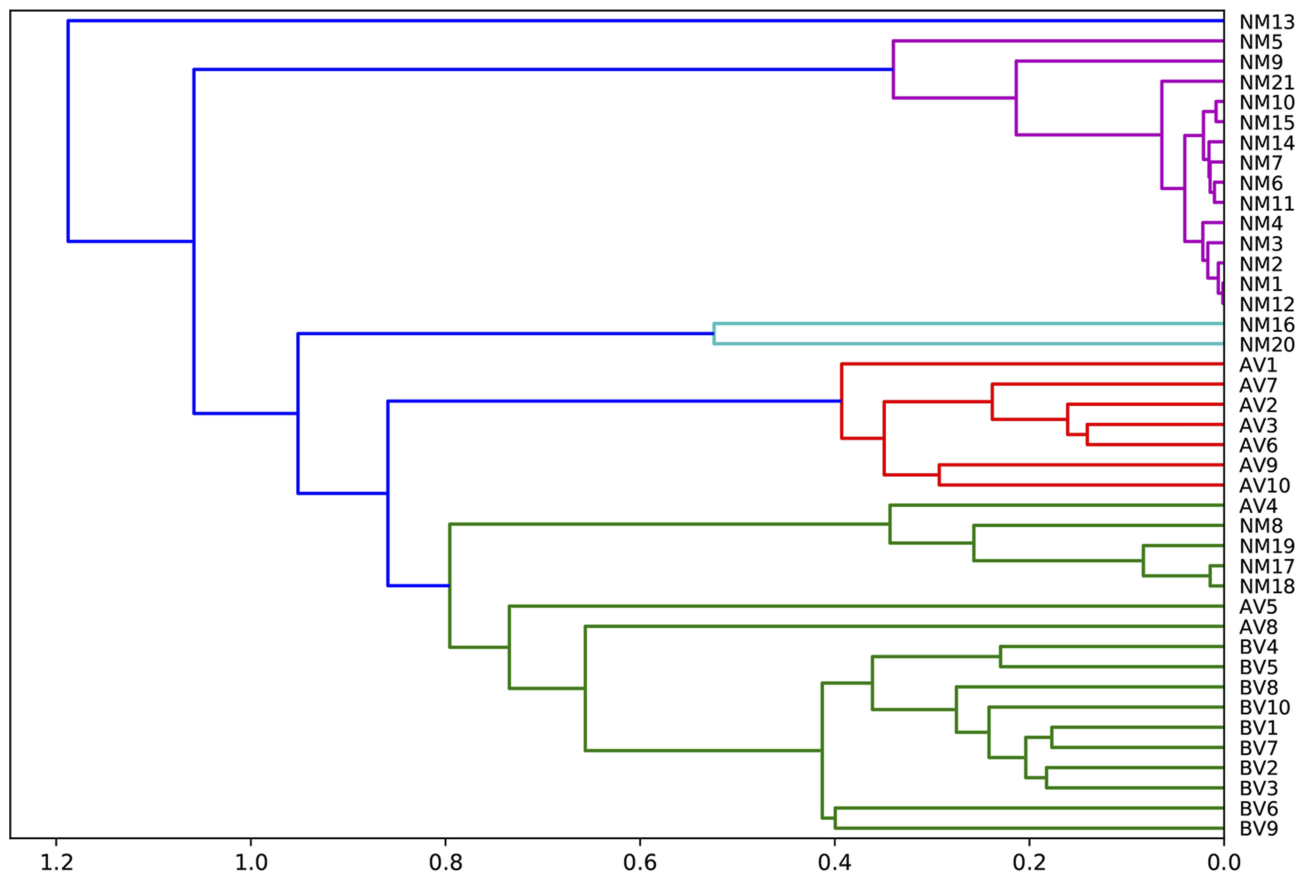


Figure 3 The dendrograms of AV, BV and NM samples. The dimensions of the tree show the distance of the samples.

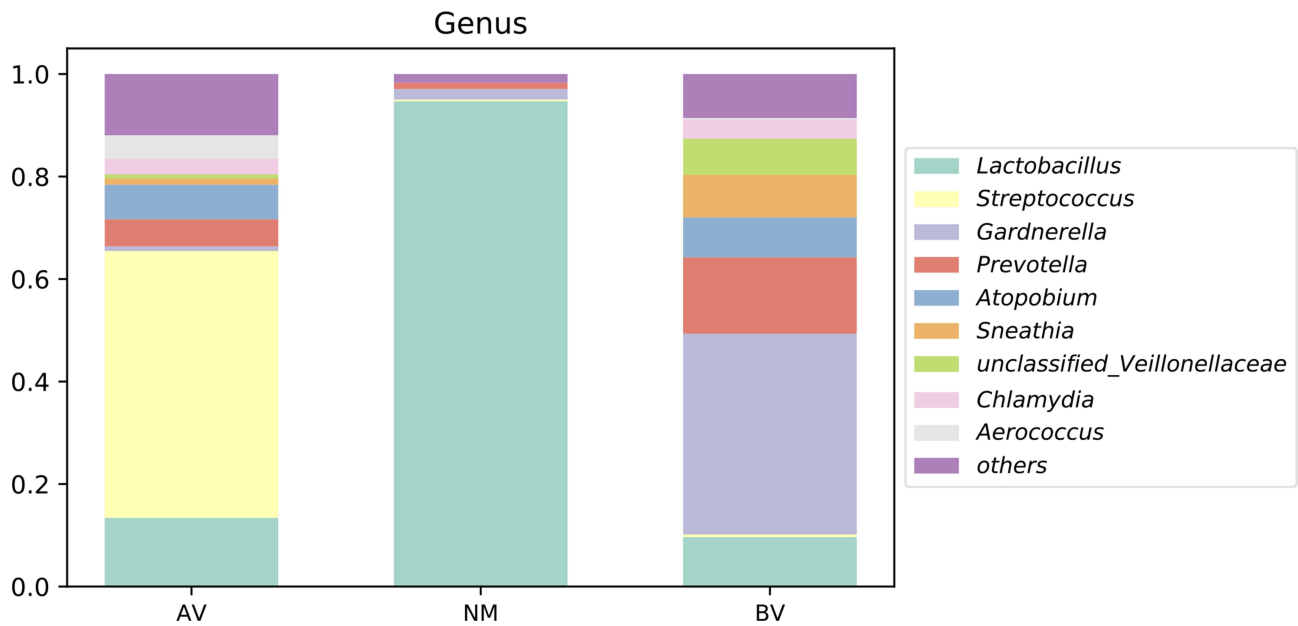


Figure 4 The average abundance of genera in AV, NM and BV samples. The total abundance of each group is 1.

Statistical Analysis

The sample number was calculated using PASS software with type I error=0.05 and target power=0.8 according to the AV microorganism proportions reported by Donders.¹

The 16S rRNA gene sequencing data were converted to proportions to determine the percentage of the microbiome. The predominant species were defined as the largest number of microorganisms, equal to the highest percentage of reads. Wilcoxon ranksum test was used to analyse the variance in abundance between and within groups.

The cytotoxicity data were expressed as the mean \pm SEM and analysed for significance by *t*-test (a P value <0.05 was used as the threshold of significance). All experiments were repeated three times.

Results

The Clinical Characteristics Of The AV Patients

The 10 patients enrolled in our study presented at least one AV symptom, such as purulent discharge, itching, dyspareunia, rotten odour and vaginal wall reddening. The most frequent symptom was purulent discharge (9/10), the second most frequent was itching (7/10), and the third was vaginal wall reddening (4/10). All patients with dominant *S. anginosus* (7/10) presented purulent discharge, and 5 of these patients complained of itching (5/7). All patients found to have vaginal wall reddening during the gynaecological examination had a greater abundance of *S. anginosus* than of other species (Table 2).

The Structure Of The Symptomatic AV, BV, And NM Vaginal Microbiome

The clustering analysis of AV, BV and NM sequencing data indicated a distinct community between three groups based on the operational taxonomic unit (OTU)-taxon-origin (Figures S5–S8). AV mostly correlated with *S. anginosus*, BV mostly correlated with *Gardnerella vaginalis*, and NM clustered with *Lactobacillus crispatus*, *L. gasseri*, *L. inners* and *L. jensenii* (Figure 2). The dendrogram showed that the distance of the AV and BV samples was larger than that of the NM samples, but most of the AV or BV samples were close to each other (Figure 3).

Streptococcus, *Lactobacillus* and *Gardnerella* were the predominant genera in AV, NM and BV, respectively (Figures 4 and S3). *Streptococcus* was significantly more abundant in AV than in BV and NM ($P<0.01$) (Figure 5).

Streptococcus

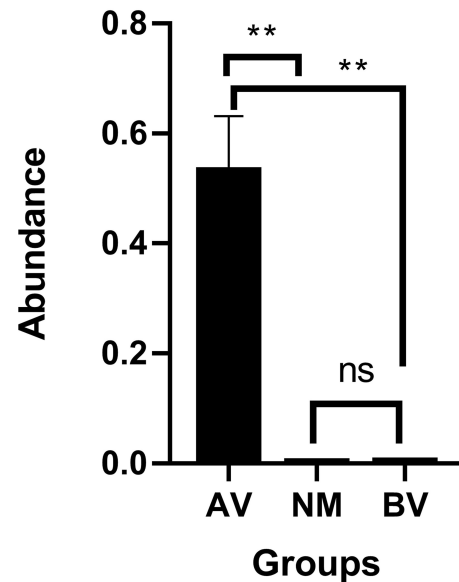


Figure 5 Comparison of the abundance of *Streptococcus* between AV, NM and BV samples. ** $p<0.01$.

S. anginosus was the dominant species in AV, as shown in Figure 6, and it was barely present in NM and BV samples (Figures 6 and S4). Compared with NM, both AV and BV were associated with a significant decrease in *Lactobacillus crispatus* (Figures 6 and S4). The comparison of the abundance of each species in AV revealed that *S. anginosus* was significantly more abundant than other species ($P<0.01$). (Figure 7)

The Cytotoxicity Of Streptococcus In Vaginal Epithelial Cells

S. anginosus (WT) was cytotoxic to vaginal epithelial cells. The cytotoxicity of the WT and the mutant was $(58.34\pm 6.712)\%$ and $(16.43\pm 6.712)\%$ respectively. Though the mutant *S. anginosus* grew at the same rate as the wild type, the cytotoxicity difference was significant ($P<0.01$) (Figures 8 and 9).

Discussion

Fertile women are the most important category of AV patients, but not all of these patients are symptomatic.⁵ A previous study found that 53.85% of AV cases involved mixed infections, including BV, trichomonas vaginitis (TV) or vulvovaginal candidiasis (VVC).¹⁴ We noticed that patients with BV, TV or VVC frequently score above

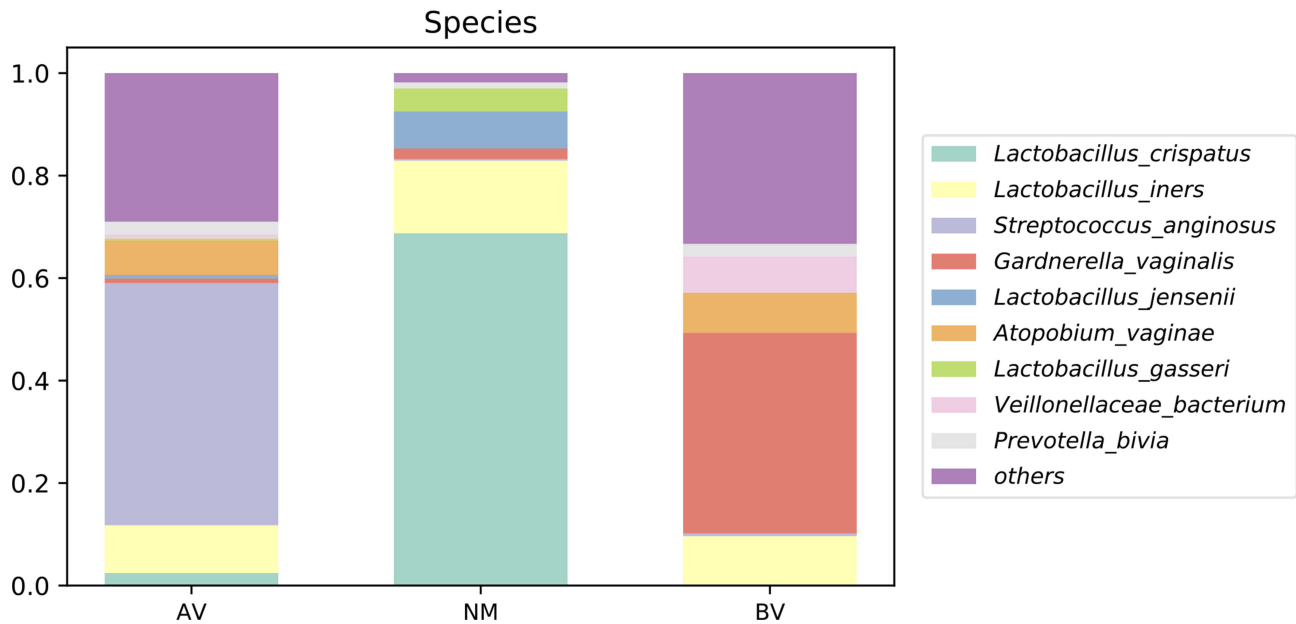


Figure 6 The average abundance of species in AV, NM and BV samples. The total abundance of each group is 1.

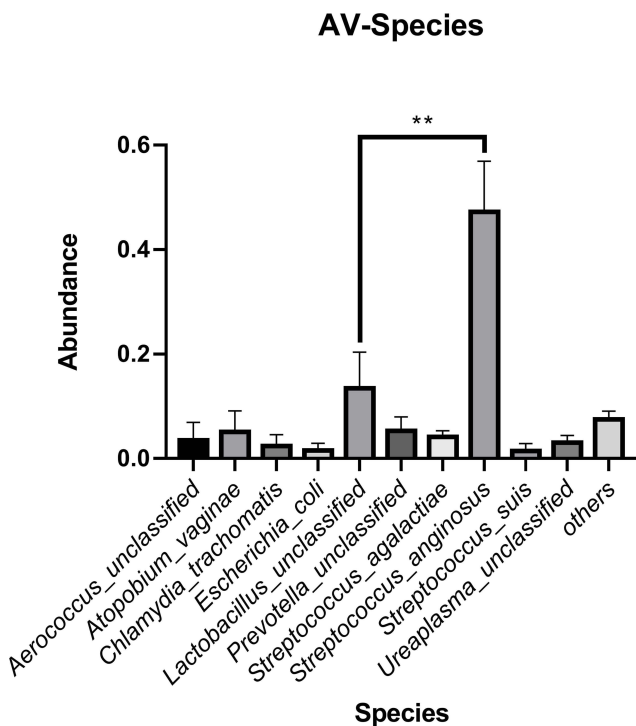


Figure 7 The average abundance of each species in the AV microbiome. *S. anginosus* was significantly more abundant than other species. ** $p < 0.01$.

3' (according to Donders' score criteria), and the treatment of other infections affects the AV score. Therefore, single-cause symptomatic AV needs further attention. We recruited symptomatic women with pure AV caused by

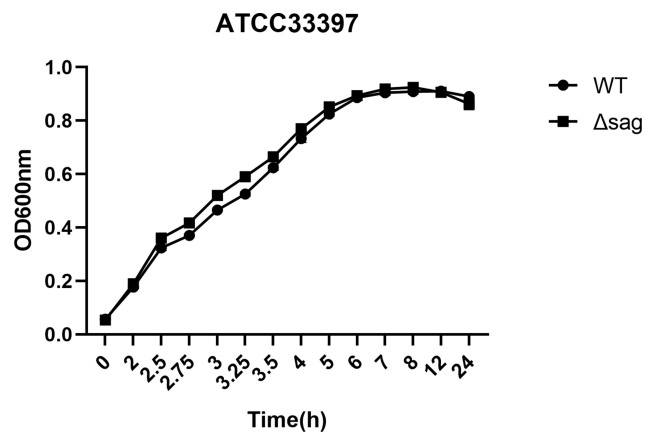


Figure 8 The growth curves of *S. anginosus* (ATCC33397) and the Δ sag mutant.

cocci and found that purulent discharge was present in 90% and itching was present in 70%, which is higher than that of all types of AV.¹

Consistent with a previous study,¹⁵ the 16S rRNA sequencing of symptomatic fertile women with AV in our study showed a dramatic predominance of *Streptococcus* spp. However, at the species level, *S. anginosus* was more abundant than *Streptococcus agalactiae* in AV patients in our study. Relative to previous studies, this is a new discovery.¹⁸

S. anginosus is a commensal strain belonging to the *S. anginosus* group (SAG) in the human oral cavity, genitourinary tract, and gastrointestinal tract that was not recognized as a pathogen until nearly 20 years. Case

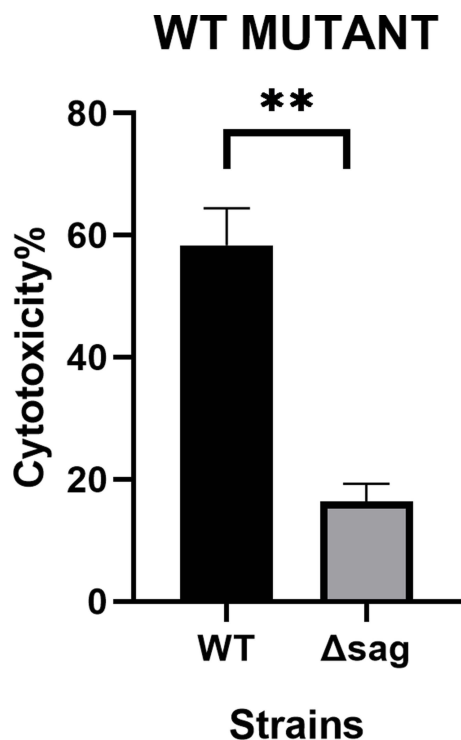


Figure 9 The cytotoxicity of *S. anginosus* (ATCC33397) and the Δ sag mutant was significantly different. ** $p < 0.01$.

reports about *S. anginosus* as an emerging pathogen associated with purulent infections have become increasingly frequent.²² *S. anginosus* accounted for 82% of the 245 patient-unique strains isolated from hospitalized patients by an automated system during a 37-month investigation in a tertiary care teaching hospital in Israel.²³ *S. anginosus* is an opportunistic pathogen that is able to survive in acid conditions,²⁴ adhere to exposed basement membranes of human and cultured epithelial cells,^{25,26} and lyse erythrocytes and some epithelial cell types.²⁷ Streptolysin S (SLS) is an important *S. anginosus* virulence factor encoded by the sag gene cluster, including sagA1/A2, B-I.^{22,27–32} *S. anginosus* ATCC33397 was cytotoxic to vaginal epithelial cells in our study, and the toxicity was significantly reduced by sag gene ablation.

All patients in whom *S. anginosus* was the dominant species, presented purulent discharge, and 5 of these patients complained of itching. Furthermore, all patients found to have vaginal wall reddening during gynaecological examination had a greater abundance of *S. anginosus* than other species. Based on the above results, we hypothesized that *S. anginosus* causes purulent discharge and vaginal wall reddening in patients with AV, which is associated with damage to the vaginal epithelial wall. Furthermore, the pathogenic characteristic of *S. anginosus* matched the AV patient symptoms.

Our results suggest that *S. anginosus* may be a pathogenic organism that causes AV and may be associated with purulent discharge. However, we should collect more symptomatic subjects to verify the predominance and isolate clinical strains to test their pathogenic genes and cytotoxicity. In addition, more research is needed to further indicate the virulence activity of SLS from *S. anginosus* in the pathogenesis of AV.

Conclusion

S. anginosus is the predominant microorganism in patients with symptomatic AV and caused the lysis of vaginal epithelial cells, which indicates that *S. anginosus* is an AV pathogen. The *S. anginosus* virulence gene sag is vital for vaginal epithelial cell lysis.

Ethics Approval

All procedures of this study involving human subjects were reviewed and approved by the Ethics Committee Board of Beijing Tsinghua Changgung Hospital in 2016 (File NO 19201-0-01). We confirm that this study was conducted in accordance with the Declaration of Helsinki.

Acknowledgments

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Author Contributions

All authors contributed to data analysis, drafting or revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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