


# Male Partners of Infertile Couples With Seminal Positivity for Markers of Bacterial Vaginosis Have Impaired Fertility

American Journal of Men's Health  
2018, Vol. 12(6) 2104–2115  
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DOI: 10.1177/1557988318794522  
journals.sagepub.com/home/jmh  


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## Abstract

To access the possibility that key markers of bacterial vaginosis (KM-BV) could affect seminal parameters and thus fertility a prospective cohort study was designed (a) to develop rapid and sensitive multiplex polymerase chain reaction (M-PCR) assays to screen 13 key markers of bacterial vaginosis (KM-BV) in semen specimens, (b) to determine the prevalence of KM-BV in semen from randomized male partners of couples seeking fertility evaluation. A total of 229 semen samples were included in the study from males who visited the Sperm Analysis Section of Brazil between October 2015 and March 2016. Eligible men were 18 years or older and had a semen analysis due fertility evaluation (after failing to conceive with their partner after 1 year of unprotected intercourse). Basic seminal parameters were analyzed, and KM-BV was detected by M-PCR assays. M-PCR assays clearly distinguished 13 KM-BV in 146 semen samples (63.8%), mainly *Gardnerella vaginalis* (50.7%). Some important associations occurred between the presence of KM-BV in semen and changes in seminal parameters. KM-BV is commonly present in the semen of males seeking fertility evaluation and could potentially play significant roles in male subfertility and/or infertility.

## Keywords

semen, infertile couples, bacterial vaginosis markers, seminal parameters

Received May 7, 2018; revised July 12, 2018; accepted July 22, 2018

Infertility is defined as the inability for couples to conceive even after  $\geq 12$  consecutive months of regular unprotected sexual intercourse (Boivin, Bunting, Collins, & Nygren, 2007; Chandra, Coppen, & Stephen, 2013). Infertility is an increasingly common condition, and male factors (exclusively or in combination with female factors) are now estimated to play a significant role in approximately 40%–50% of infertility cases (Carr, 2013; Rittenberg & El-Toukhy, 2010). Despite contemporary therapies, which have undoubtedly increased the likelihood of conception among couples suffering from male infertility, these solutions often overlook the absence of a defined etiological or pathophysiological diagnosis. Unfortunately, male infertility is still considered “idiopathic” in a large proportion of cases (Carr, 2013; Rittenberg & El-Toukhy, 2010; Weng et al., 2014).

Since 2000, the World Health Organization (WHO) recognized the role of genital tract infections in human infertility (Gimenes et al., 2014; Rowe & Comhaire, 2000). Most Sexually transmitted diseases, such as *Chlamydia*

*trachomatis*, *Neisseria gonorrhoeae* and human papillomavirus can target different tissues along the male genital tract, causing functional alterations that can result in reduced fertility or even infertility. In particular, pathological bacterial strains that are present in semen can directly affect sperm quality by reducing motility (Rybar et al., 2012), inducing apoptosis or necrosis (Villegas, Schulz, Soto, & Sanchez, 2005), and altering sperm morphology (Isaiah, Nche, Nwagu, & Nnanna, 2011). Although in the past 6 years it has become accepted that bacterial infections, such as *Chlamydia trachomatis* and *Neisseria*

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*gonorrhoeae* can lead to changes in male fertility (Abusarah, Awwad, Charvalos, & Shehabi, 2013; Isaiah et al., 2011; Pajovic, Radojevic, Vucovik, & Stjepcevic, 2013), little is known about the impact of most bacterial infections on seminal parameters.

Since the beginning of 2000s, it is becoming more evident that countless microorganisms coexist in the human body as part of a complex community known as the microbiome. The microbiome has multiple implications in diverse multifactorial diseases, such as obesity, Crohn's disease, asthma, and infertility (Althani et al., 2016; Franasiak & Scott, 2015; Morgan, Segata, & Huttenhower, 2013). In this scope, the female reproductive tract has been more intensively studied in healthy and diseased individuals. It has been established that *Lactobacillus* spp. have an important role in controlling the overgrowth of other bacteria (Althani et al., 2016; Braundmeier et al., 2001; Franasiak & Scott, 2015). An altered microbiota is associated with bacterial vaginosis (BV), the most common genital tract syndrome in women. BV is associated with an increased risk of perinatal complications (Foxman et al., 2014), pelvic inflammatory disease (Peipert et al., 2001), and HIV acquisition and transmission (Hay, 2014). The etiology of BV is not completely understood, and the syndrome is considered to be an ecological disorder of the vaginal microbiota. BV is characterized by a reduction in lactic acid-producing bacteria (mainly *Lactobacillus*) and an increase in the number and diversity of facultative and strictly anaerobic bacteria, mainly *Gardnerella vaginalis* (Bautista et al., 2016; Hay, 2014; Ravel et al., 2011). Over the past 6 years studies using modern molecular techniques on vaginal specimens have dramatically increased the number of bacterial species known to inhabit the vaginal environment. Many of these bacteria, mainly the anaerobic, have been identified to be key markers for BV (KM-BV) and/or highly associated with BV, including *Atopobium vaginae*; *Bacteroides fragilis*; Clostridium-like bacterial vaginosis-associated bacteria (BVAB) 1, 2, and 3; *Sneathia* spp.; *Megasphaera* type 1; *Mycoplasma hominis*; *Mycoplasma genitalium*; *Mobiluncus* spp.; and *Ureaplasma urealyticum* (Fethers et al., 2012; Foxman et al., 2014; Malaguti, Bahls, Uchimura, Gimenes, & Consolaro, 2015; Shipitsyna et al., 2013; Srinivasan et al., 2012).

Much less attention has been given to the male reproductive system, particularly the characterization of bacterial communities in the seminal plasma (Franasiak & Scott, 2015; Monteiro et al., 2018). It is reasonable to suspect that bacteria involved in female reproductive tract diseases, such as KM-BV, may contribute to male genital microbiota and affect reproductive outcomes. Despite its importance for men and their partners, little is known about the prevalence and role of KM-BV in the male reproductive tract. Furthermore, the significance of

bacteriospermia in asymptomatic men remains unclear. Therefore, this work presents a prospective cohort study (a) to develop rapid and sensitive multiplex PCR assays to screen 13 key markers of BV (KM-BV) in semen specimens, (b) to determine the prevalence of KM-BV in the semen of randomized male partners of couples seeking fertility evaluation, and (c) to access the possibility that KM-BV could affect seminal parameters and thus fertility.

## Materials and Methods

### Study Population

A total of 285 men attended the Sperm Analysis Section of Brazil, between October 2015 and March 2016. As part of a work-up for conjugal infertility investigations (after failing to conceive with their partner after 1 year of unprotected intercourse), all participants had requested semen analysis for medical action. Men who presented with any symptoms of genitourinary infections, had received antibiotics within the previous 3 months, had reproductive system abnormalities (e.g., varicocele), or had undergone infertility therapy in the preceding year were excluded from the study. In addition, subjects who tested positive for *C. trachomatis*, *N. gonorrhoeae*, and HPV were excluded from the study.

All procedures performed in the study were in accordance with the ethical standards of the Committee for Ethics in Research Involving Humans at Brazil (report no. 1163409/2015) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All subjects were aged at least 18 years. Participation was voluntary, and patients were properly informed about the aim of the study. At recruitment, written informed consent was obtained from all study subjects.

### Semen Collection and Analysis

Prior to semen analysis, all subjects were asked to abstain from sexual intercourse or masturbation for 3–5 days before presenting for semen collection in the laboratory. Before semen collection, subjects were instructed to wash their hands with soap. Subjects urinated and washed the glans penis and coronal sulcus with soap and water before samples were collected. Semen was collected via masturbation and ejaculated directly into sterile standard containers previously ascertained to have no cytotoxic effects on human spermatozoa. To prevent sample contamination, contact was avoided with the interior of the sterile wall of the container. Freshly collected semen was immediately incubated at 37°C for 15–60 min for liquefaction. Samples were then homogenized, and 300 µL of

the sample was transferred to tubes containing 1.0 ml of sterile 0.9% NaCl solution. Samples were immediately stored at  $-80^{\circ}\text{C}$  until DNA extraction.

Detection of basic semen parameters was performed according to the WHO (2010) criteria to determine the following variables: seminal volume, pH, viscosity, sperm concentration, vitality, total progressive motility (category [a + b]), rapid progressive motility (category [a]) and morphology (normal forms), leukocytospermia, and hematospermia. Semen was considered to be acidic when pH was  $<$  and alkaline when pH was  $>$  8.2. Hypospermia was defined as seminal volume  $<$  1.5 mL, oligozoospermia was defined as sperm concentration  $<$   $15 \times 10^6/\text{mL}$ , asthenozoospermia was defined as sperm motility  $<$  50% (category [a + b]), necrozoospermia was defined as sperm vitality  $<$  58%, teratozoospermia was defined as normal morphology  $<$  30%, leukocytospermia was defined as leukocyte concentration  $>$   $1 \times 10^6/\text{ml}$ , and hematospermia was defined as erythrocyte concentration  $>$   $1 \times 10^6/\text{ml}$  (WHO, 2010).

### Genomic DNA Extraction and Purification

To remove any polymerase chain reaction (PCR) inhibitors from the semen, samples were incubated for 15 min with proteinase K in phosphate-buffered saline and then centrifuged. Extraction of genomic DNA was performed using a Purelink® Viral RNA/DNA Mini Kit (Invitrogen, CA, USA), according to manufacturer's instructions. Measurement of the quantity and quality of purified DNA was performed by spectrophotometry (NanoDrop 2000 Spectrophotometer, Thermo Scientific, Wilmington, USA).

### Multiplex PCR (M-PCR) Assays

M-PCR assays proposed here were previously developed for use on vaginal specimens (Malaguti, Bahls, Uchimura, Gimenes, & Consolaro, 2015). To adapt and validate these assays for semen specimens, 13 primers were selected based on published papers (Ferris, Mastzal, & Martin, 2004; Fredricks, Fiedler, Thomas, Mitchel, & Marrazzo, 2009; Ingianni, Petruzezzli, Morandotti, & Pompei, 1997; McIver et al., 2009; Muvunyi et al., 2011; Nassar, Abu-Elamreen, Shubair, & Sharif, 2008; Obata-Yasuoka, Ba-Thein, Hamada, & Haiashi, 2002; Riley, Samadpour, & Krieger, 1991; Tiveljung, Forsum, & Monstein, 1996). Specificity was checked against all sequences in the GenBank using SeqSearch, and primers were aligned using the Clustal X program (v. 1.81, NCBI, Bethesda, MD). Subsequently, all primers were evaluated by performing a BLAST analysis against the sequences in NCBI database. The primers were approved when no mismatches within the critical regions of the primers

(e.g., no mismatch at the 3' end of a primer) were identified. All selected primers were designed to have similar physical characteristics to allow for simultaneous amplification in a multiplex reaction without the loss of sensitivity and easy separation using gel electrophoresis as follows: melting temperature  $-55^{\circ}\text{C}$ – $65^{\circ}\text{C}$ ; length 18–26 base pairs (bp), and amplicon size 80–842 bp (Supplementary Table 1).

To assess the specificity of these primers, they were tested in a single-target PCR (sPCR) or an M-PCR assay with different samples. Upon amplification of clinical samples that tested positive for any of the 13 bacteria, no cross-reactivity was observed among the primers.

Different parameters ( $\text{MgCl}_2$  and primers concentrations, annealing and extension temperatures, and number of cycles) were tested in different combinations. Different annealing temperatures for the primers were identified, and the analysis was split into three M-PCR assays. Assay 1 was standardized to detect six KM-BV: *G. vaginalis*, *M. curtisii*, *B. fragilis*, *Mycoplasma hominis*, *U. urealyticum* and *Megasphaera* type 1. Assay 2 was standardized to detect three KM-BV: *A. vaginae*, BVAB1, and BVAB2. Assay 3 was standardized to detect four KM-BV: *M. mulieris*, BVAB3, *S. sanguinegens*, and *Mycoplasma genitalium*.

The optimized protocol for each M-PCR assay was a mixture of 25  $\mu\text{L}$  containing 2.5 mM aliquots of each dNTP, 0.6 mM  $\text{MgCl}_2$ , 25 mM aliquots of each primer, 5  $\mu\text{L}$  of extracted DNA (50 ng of total sample), and 1 U of platinum Taq DNA polymerase (Invitrogen, CA, USA). PCR conditions comprised 35 amplification cycles of denaturation for 10 min at  $94^{\circ}\text{C}$ , annealing for 1 min with variable temperature depending on the assay ( $55^{\circ}\text{C}$ ,  $62^{\circ}\text{C}$ , and  $63^{\circ}\text{C}$  for assays 1, 2, and 3, respectively), and extension for 1 min at  $72^{\circ}\text{C}$  and a final extension for 10 min at  $72^{\circ}\text{C}$  (Thermal cycler, Biosystem, CA, USA). Resulting M-PCR products were electrophoresed in 8% polyacrylamide gel using 1X TBE buffer (45 mM Tris-borate, 1 mM EDTA, pH 8.0). All gels were stained after running with 1 mg/mL ethidium bromide and photographed under UV light (UVP BioImaging Systems, Upland, CA, USA).

### Single-Target PCR (sPCR) Assays

For standardization and validation of the detection by M-PCR, sPCR was also performed for the 13 bacteria in all samples studied and in positive controls using the same primers used for the M-PCR. sPCR (the gold standard) is generally more sensitive than M-PCR, and it prevents cross-reactions, which can occur during M-PCR (McIver et al., 2009). The sPCR assay comprised 15  $\mu\text{L}$  containing 2.5 mM aliquots of each dNTP, 0.6 mM of  $\text{MgCl}_2$ , 25 mM aliquots of each primer, 5  $\mu\text{L}$  of extracted DNA (50 ng), and 1 U of Taq DNA polymerase (Invitrogen, CA, USA). sPCR conditions were kept identical to those in M-PCR

assays 1, 2, and 3. Amplification fragments were electrophoresed in a 1.5% agarose gel stained with 1 mg/mL ethidium bromide.

Co-amplification of the human  $\beta$ -globin gene using specific primers GH20 (5'-GAAGAGCCAAGG-ACAGGTAC-3') and PC04 (5'-CAACTTCATCCAC GTTCACC-3') was performed by sPCR and M-PCR for all semen and control samples. Co-amplification of the  $\beta$ -globin gene was performed for assessing inhibition, sample adequacy, and integrity. In particular, it was used as an internal control for amplification to ensure that amplifiable DNA was successfully extracted from the samples and controls. Finally, it was used to check for PCR inhibitors (Fredricks & Relman, 1999) under the same conditions as M-PCR and sPCRs.

Two types of controls, "no-DNA" (negative control) and "positive DNA" (positive control), were also included in each reaction series (M-PCR, sPCR, and  $\beta$ -globin PCR). These controls were run for all studied KM-BV that were derived from positive clinical samples previously detected and identified using reference methods, including culture and/or sPCR.

### Statistical Analysis

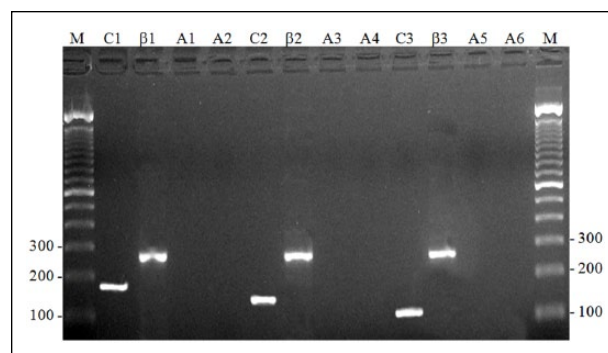
For validation of M-PCR assays, was calculated the sensitivity [(true positive/(true positive + false negative))\*100], specificity {[true negative/(true negative + false positive)]\*100}, positive predictive value {[true positive/(true positive + false positive)]\*100}, and negative predictive value {[true negative/(true negative + false negative)]\*100}. Then, the results from M-PCR were compared with those from sPCR (gold standard).

Seminal parameters were compared between total KM-BV-positive and -negative groups using the  $\chi^2$  test. Mean values for age and seminal parameters were compared in the total study population and in strata of KM-BV presence. KM-BV-negative and mutually exclusive KM-BV-positive subgroups were compared by one-way analysis of variance (ANOVA). Two-sided  $p \leq .05$  was considered statistically significant. Statistical analysis was performed using GraphPad Prism 6.0 (San Diego, CA, USA).

### Results

After application of the inclusion and exclusion criteria of patients for the study, which have been described previously, a total of 229 men were included. The patient age range was 18–52 years, with a mean age and standard deviation of  $32.87 \pm 6.6$  years.

For validation of M-PCR assays, 81 semen samples were used. The M-PCR assay clearly distinguished and correctly identified KM-BV analyzed in semen samples,



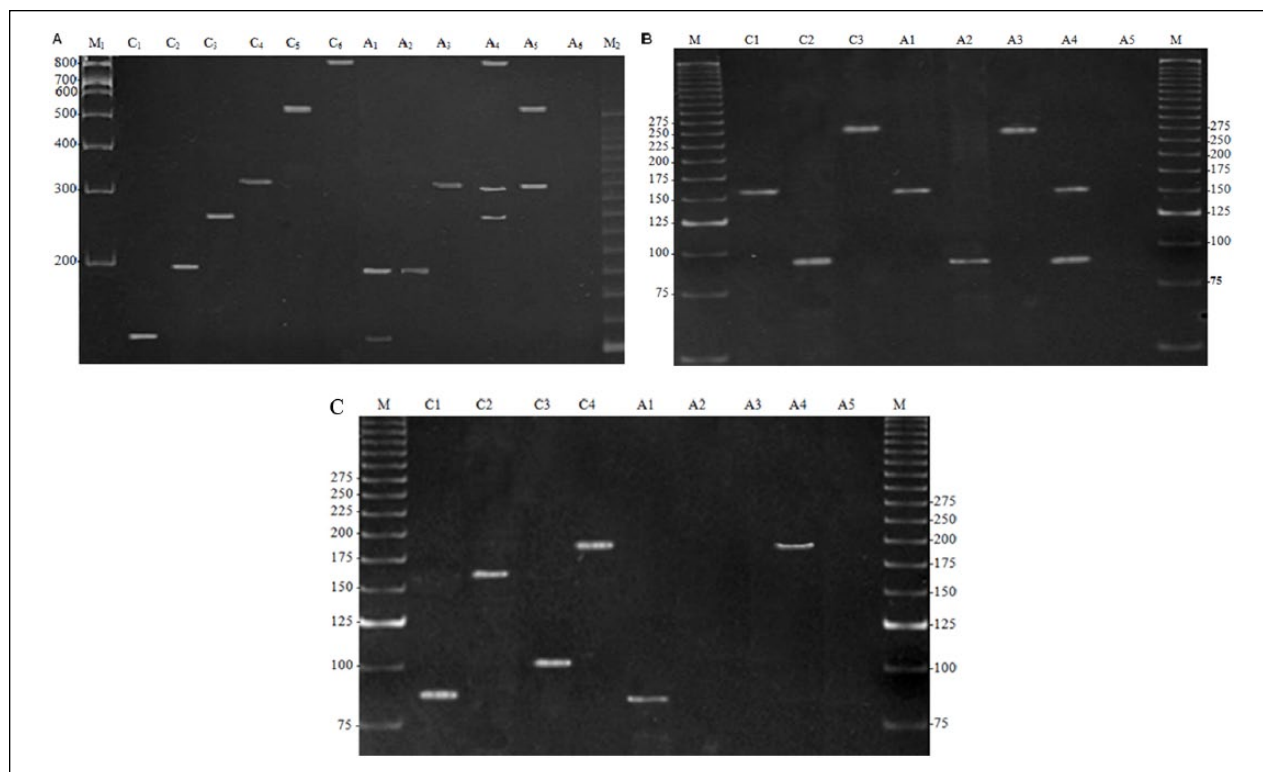
**Figure 1.** Electrophoretic analysis of amplified fragments using M-PCR in 2% agarose gel.

C1, positive control for *Mycoplasma genitalium* (193 base pairs-bp);  $\beta$ 1, *M. genitalium*-positive  $\beta$ -globin (268 bp); A1, negative sample for *M. genitalium*; A2, negative control; C2, positive control for BVAB3 (160 bp);  $\beta$ 2, BVAB3-positive  $\beta$ -globin (268 bp); A3, negative sample for BVAB3; A4, negative control; C3, positive control for *Sneathia sanguinegens* (102 bp);  $\beta$ 3, *S. sanguinegens*-positive  $\beta$ -globin (268 bp); A5, negative sample for *S. sanguinegens*; A6, negative control; and M, molecular weight marker (100 bp).

whether single agent (1 KM-BV) or coinfection ( $\geq 2$  KM-BV). In addition, false positive results were not detected. BVAB3 and *S. sanguinegens* were not detected in semen samples analyzed in the study, but their positive controls and  $\beta$ -globin were correctly amplified in M-PCR and sPCR assays (Figure 1). Thus, parameters of the M-PCR assay were established considering positive controls for both bacteria. Figure 1 also shows amplification of the positive control and  $\beta$ -globin of *M. genitalium*, which was detected in a small number of samples in the present study. The overall agreement of M-PCR results with sPCR was 99.3%; validation parameters were as follows: 96.79% sensitivity, 100.00% specificity, 100.00% PPV, 99.78% NPV, and 99.80% ACC (Supplementary Table 2).

When the agents were individually analyzed, M-PCR reported to have 100.0% specificity and PPV for all bacterial agents. For *M. curtisii*, *M. hominis*, *G. vaginalis*, *B. fragilis*, *A. vaginae*, BVAB1, BVAB2, BVAB3, *Mycoplasma genitalium*, *M. mulieris*, and *S. sanguinegens*, M-PCR values of 100% were observed for all other parameters. For both *U. urealyticum* and *M. type 1*, M-PCR showed 98.60% NPV and 98.70% ACC. These bacteria differed in sensitivity; 83.30% for *U. urealyticum* and 75.0% for *M. type 1*. In four semen samples (9.5%), coinfections were detected in both M-PCR and sPCR assays (100% agreement with sPCR) (Supplementary Table 2). Figure 2A–C shows M-PCR amplification fragments of positive semen samples for different KM-BV in an 8% polyacrylamide gel.

KM-BVs were detected in 146 of the 229 semen samples (63.8%), as a single agent or in coinfections. The



**Figure 2.** Electrophoretic analysis of amplified fragments using multiplex polymerase chain reaction (M-PCR) in 8% polyacrilamide gels.

(A) Assay 1. Positive Controls: C1, *Mobiluncus curtisii* (130 base pairs–bp); C2, *Megasphaera* type I (211 bp); C3: *Mycoplasma hominis* (270 bp); C4, *Gardnerella vaginalis* (330 bp); C5, *Ureaplasma urealyticum* (541 bp); C6, *Bacteroides fragilis* (842 bp); A1, positive sample for *M. type I* (211 bp) and *M. curtisii* (130 bp); A2, positive sample for *M. type I* (211 bp); A3, positive sample for *G. vaginalis* (330 bp); A4, positive sample for *B. fragilis* (842 bp), *G. vaginalis* (330 bp) and *M. hominis* (270 bp); A5, positive sample for *U. urealyticum* (541 bp) and *G. vaginalis* (330 bp); A6; negative control; M1, molecular weight marker (100 bp); and M2, molecular weight marker (25 bp). (B) Assay 2. Positive Controls: C1, *Atopobium vaginae* (155 bp); C2, BVAB1 (90 bp); C3, BVAB2 (260 bp); A1, positive sample for *A. vaginae* (155 bp); A2, positive sample for BVAB1 (90 bp); A3, positive sample for BVAB2 (260 bp); A4, positive sample for BVAB1 (90 bp) and *A. vaginae* (155 bp); A5, negative control; and M, molecular weight marker (25 bp). (C) Assay 3. Positive Controls: C1, *Mobiluncus mulieris* (80 bp); C2, BVAB3 (160 bp); C3, *Sneathia sanguinegens* (102 bp); C4, *M. genitalium* (193 bp); A1, positive sample for *M. mulieris* (80 bp); A2, negative sample for BVAB3; A3, negative sample for *S. sanguinegens*; A4, positive sample for *M. genitalium*; A5, negative control; and M: molecular weight marker (25 bp).

most prevalent KM-BV was *G. vaginalis* ( $n = 116$  samples, 50.7%) as a single agent ( $n = 10$ , 43.6%) and in coinfections ( $n = 14$ , 6.11%). Exclusive infection by KM-BV other than *G. vaginalis* occurred ( $n = 25$ , 10.92%) (Table 1) as follows: *M. curtisii* ( $n = 12$ , 5.24%); *U. urealyticum* ( $n = 9$ , 3.93%); *M. type 1* and *M. hominis* ( $n = 7$ , 3.10% each); *M. mulieris* ( $n = 4$ , 1.80%); BVAB2, *A. vaginae*, and *B. fragilis* ( $n = 3$ , 1.31% each); and *M. genitalium* and BVAB1 ( $n = 2$ , 0.90% each). As previously stated, BVAB3 and *S. sanguinegens* were not detected (Table 2).

KM-BVs were detected as a single agent in 127 semen samples (55.5% of total samples and 87% of total KM-BV-positive samples). KM-BV coinfections were detected in 19 semen samples (8.6% of total semen samples and 13.01% of KM-BV-positive samples; Table 2).

Table 3 displays the correlation between KM-BV-positive semen samples and adverse effects on seminal

**Table 1.** Presence of KM-BV in Total Semen Samples ( $n = 229$ ).

KM-BV in semen	<i>n</i>	Percentage (%) <sup>a</sup>
Total KM-BV	146	63.70
Exclusively <i>G. vaginalis</i>	102	44.50
Exclusively other KM-BV non <i>G. vaginalis</i>	25	10.92
Only one BV-TM	127	55.50
BV-TM coinfections	19	8.30

Note. KM-BV = key markers of bacterial vaginosis. <sup>a</sup>Percentage not sum to total due to rounding.

parameters. It also presents results of bacteria that individually showed association with semen alterations. Total KM-BV-positive samples showed a tendency to be correlated with teratozoospermia ( $p = .08$ ). Individual KM-BV bacteria that showed significant association with some

**Table 2.** Total Individual or Simultaneous KM-BV Detected by M-PCR Assays.

KM-BV detected	Overall positivity	In total semen samples (n = 229)	In all KM-BV positive semen samples (n = 146)
	n	(%)	(%)
<b>Overall KM-BV detection</b>	<b>146</b>	<b>63.7</b>	<b>100.0</b>
<i>Gardnerella vaginalis</i>	116	50.65	79.5
<i>Mobiluncus curtisii</i>	12	5.24	8.20
<i>Ureaplasma urealyticum</i>	9	3.93	6.20
<i>Mycoplasma hominis</i>	7	3.10	4.80
<i>Megasphaera</i> type I	7	3.10	4.80
<i>Mobiluncus mulieris</i>	4	1.80	2.70
<i>Atopobium vaginae</i>	3	1.31	2.0
BVAB2	3	1.31	2.0
<i>Bacteroides fragilis</i>	3	1.31	2.0
<i>Mycoplasma genitalium</i>	2	0.90	1.40
BVAB1	2	0.90	1.40
<b>KM-BV single infection</b>	<b>127</b>	<b>55.50</b>	<b>87.00</b>
<i>Gardnerella vaginalis</i>	102	44.50	70.00
<i>Mobiluncus curtisii</i>	7	3.06	4.80
<i>Mycoplasma hominis</i>	5	2.20	3.42
<i>Ureaplasma urealyticum</i>	4	1.75	2.74
<i>Mobiluncus mulieris</i>	4	1.75	2.74
<i>Megasphaera</i> type I	2	0.87	1.37
<i>Bacteroides fragilis</i>	2	0.87	1.37
<i>Mycoplasma genitalium</i>	1	0.44	0.68
<b>KM-BV coinfections</b>	<b>19</b>	<b>8.6</b>	<b>13.01</b>
<i>Gardnerella vaginalis</i> + <i>Mobiluncus curtisii</i>	4	1.75	2.74
<i>Gardnerella vaginalis</i> + <i>Megasphaera</i> type I	4	1.75	2.74
<i>Gardnerella vaginalis</i> + <i>Ureaplasma urealyticum</i>	3	1.31	2.05
<i>Gardnerella vaginalis</i> + <i>Atopobium vaginae</i>	1	0.44	0.68
<i>Ureaplasma urealyticum</i> + <i>Mycoplasma hominis</i>	1	0.44	0.68
<i>Ureaplasma urealyticum</i> + BVAB1	1	0.44	0.68
BVAB1 + <i>Atopobium vaginae</i>	1	0.44	0.68
BVAB2 + <i>Mobiluncus curtisii</i>	1	0.44	0.68
<i>Gardnerella vaginalis</i> + <i>Bacteroides fragilis</i> + <i>Mycoplasma hominis</i>	1	0.44	0.68
<i>Gardnerella vaginalis</i> + <i>Megasphaera</i> type I + BVAB2	1	0.44	0.68
<i>Atopobium vaginae</i> + BVAB2 + <i>Mycoplasma genitalium</i>	1	0.44	0.68

Note. M-PCR = multiplex polymerase chain reaction; KM-BV = key markers of bacterial vaginosis; BVABs 1, 2 and 3 = bacterial vaginosis-associated bacteria 1, 2, and 3.

abnormal semen parameters were as follows: *G. vaginalis* was associated with increased seminal viscosity ( $p = .03$ ), *B. fragilis* was associated with reduced seminal viscosity ( $p = .04$ ) and with increased seminal coagulation time ( $p = .003$ ), *U. urealyticum* was associated with teratozoospermia ( $p = .022$ ), and *M. mulieris* was associated with decreased seminal viscosity ( $p = .01$ ) ( $\chi^2$  test, Table 3).

Table 4 presents mean participant ages and seminal parameters in the total study population and in strata by

KM-BV positivity (total KM-BV-negative samples and mutually exclusive KM-BV-positive subgroups). The presence of KM-BV was not related to the mean age of the participants. The following significant associations were observed: *B. fragilis* was associated with increased coagulation time ( $p = .0001$ ); *A. vaginae* was associated with decreased coagulation time ( $p = .0001$ ); and BVAB1 was associated with increased mean semen pH ( $p = .006$ ), increased coagulation time ( $p = .0001$ ), and

**Table 3.** Correlation Between Testing KM-BV Positivity and Changes to Seminal Parameters.

Variables	Total KM-BV				G. vaginalis				B. fragilis				U. urealyticum				M. mulieris			
	+		-		+		-		+		-		+		-		+		-	
	n(%)	n(%)	n(%)	n(%)	p value	n(%)	n(%)	p value	n(%)	n(%)	p value	n(%)	n(%)	p value	n(%)	n(%)	p value	n(%)	n(%)	p value
Hypo spermia	11 (7.4%)	10 (12%)	6 (5.1%)	15 (13.2%)	ns	0 (0%)	21 (9.3%)	ns	0 (0%)	21 (9.3%)	ns	0 (0%)	21 (9.5%)	ns	0 (0%)	21 (9.3%)	ns	0 (0%)	21 (9.3%)	ns
Low Viscosity	23 (15.7%)	17 (20.5%)	6 (5.1%)	34 (30.1%)	ns	3 (100%)	37 (16.3%)	.04*	2 (22%)	38 (17%)	ns	4 (100%)	36 (16%)	ns	4 (100%)	36 (16%)	ns	4 (100%)	36 (16%)	.01*
High Viscosity	27 (18.5%)	10 (12%)	26 (22%)	11 (9.7%)	.03*	0 (0%)	37 (16.4%)	ns	1 (11%)	36 (16%)	ns	0 (0%)	36 (16%)	ns	0 (0%)	37 (16.4%)	ns	0 (0%)	37 (16.4%)	ns
pH	2 (1.3%)	1 (1.2%)	2 (1.7%)	1 (0.9%)	ns	0 (0%)	3 (1.3%)	ns	0 (0%)	3 (1.3%)	ns	0 (0%)	3 (1.3%)	ns	0 (0%)	3 (1.3%)	ns	0 (0%)	3 (1.3%)	ns
Abnormal coagulation	3 (2%)	2 (2.4%)	2 (1.7%)	3 (2.6%)	ns	2 (66%)	3 (1.32%)	.003*	0 (0%)	5 (2.3%)	ns	1 (25%)	4 (1.77%)	ns	1 (25%)	4 (1.77%)	ns	1 (25%)	4 (1.77%)	ns
Low Progressive motility	54 (37%)	26 (31%)	41 (35%)	39 (34.5%)	ns	0 (0%)	80 (35.4%)	ns	6 (66%)	74 (33%)	ns	1 (25%)	79 (35%)	ns	1 (25%)	79 (35%)	ns	1 (25%)	79 (35%)	ns
Necro spermia	63 (43%)	33 (40%)	46 (39%)	50 (44%)	ns	3 (100%)	93 (41.1%)	ns	7 (77%)	89 (40%)	ns	2 (50%)	98 (43%)	ns	2 (50%)	98 (43%)	ns	2 (50%)	98 (43%)	ns
Oligozoospermia	41 (28%)	21 (25%)	32 (27%)	30 (26%)	ns	1 (33%)	61 (27%)	ns	4 (44%)	58 (26%)	ns	0 (0%)	62 (27.5%)	ns	0 (0%)	62 (27.5%)	ns	0 (0%)	62 (27.5%)	ns
Leukospermia	10 (6.84%)	2 (2.4)	8 (6.8%)	4 (3.5%)	ns	0 (0%)	12 (5.3%)	ns	0 (0%)	12 (5.4%)	ns	0 (0%)	12 (5.4%)	ns	0 (0%)	12 (5.3%)	ns	0 (0%)	12 (5.3%)	ns
Hematospermia	1 (0.6%)	3 (2.9%)	1 (0.8%)	3 (2.6%)	ns	0 (0%)	4 (1.7%)	ns	0 (0%)	4 (1.8%)	ns	0 (0%)	4 (1.8%)	ns	0 (0%)	4 (1.8%)	ns	0 (0%)	4 (1.8%)	ns
Teratospermia	27 (18.5%)	7 (8.4%)	19 (16.4%)	15 (13.2%)	.08	0 (0%)	34 (15%)	ns	5 (55%)	29 (13.2%)	.022*	0 (0%)	34 (15.1%)	ns	0 (0%)	34 (15.1%)	ns	0 (0%)	34 (15.1%)	ns

Note. KM-BV, key marker of bacterial vaginosis; ns: not statistically significant; +, positive; -, negative. Present data are not means. p values obtained by  $\chi^2$  test.  
\*p values < .05 were considered statistically significant.

**Table 4. Mean Participant Age Seminal Parameters in the Study Population (n = 229) and in Strata by KM-BV Positivity.**

Semen parameters	Total KM-BV+ (n = 146; 63.7%)	<i>G. vaginalis</i> + (n = 116; 50.65%)	<i>M. curvispiris</i> + (n = 12; 5.24%)	<i>B. fragilis</i> + (n = 3; 1.31%)	<i>M. hominis</i> + (n = 7; 3.10%)	<i>U. urealyticum</i> + (n = 9; 3.93%)	<i>M. type 1</i> + (n = 7; 3.10%)	<i>A. vaginae</i> + (n = 3; 1.31%)	BVABI+ (n = 2; 0.90%)	BVAB2+ (n = 3; 1.31%)	<i>M. miferis</i> + (n = 4; 1.80%)	<i>M. genitalium</i> + (n = 2; 0.90%)	Total KM-BV- (n = 86; 36.3%)	p
Age (y)	33.8 (32.8–34.9)	34.5 (33.4–35.7)	28.6 (25.5–31.8)	32 (12.1–51.8)	35.4 (29.7–41.1)	35.2 (31.4–39)	29.5 (25.9–33.2)	26.3 (8.9–43.7)	26.5 (8.8–49.1)	27.3 (15.5–39)	31.2 (24.3–38.1)	26 (6.2–11.5)	32.4 (31–33.9)	.13
Volume of Semen (ml)	3.5 (0.3–3.8)	3.8 (3.5–4.1)	3.4 (2.4–4.3)	2.4 (0.8–4)	3.5 (1.5–5.3)	2.7 (2.1–3.2)	2.6 (1.2–4)	2.4 (0.6–5.4)	1.5 (0.23–5.3)	2 (0.6–3.5)	2.6 (0.9–4.3)	2.8 (1.8–23.8)	3.5 (3.1–3.8)	.058*
pH	7.7 (7.6–7.7)	7.6 (7.6–7.7)	7.6 (7.4–7.7)	7.8 (7–8.7)	7.8 (7.4–8.1)	7.8 (7.5–8.1)	7.7 (7.4–8)	7.8 (7.2–8.5)	8.1 (7.5–8.7)	7.7 (6.5–9)	8.1 (7.8–8.2)	8.2 (6.9–9.4)	7.7 (7.6–7.8)	.006*
Sperm (million/ml)	36 (31.2–42.3)	32 (26–37)	38 (11.5–65.2)	52.6 (12.4–86.2)	48.2 (19.2–77.4)	39.9 (26.2–77.2)	58 (26.3–89.7)	38.7 (12–97)	51.2 (5.3–63.2)	90.6 (65.2–116)	55.8 (42.9–154)	57.3 (44.6–97)	32.5 (24.1–40.8)	.15
Progressive motility (%)	43.5 (39.6–47.4)	45.7 (41.1–50.3)	37.1 (18.3–56)	41.6 (27.3–56)	42.5 (17.7–67.3)	28.3 (7.3–49.3)	48.8 (31.4–66.2)	32 (1.53–79.3)	32.5 (1.89–50.1)	53 (18.8–87.1)	46.2 (22.4–70.1)	37.5 (1.2–98.7)	43.4 (37.8–49.1)	.9
Vitality (%)	58.1 (53.7–62.3)	60.2 (55.2–65.3)	55.2 (34.3–76.1)	51 (41–60.9)	58.4 (35.3–81.5)	40 (18–62)	64.2 (43.9–84.6)	53 (3.3–102)	48 (26.1–96)	71 (42.3–99.6)	55.7 (26.6–84.8)	51 (1.9–99.7)	57.2 (50.9–63.6)	.8
Coagulation time (min)	25.1 (24.7–25.5)	25 (24.7–25.3)	24.6 (23.6–25.7)	30 (14.5–45.5)	24.7 (32.1–26.2)	24.3 (23.3–25.3)	24.5 (21.6–27.5)	20 (2–37.9)	18.5 (0.6–101)	25 (2.5–25)	28.5 (15.5–41.5)	20 (0.8–28)	25.1 (24.7–25.5)	.0001*
Leukospermia (10 <sup>6</sup> /ml)	357.3 (278.6–436)	530.9 (279.5–782.3)	320 (120.7–386.8)	83.3 (11.6–155)	264.3 (107–421.6)	320 (224.3–415.7)	287.5 (157.7–417.3)	300 (–130.3–730.3)	175 (–778–1128)	350 (134.9–565.1)	100 (0–0)	150 (–485.3–785.3)	404 (329.5–478.6)	.8

Note. KM-BV, key markers of bacterial vaginosis. Presented data are means (95% CI); p value was obtained by one-way ANOVA, comparing the negative group (control) with the positive mutually excluded groups. \*p values <.05 were considered statistically significant.



decreased mean seminal volume ( $p = .058$ ). *M. mulieris* was associated with increased mean semen pH ( $p = .006$ ). *M. genitalium* was associated with increased mean semen pH ( $p = .006$ ) and increased coagulation time ( $p = .0001$ ) (one-way ANOVA, Table 4).

## Discussion

To the best of the knowledge, this study was the first to use M-PCR assays for determining the prevalence of several KM-BV in the semen of randomized male partners of couples seeking fertility evaluation. It was also the first to examine the possibility that KM-BV could affect seminal parameters and thus fertility. The results presented high prevalence of KM-BV, particularly *G. vaginalis*, in semen. Furthermore, were demonstrated some important associations between the presence of KM-BV in semen and changes in seminal parameters. These changes potentially play significant roles in male subfertility and/or infertility.

In total, a high prevalence of KM-BV was detected in semen samples from male partners of infertile couples (63.8%). In addition, individual analysis of each KM-BV revealed that 50.6% of the total samples were positive for *G. vaginalis*, as a single infection or via coinfections. This prevalence was much higher than that described previously by De Francesco, Negrini, Ravizzola, Galli, and Manca (2011) which reported that *G. vaginalis* was also the most prevalent bacterium in their semen culture; however, it was recorded in only 6.32% samples. Andrade-Rocha (2009) also detected *G. vaginalis* at a lower prevalence (22.2%) than this study. However, contrary to the results presented here, De Francesco et al. (2011) reported significant association with the presence of leukocytes only in patients with seminal infection by *G. vaginalis*. Additionally, these authors reported reduction in sperm concentration, motility, and morphology compared with the controls with *G. vaginalis* infection. Similarly, this study identified an association between total KM-BV and teratozoospermia and an association between *U. urealyticum* infection and teratozoospermia. However, an association of teratozoospermia specifically with *G. vaginalis* infection was not observed.

Furthermore, substantial research has associated *U. urealyticum* and *Mycoplasma hominis* infections with deterioration of semen parameters (De Francesco et al., 2011; Potts et al., 2000; Reichart, Kahane, & Bartoov, 2000). In a report by Gdoura et al. (2007) the prevalence of mixed species of mycoplasmas and ureaplasmas in semen was 6.7% comparable to that demonstrated in this study. However, when comparing infected and uninfected samples, Al-Sweih, Al-fadli, Omu, and Rotimi (2012) showed an association between *Mycoplasma hominis* infection and higher leukocyte count, lower sperm

vitality, and reduced progressive motility, respectively. These associations were not demonstrated in this study.

Two other studies support the hypothesis that *G. vaginalis* may negatively influence seminal fertility parameters. Virecoulon et al. (2005) identified that *G. vaginalis* colonizes semen from men attending infertility clinics in high proportions (44%). Mändar, (2013) reported that the predominance of *G. vaginalis* in the female partner was significantly related to inflammation in the male genital tract. On the other hand, the results presented here indicated that *G. vaginalis* semen positivity may be associated with increased seminal viscosity, suggesting that the infection affects prostate function and alters coagulation–liquefaction of semen.

The second most frequent KM-BV was *M. mulieris* (5.26%). *M. curtisii* was detected at a much lower prevalence (1.4%). To the best of the knowledge, this was the first molecular analysis of *M. curtisii* and *M. mulieris* in semen samples. The results indicated that *M. mulieris* semen infection is correlated with decreased seminal viscosity and increased mean pH. This suggests that *M. mulieris* disrupts function of seminal vesicles and is responsible for alterations in the coagulation–liquefaction of semen, as observed in *G. vaginalis* infection.

*U. urealyticum* was detected in 4.56% samples, *M. type 1* in 3.85% samples, *Mycoplasma hominis* in 2.8%, BVAB2 and *M. mulieris* in 1.4% each, *B. fragilis* and *A. vaginae* in 1.05% each, and BVAB1 in 0.7% of all samples. Furthermore, other KM-BV that showed an association with impairment of seminal parameters were as follow: *B. fragilis* with reduced seminal viscosity and increased coagulation time; BVAB1 with increased mean semen pH, increased coagulation time, and reduced mean seminal volume; *M. genitalium* with increased mean semen pH and increased coagulation time; and *A. vaginae* with decreased coagulation time.

A few important points deserve further discussion. Increased viscosity and abnormal coagulation times observed in *B. fragilis* infections may be due to prostatic gland infection. Benway and Moon (2008) previously reported isolation of *B. fragilis* in patients with prostatic abscesses. Therefore, among KM-BV, it may be important to study *B. fragilis* in isolation. *Atopobium* spp. was not associated with urethritis but was detected at a higher frequency (8.1%) by Manhart et al. (2013) than in this study (1.31%). *Megasphaera* spp. was only detected in men with urethritis, but at a lower frequency (0.4%) than presented in this study (3.10%).

Although total prevalence of KM-BVs in semen was high, the low presence of non-*G. vaginalis* bacteria may have influenced the results of this study. Furthermore, even though positive controls and the  $\beta$ -globin gene were correctly amplified in M-PCR and sPCR assays, BVAB3 and *S. sanguinegens* were not detected in the analyzed

semen samples. This suggests that negative results for BVAB3 and *S. sanguinegens* were true negatives. Contrary, Manhart et al. (2013) detected *Sneathia* spp. and BVAB3 in semen samples. In their study, infection with *Sneathia* spp. was significantly associated with urethritis and BVAB3 was only detected in men with urethritis. To establish the effects of low prevalence non-*G. vaginalis* KM-BVs in male subfertility or infertility, it is necessary that future studies have a greater sample size.

Overall, the results presented here indicate most KM-BVs, as single infections or via coinfections, seem to affect the sperm and seminal fluid, that is, two main components related to male fertility. In particular, male fertility depends on two major components of semen: healthy spermatozoa (as measured by vitality, motility, and morphology) and composition of the seminal fluid, which is important for sperm function. Secretory products from seminal vesicles and the prostate are crucial for sperm motility, viability, and chromatin stability. They are very important for semen coagulation and liquefaction. Seminal vesicles secrete the major fraction of the ejaculate ( $\approx 60\%$ ) (WHO, 2010) and produce compounds, such as fructose, semenogelin-I (the predominant component of the coagulum), and sperm motility stimulators, that play significant roles in seminal physiology (Bukovsky, Thaler, & McIntyre, 1991; Gonzales, 2001; Robert & Gagnon, 1995). Seminal hyper-viscosity suggests deficient secretory activity in the seminal vesicles. The prostate secretes 30%–35% of the ejaculate and produces several compounds that are available for analysis in the seminal plasma, including enzymes for semen liquefaction. Reduced levels of prostate markers are indicative of glandular dysfunction, often associated with abnormalities in pH ( $\geq 7.8$ ), volume (decreased or increased), liquefaction, and/or viscosity (WHO, 2010).

Considering the findings of this study and the current evidence in literature, it can be reasonably hypothesized the mechanisms underlying the effects of KM-BV on the spermatozoa and seminal fluid: (a) bacterial gametotoxicity hampering spermatogenesis, increasing the number of apoptotic cells, and altering the sperm morphology; (b) inflammatory response to KM-BV causing pathologic lesions, leading to seminal vesicle and/or prostate dysfunction and changes in seminal fluid composition; (c) bacterial colonization and/or infection inducing an immune response against KM-BV that may cross-react with the host's tissues affecting male genital tract, spermatogenesis, and seminal fluid; and (d) bacterial activity resulting in epigenetic and DNA damage during spermatogenesis, among many other reasons.

Limitations of this study include absence of data on mixed antiglobulin reaction testing, which was not conducted during each primary semen analysis. This test was performed upon the physician's request only. Second, as

this study did not include an analysis of medical records, it does not include data on the female partner's characteristics, such as age, hormone levels, tubes, and pelvic status. In addition, other male partner characteristics, such as clinical analysis of the prostate and seminal vesicles, are not included. Furthermore, semen results were taken from only one semen analysis per subject and did not include a control group of normally fertile subjects. Finally, although the detection of KM-BV was associated with changes in seminal parameters, this association happened with a small number of patients in each subgroup. However, these results may encourage further studies to evaluate the influence of these 13 KM-BV on semen and changes in seminal parameters.

## Conclusions

This study identified that KM-BV is commonly present in the semen of males seeking fertility evaluation. Furthermore, were demonstrated some important associations between the presence of KM-BV in semen and changes in seminal parameters. Therefore, screening for KM-BV in the semen should be considered as a diagnostic tool in cases of unexplained infertility, assisted reproduction, and for semen donation to prevent unnecessary bacterial transmission to the female genital tract.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by grants from the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Brazilian Government.

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