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# Identification of novel microbes associated with pelvic inflammatory disease and infertility

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## ABSTRACT

**Objectives** As pelvic inflammatory disease (PID) aetiology is not completely understood, we examined the relationship between select novel bacteria, PID and long-term sequelae.

**Methods** Fastidious bacterial vaginosis (BV)-associated bacteria (*Sneathia (Leptotrichia) sanguinegens*, *Sneathia amnionii*, *Atopobium vaginae* and BV-associated bacteria 1 (BVAB1)), as well as *Ureaplasma urealyticum* and *Ureaplasma parvum* were identified in cervical and endometrial specimens using organism-specific PCR assays among 545 women enrolled in the PID Evaluation and Clinical Health study. Risk ratios and 95% CIs were constructed to determine associations between bacteria, histologically confirmed endometritis, recurrent PID and infertility, adjusting for age, race, gonorrhoea and chlamydia. Infertility models were additionally adjusted for baseline infertility.

**Results** Persistent detection of BV-associated bacteria was common (range 58% for *A. vaginae* to 82% for BVAB1) and elevated the risk for persistent endometritis (RR<sub>adj</sub> 8.5, 95% CI 1.6 to 44.6) 30 days post-cefoxitin/doxycycline treatment, independent of gonorrhoea and chlamydia. In models adjusted for gonorrhoea and chlamydia, endometrial BV-associated bacteria were associated with recurrent PID (RR<sub>adj</sub> 4.7, 95% CI 1.7 to 12.8), and women who tested positive in the cervix and/or endometrium were more likely to develop infertility (RR<sub>adj</sub> 3.4, 95% CI 1.1 to 10.4). Associations between ureaplasmas and PID sequelae were modest.

**Conclusions** To our knowledge, this is the first prospective study to demonstrate that *S. sanguinegens*, *S. amnionii*, BVAB1 and *A. vaginae* are associated with PID, failure of the Centers for Disease Control and Prevention-recommended treatment to eliminate short-term endometritis, recurrent PID and infertility. Optimal antibiotic regimens for PID may require coverage of novel BV-associated microbes.

## INTRODUCTION

Pelvic inflammatory disease (PID), infection and inflammation of the uterine lining (endometritis) and fallopian tubes (salpingitis), is a frequent condition among young women that often results in tubal factor infertility, chronic pelvic pain and recurrent PID.<sup>1</sup> Tubal factors account for approximately a quarter of infertility with a significant proportion linked to prior PID.<sup>2</sup> A primary treatment option is in vitro fertilisation, which is costly and

results in lower birth rates than among women with other forms of infertility, particularly if hydro-salpinx is present.<sup>3</sup>

Although PID is a recognised complication of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infections,<sup>4,5</sup> the aetiology of up to 70% of cases is unknown. One clue about the trigger for non-gonococcal/non-chlamydial cases is the association between PID and bacterial vaginosis (BV), a condition marked by vaginal overgrowth of anaerobic bacterial species.<sup>6</sup> Anaerobic Gram-negative rods, frequently present in BV flora, have been cultured from the upper genital tract in women with PID.<sup>7</sup> Recent application of culture-independent broad-range PCR and bacterial 16S rRNA gene sequencing has identified specific, novel bacterial species in BV.<sup>8</sup> Gram-negative anaerobes *Sneathia (Leptotrichia) sanguinegens/amnionii* have been linked in case reports to postpartum fever,<sup>9</sup> endometritis,<sup>9</sup> tuboovarian abscesses,<sup>10</sup> amnionitis and preterm labour.<sup>11</sup> The Gram-positive anaerobe *Atopobium vaginae* has been associated with tuboovarian abscess, tubal factor infertility,<sup>12</sup> endometritis<sup>13</sup> and fetal death.<sup>14</sup> Totten *et al* identified bacterial 16S sequences in the fallopian tubes of 24% of women with salpingitis but in none of controls,<sup>15</sup> including phylotypes closely related to *Sneathia* and *A. vaginae*.

Emerging evidence implicates *Mycoplasma genitalium* as a significant aetiological agent of PID,<sup>16,17</sup> yet less is known about the role of other mollicutes, including ureaplasmas, in PID. Undifferentiated *Ureaplasma* spp. have been cross-sectionally associated with PID and infertility,<sup>18</sup> although not consistently.<sup>6</sup> Recently, the two biovars of *Ureaplasma urealyticum* have been classified as separate species:<sup>19</sup> *U. urealyticum*, which has been associated with urethritis in men and *Ureaplasma parvum* that has not.<sup>20</sup>

As there have been no prospective studies examining the role of recently identified fastidious BV-associated bacteria or the newly differentiated ureaplasma species in PID or its sequelae, we examined the relationships among the BV-associated bacteria *S. sanguinegens*, *S. amnionii*, *A. vaginae*, BVAB1, as well as *U. urealyticum* and *U. parvum*, and the outcomes of histologically confirmed endometritis, post-treatment endometritis, recurrent PID, infertility and chronic pelvic pain in a cohort of women with PID followed for a mean of 7 years.



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## MATERIALS AND METHODS

### Patient population

Women who participated in the PID Evaluation and Clinical Health (PEACH) study and had archived cervical and endometrial specimens were studied. The PEACH study methods have been described in detail elsewhere.<sup>4</sup> Briefly, 831 women aged 14–37 years with clinically suspected PID (pelvic pain <30 days duration, pelvic organ tenderness and leucorrhoea, mucopurulent cervicitis or untreated cervicitis) were recruited between March 1996 and February 1999 from emergency departments, OB/GYN clinics, STD clinics and private practices at 13 US clinical sites and randomised to inpatient or outpatient cefoxitin and doxycycline treatment.

### Endometrial biopsies and microbiological studies

At baseline and 30 days, an endometrial biopsy was obtained for histology, chlamydial PCR (Roche Diagnostics), *M. genitalium* PCR<sup>16</sup> and gonococcal culture. A modification<sup>21</sup> of the criteria proposed by Kiviat *et al.*<sup>5</sup> was used to diagnose endometritis, defined by at least five neutrophils in the endometrial surface epithelium in the absence of menstrual endometrium and/or at least two plasma cells in the endometrial stroma. Cervical swabs were used for *N. gonorrhoeae* culture and *C. trachomatis* and *M. genitalium*<sup>16</sup> PCR.

For this substudy, previously frozen endometrial biopsy (N=609) and cervical swab (N=691) specimens stored in –80°C freezers were thawed, purified using the MasterPure DNA purification kit for patient specimens (Epicentre) and tested by species-specific PCR assays. The preservation of bacterial DNA suitable for PCR analysis of specimens stored long term under these conditions has been previously demonstrated.<sup>22</sup> Specimens and endometrial histology were available from 545 women, with occasional missing assessments of specific bacterium. PCR assays for *S. sanguinegens/ammionii*, *A. vaginae*, and BVAB1 were performed as previously described,<sup>23 24</sup> and the *U. urealyticum* and *U. parvum* assays were performed using a modification of the procedure of Xiao *et al.*<sup>25</sup> Separate tests were performed for each specie and contained 1× PCR buffer (Mg<sup>++</sup> free, Promega), 5 mM MgCl<sub>2</sub>, 5U Taq DNA polymerase; 200 μM of each of the four dNTPs and the primers described by Xiao *et al.*<sup>25</sup> (0.2 and 0.3 μM for the forward primers of *U. urealyticum* and *U. parvum*, respectively, and 0.5 μM for both reverse primers) and 2 μL of purified patient specimen in a total volume of 100 μL. PCR was performed in the Vii7 real-time PCR system (Applied Biosystems) under amplification conditions of: initial denaturation (95°C for 10 min) and the following cycling programme (40 cycles): denaturation: 95°C for 15 s, annealing at 55°C for 50 s and extension at 72°C for 45 s, final elongation at 72°C for 10 min, and a final soak set at 4°C indefinitely. *U. urealyticum* and *U. parvum* PCR assay specificities were confirmed by detection of the appropriate sized PCR product (152 bp for *U. urealyticum* and *U. parvum*) on agarose gels and reactivity with the *U. urealyticum* biotin labelled UU127#1 probe (5'-biotin-ACACGAGTATGGATGAAATCAAAATCATCAAA-3')<sup>25</sup> and *U. parvum* UP063#1 probe (5'-biotin-CCCATTTTCAGCCATGGTGCCATCA-3')<sup>25</sup> on Southern blots hybridised at 58°C and 60°C, respectively, then visualised on Kodak BioMax XAR film after reaction in the chemiluminescence assay using the ECL kit (Amersham). Inhibition testing was performed using the *M. genitalium* internal control assay<sup>26</sup> on every 10th specimen negative for all assays; no inhibition was detected.

### Follow-up

Participants were monitored with in-person visits at 5 and 30 days and telephone interviews every 3–4 months until June

2004, at which point we had follow-up information for 541 women, representing a mean follow-up of 84 months. Infertility was defined when a sexually active woman with at least 12 months of follow-up did not report conception (positive urine or blood test or doctor's diagnosis) despite rare or no use of a contraceptive method. Chronic pelvic pain was defined as pain reported during at least two consecutive interviews, suggesting a minimum of 6 months duration. Recurrent PID was self-reported and confirmed in 76% of medical records.<sup>4</sup> Since few women experienced an ectopic pregnancy (N=6), we did not include as an outcome.

### Statistical methods

In our primary analysis, cross-sectional associations between bacteria and endometritis were determined using logistic regression models, adjusted for age and race. Logistic regression was used to construct relative risks and 95% CIs between each bacterium and endometritis at 30 days, recurrent PID, infertility and chronic pelvic pain, and included age, and race as explanatory variables. Models predicting infertility and post-treatment endometritis were adjusted for age, race, gonorrhoea, chlamydia, self-reported infertility at baseline and self-reported partner treatment, respectively. In exploratory analyses, all the above models were additionally analysed in a subset of women negative for both chlamydia and gonorrhoea (see web appendix). Subsequent exploratory models included terms representing interactions between BV-associated bacteria and chlamydia/gonorrhoea. Analyses were conducted first using both cervical and endometrial PCR results combined and were then repeated using only endometrial PCR. SPSS V20.0 for Windows was used for statistical comparisons.

## RESULTS

*S. sanguinegens* (54%), *S. amnionii* (66%), *A. vaginae* (83%), BVAB1 (65%), *U. urealyticum* (30%) and *U. parvum* (58%) were commonly isolated from the cervix and/or endometrium. As expected, *S. sanguinegens*, *S. amnionii*, *A. vaginae* and BVAB1 were significantly associated with BV determined by Nugent's<sup>27</sup> and Amsel's<sup>28</sup> criteria (p<0.0001 for all comparisons). Cervical identification was strongly associated with endometrial identification, suggesting lower to upper genital tract ascension (*S. sanguinegens*: OR 9.6, 5.8–15.9; *S. amnionii*: OR 12.1, 7.8–19.0; *A. vaginae*: OR 4.3, 2.8–6.7; BVAB1: OR 2.8, 2.0–4.1; *U. urealyticum*: OR 20.3, 9.5–43.0; *U. parvum*: OR 13.1, 6.7–25.8). Women testing positive for each of these bacteria in the cervix were significantly more likely to test positive for each of the other bacteria (p<0.05 for all comparisons) with the exception of *U. parvum*, which was not associated with any other bacteria. Similarly, endometrial identification of each bacteria was linked to endometrial detection of the other bacteria (p<0.05 for all comparisons). The exception was BVAB1, which was not associated with ureaplasma bacteria. *S. sanguinegens* and *S. amnionii*, two closely related Gram-negative anaerobes, co-occurred in the endometrium in 97% of samples testing positive for *S. sanguinegens* and in 52% of samples testing positive for *S. amnionii*. As *S. sanguinegens*, *S. amnionii*, *A. vaginae* and BVAB1 thus constituted a microbial community (as previously reported among women with BV),<sup>29</sup> we termed them 'fastidious BV-associated bacteria' and grouped them together in remaining analyses.

Women testing positive versus negative for any or all four fastidious BV-associated bacteria in the endometrium at baseline were at significantly elevated risk for endometritis (table 1). Cervical and/or endometrial fastidious BV-associated bacteria

**Table 1** Associations between selected fastidious bacterial vaginosis-associated bacteria (BVAB)\*, ureaplasma bacteria and histologically confirmed endometritis

	Endometritis+ n=258 n/N (%)	Endometritis– n=287 n/N (%)	Adjusted OR‡ (95% CI)
BVAB* (1 or more +)			
Cervix and/or endometrium	233/258 (90.3)	250/287 (87.1)	1.1 (0.6 to 2.0)
Endometrium	183/247 (74.1)	166/270 (61.5)	1.6 (1.1 to 2.4)
BVAB* (all +)			
Cervix and/or endometrium	93/118 (78.8)	81/118 (68.6)	1.3 (0.7 to 2.6)
Endometrium	27/93 (29.0)	18/129 (14.0)	2.0 (1.0 to 4.0)
<i>Ureaplasma urealyticum</i>			
Cervix and/or endometrium	75/247 (30.4)	70/271 (25.8)	1.2 (0.8 to 1.8)
Endometrium	24/254 (9.4)	22/281 (7.8)	1.2 (0.6 to 2.2)
<i>Ureaplasma parvum</i>			
Cervix and/or endometrium	128/252 (50.8)	164/276 (59.4)	0.7 (0.5 to 1.0)
Endometrium	47/254 (18.5)	49/281 (17.4)	1.1 (0.7 to 1.7)

\**Sneathia sanguinegens*, *Sneathia amnionii*, *Atopobium vaginae* and BVAB1.

Comparison group consists of women who tested negative for all four bacteria. In model of 'all positive', women testing positive for some but not all bacteria are excluded.

†≥5 surface epithelium neutrophils per ×400 field absent of menstrual endometrium and/or ≥2 stromal plasma cells per ×120 field.

‡Adjusted for age and race.

**Table 2** Associations between selected fastidious bacterial vaginosis-associated bacteria (BVAB)\*, ureaplasma bacteria and histologically confirmed endometritis 30 days following treatment for pelvic inflammatory disease

	Endometritis+ n=175 n/N (%)	Endometritis– n=238 n/N (%)	Adjusted RR‡ (95% CI)
BVAB (1 or more +)*			
Cervix and/or endometrium	149/175 (85.1)	199/238 (83.6)	1.1 (0.6 to 2.2)
Endometrium	111/169 (65.7)	140/234 (59.8)	1.2 (0.7 to 2.0)
BVAB (all positive)*			
Cervix and/or endometrium	37/63 (58.7)	32/71 (45.1)	2.3 (0.9 to 5.9)
Endometrium	11/69 (15.9)	5/99 (5.1)	5.7 (1.4 to 23.3)
<i>Ureaplasma urealyticum</i>			
Cervix and/or endometrium	40/167 (24.0)	49/231 (21.2)	1.0 (0.5 to 1.7)
Endometrium	15/170 (8.8)	11/235 (4.7)	1.7 (0.6 to 4.3)
<i>Ureaplasma parvum</i>			
Cervix and/or endometrium	69/169 (40.8)	70/234 (29.9)	1.9 (1.2 to 3.2)
Endometrium	27/170 (15.9)	31/235 (13.2)	2.0 (1.0 to 3.9)

\**Sneathia sanguinegens*, *Sneathia amnionii*, *Atopobium vaginae* and BVAB1.

Comparison group consists of women who tested negative for all four bacteria. In model of 'all positive', women testing positive for some but not all bacteria are excluded.

†≥5 surface epithelium neutrophils per ×400 field absent of menstrual endometrium and/or ≥2 stromal plasma cells per ×120 field.

‡Adjusted for age, race and self-reported partner treatment.

and chlamydia and/or gonorrhoea were highly correlated ( $p < 0.001$ ), with 36% of women testing positive for both. In a subset of women testing negative for chlamydia and gonorrhoea, results were attenuated and no models were statistically significant (see online supplementary table S1). However, the OR for endometritis associated with at least two fastidious BV-associated bacteria was significantly higher in women testing positive for chlamydia and/or gonorrhoea than that in women testing negative ( $OR_{adj}$  3.6, 95% CI 1.4 to 9.2). The relationship between a positive test for all four BV-associated bacteria and endometritis was significant after adjustment for *M. genitalium* ( $OR_{adj}$  5.1, 95% CI 1.2 to 21.7). *U. urealyticum* and *U. parvum* were not significantly associated with endometritis.

Persistent bacterial detection after cefoxitin and doxycycline treatment was common, with 46% of women with *S. sanguinegens*, 55% with *S. amnionii*, 82% with BVAB1, 58% with *A. vaginae*, 46% with *U. urealyticum* and 49% with *U. parvum*-positive baseline tests remaining positive at the 30-day visit. In comparison, continuance of *N. gonorrhoeae* and *C. trachomatis* occurred among only 8% and 10% of the cohort. Endometritis persistence 30 days post-treatment befell 43% of women despite receipt of the Centers for Disease Control and Prevention (CDC)-recommended cefoxitin and doxycycline.<sup>4</sup> Persistent endometritis was strongly linked to endometrial re-detection of the combined fastidious BV-associated bacteria (table 2,  $RR_{adj}$  5.7, 95% CI 1.4 to 23.3). This relationship remained significant after excluding women with gonorrhoea and chlamydia (see online supplementary table S2,  $RR_{adj}$  8.5, 95% CI 1.6 to 44.6). Women who tested positive for both fastidious BV-associated bacteria and chlamydia and/or gonorrhoea did not have a further elevated risk of persistent endometritis. Endometrial *U. urealyticum* and *U. parvum* were associated, albeit marginally, with persistent endometritis.

Long-term sequelae rates in the cohort were high among women testing positive for fastidious BV-associated bacteria (table 3). Endometrial detection of all four fastidious BV-associated bacteria was associated with recurrent PID. Similarly, detection of all BV-associated bacteria was significantly predictive of recurrent PID in subsets of women who tested negative for chlamydia and gonorrhoea (see online supplementary table S3,  $RR_{adj}$  4.7, 95% CI 1.7 to 12.8). Detection of all four fastidious BV-associated bacteria in the cervix and/or endometrium was also associated with subsequent infertility, although risks were attenuated when these microbes were identified only in the endometrium. Similarly, women testing positive for all BV-associated bacteria were significantly more likely to develop infertility in subsets of women without gonorrhoea or chlamydia (see online supplementary table S3,  $RR_{adj}$  3.4, 95% CI 1.1 to 10.4). In contrast to these novel bacteria, women with chlamydial infection had a marginal elevation in infertility risk ( $RR_{adj}$  1.9, 95% CI 0.9 to 4.2) and those with gonococcal infection had no increased infertility risk ( $RR_{adj}$  1.1, 95% CI 0.6 to 2.2). Neither *U. urealyticum* nor *U. parvum* were consistently associated with long-term morbidity.

## DISCUSSION

In our study of 545 women with clinically suspected PID, women who tested positive for *S. sanguinegens*, *S. amnionii*, *A. vaginae* or BVAB1 were more likely to have endometritis at PID diagnosis and experience recurrent PID and subsequent infertility. Women who tested positive for all four BV-associated bacteria in the endometrium had a twofold increased likelihood of having histologically confirmed endometritis, were nearly six times as likely to experience persistent endometritis after PID treatment and were four times as likely to have recurrent PID.

**Table 3** Long-term sequelae following pelvic inflammatory disease (PID) with selected fastidious bacterial vaginosis-associated bacteria (BVAB)\* and ureaplasma bacteria

	Cervical and/or endometrial BVAB 1 or more+ N=629 n (%)	Cervical and/or endometrial BVAB All negative N=62 n (%)	Adjusted RR† (95% CI)	Endometrial BVAB 1 or more+ N=418 n (%)	Endometrial BVAB All negative N=191 n (%)	Adjusted RR† (95% CI)
Recurrent PID	126 (21.1)	9 (15.0)	1.4 (0.6 to 2.9)	98 (24.3)	28 (15.8)	1.6 (1.0 to 2.6)
Infertility	117 (18.9)	6 (9.7)	2.3 (1.0 to 5.6)	84 (20.4)	30 (16.0)	1.4 (0.9 to 2.3)
Chronic pelvic pain	254 (42.0)	28 (46.7)	1.0 (0.5 to 1.7)	160 (39.7)	87 (47.8)	0.8 (0.6 to 1.2)
	Cervical and/or endometrial BVAB All positive N=229 n (%)	Cervical and/or endometrial BVAB All negative N=62 n (%)	Adjusted RR* (95% CI)	Endometrial BVAB All positive N=55 n (%)	Endometrial BVAB All negative N=191 n (%)	Adjusted RR† (95% CI)
Recurrent PID	51 (23.3)	9 (15.0)	1.5 (0.7 to 3.5)	22 (41.5)	28 (15.8)	3.9 (1.9 to 8.2)
Infertility	54 (24.0)	6 (9.7)	3.8 (1.4 to 10.2)	11 (20.0)	30 (16.0)	1.5 (0.6 to 3.4)
Chronic pelvic pain	79 (35.6)	28 (46.7)	0.7 (0.4 to 1.3)	25 (45.5)	87 (47.8)	1.1 (0.6 to 2.1)
	Cervical and/or endometrial UU+ N=179 n (%)	Cervical and/or endometrial UU– N=421 n (%)	Adjusted RR* (95% CI)	Endometrial UU + N=53 n (%)	Endometrial UU– N=554 n (%)	Adjusted RR† (95% CI)
Recurrent PID	42 (25.1)	79 (19.6)	1.4 (0.9 to 2.1)	15 (30.6)	111 (20.9)	1.7 (0.9 to 3.2)
Infertility	33 (18.9)	79 (19.0)	1.1 (0.7 to 1.8)	9 (17.3)	105 (19.3)	0.9 (0.4 to 2.0)
Chronic pelvic pain	82 (47.4)	165 (40.6)	1.4 (1.0 to 2.1)	28 (53.8)	219 (41.1)	1.8 (1.0 to 3.3)
	Cervical and/or endometrial UP+ N=371 n (%)	Cervical and/or endometrial UP– N=264 n (%)	Adjusted RR* (95% CI)	Endometrial UP+ N=115 n (%)	Endometrial UP– N=492 n (%)	Adjusted RR† (95% CI)
Recurrent PID	73 (20.6)	53 (21.4)	1.0 (0.7 to 1.5)	28 (25.0)	98 (21.0)	1.3 (0.8 to 2.1)
Infertility	66 (18.0)	48 (18.5)	0.9 (0.6 to 1.4)	18 (15.8)	96 (19.9)	0.8 (0.5 to 1.4)
Chronic pelvic pain	150 (42.1)	113 (44.1)	0.9 (0.7 to 1.3)	52 (46.4)	195 (41.2)	1.3 (0.8 to 1.9)

\**Sneathia sanguinegens*, *Sneathia amnionii*, *Atopobium vaginae*, and BVAB1. Comparison group consists of women who tested negative for all four bacteria. In model of 'all positive', women testing positive for some but not all bacteria are excluded.

†All models are adjusted for age and race. Infertility model additionally adjusted for self-reported infertility at baseline.

Further, women who tested positive for all four BVAB in the cervix and/or endometrium were nearly four times as likely to experience subsequent infertility.

To our knowledge, this is the first cohort study to show that molecularly identified BV-associated bacteria are associated with PID and its sequelae. Results are consistent with our prior culture study demonstrating that anaerobic black-pigmented Gram-negative rods are associated with endometritis, independent of chlamydia and gonorrhoea.<sup>6</sup> Additionally, we previously showed that a cluster of cultured vaginal BV-associated organisms (absence of hydrogen peroxide-producing lactobacillus, presence of *G. vaginalis*, *Mycoplasma hominis*, anaerobic Gram-negative rods and undifferentiated ureaplasmas) was associated with a twofold risk of incident PID.<sup>30</sup> Mirroring results among men with urethritis,<sup>20</sup> *U. parvum* was not associated with any outcomes in this study, while the relationship between *U. urealyticum* and endometritis was modest.

Half or more of women testing positive for these select bacteria had persistent infection 30 days post-cefoxitin/doxycycline treatment. Women who tested positive for all four BV-associated bacteria in the endometrium were nearly four times as likely to

experience short-term treatment failure. Moreover, women testing positive for fastidious BV-associated bacteria at baseline were nearly four times more likely than those without these pathogens to develop recurrent PID.

Whether *Sneathia* spp. and BVAB1 are sensitive to antibiotics is unclear due to the difficulty in culturing these bacteria, yet the high rates of persistent infection post-treatment in our study suggest that cefoxitin and doxycycline constitute suboptimal treatment. *A. vaginae* has been reported to be susceptible to cefoxitin.<sup>12</sup> Although it was often identified 30 days post-treatment in our study, it was not associated with persistent endometritis. The high rates of *U. urealyticum* and *U. parvum* persistence were predictable, as ureaplasmas have been shown to express tetracycline resistance<sup>1</sup> and contain *tetM* genes that encode such resistance.<sup>w2</sup>

Women testing positive for fastidious BV-associated bacteria at PID diagnosis were nearly four times as likely to develop subsequent infertility, even after adjustment for confounders. Our results extend previous culture studies demonstrating that BV categorised by Gram stain is associated with tubal factor infertility among women undergoing in vitro fertilisation.<sup>w3</sup>

Major strengths of our study are the prospective design and the characterisation of specific BV-associated microorganisms. As our study is limited to four selected BV-associated bacteria measured using targeted qualitative PCR, further research examining concentrations of a broader phylogenetic range of BV-associated microbes among PID and infertility patients using high-throughput sequencing and quantitative real-time PCR assays is warranted. Further, it is possible that transcervical sampling of the endometrium may have resulted in contamination of endometrial biopsy specimens by vaginal or cervical microorganisms. However, as possible contamination would not be expected to differ by the study outcomes, it is unlikely to be a major source of bias. Further, associations between cervical PCR and outcomes should be unaffected. Lastly, our study is limited by the lack of a truly unaffected control group of women. All women enrolled in the PEACH study had clinically suspected PID. Thus, our comparison groups of women with negative PCR comprised women who presented with characteristics consistent with PID. This may have biased our findings towards the null.

Our results must be confirmed in other prospective studies as they highlight the concerning possibility that currently recommended antibiotics do not treat a range of BV-associated organisms associated with PID sequelae. Our findings highlight the need for commercially available and affordable multiplex PCR-based genital tract microorganism tests<sup>w4</sup> of BV-associated bacteria for incorporation in screening and treatment programmes to prevent PID. Such tests could also be included in standard work-up of PID to determine microbiological aetiology and allow clinicians to tailor patient treatment. For example, as our study found that *A. vaginae* persisted post-cefoxitin therapy, other CDC-recommended PID antibiotics active against *A. vaginae*<sup>w5</sup> such as clindamycin or ampicillin/sulbactam might be used. Recent evidence suggests that the broad spectrum antibiotic nifuratel is effective against *C. trachomatis* and *Mycoplasma* spp.<sup>w6</sup> as well as *G. vaginalis* and *A. vaginae*, without affecting lactobacilli.<sup>w7</sup> However, no clinical trials of nifuratel for PID treatment have been conducted to date. The CDC treatment guidelines<sup>w8</sup> suggest optional metronidazole inclusion for additional anaerobic coverage. However, as *A. vaginae* and *Sneathia* spp. have been shown to be metronidazole resistant in some studies,<sup>12 w5</sup> clinical trials including metronidazole with other antimicrobials for mitigating short-term and long-term PID sequelae are needed.

We previously reported that *C. trachomatis* and *N. gonorrhoeae*<sup>6</sup> are associated with endometritis in this cohort. We now demonstrate that fastidious BV-associated bacteria and chlamydia/gonorrhoea in combination elevates endometritis likelihood three-and-a-half-fold. As BV-associated bacteria and chlamydia/gonorrhoea were frequently co-present, and as the relationship between BV-associated bacteria and endometritis was identified primarily among women with chlamydia and/or gonococcal infection, this suggests that chlamydia/gonorrhoea may pave the way for anaerobic bacteria ascension into the upper genital tract where they can persist post-PID treatment and cause recurrent PID and infertility, as demonstrated in our study. Alternatively or in addition, it is possible that persistent anaerobic infection may increase the risk of recurrent chlamydial or gonococcal infection.

That infertility was only marginally associated with chlamydial PID and not associated with gonococcal PID suggests that, in contrast to older studies that strongly implicated these bacteria in infertility, women in our study received timely and effective

antibiotics for these pathogens. Indeed, approximately 90% of our patients were free of gonorrhoea/chlamydia at the 30-day follow-up. Moreover, the combination of gonorrhoea/chlamydia plus fastidious BV-associated bacteria produced no additional degree of persistent endometritis and long-term PID sequelae beyond the effect from BV-associated bacteria alone.

Currently, the approach for PID treatment is empiric, using broad spectrum antibiotics to target a range of pathogens but largely directed towards eliminating *N. gonorrhoeae* and/or *C. trachomatis*. However, approximately 60% of women on our cohort had non-gonococcal, non-chlamydial PID.<sup>4</sup> Fastidious BV-associated bacteria, commonly found in the endometrium at baseline, often persisted after CDC-recommended PID treatment as did endometritis. A sizeable proportion of women receiving timely, standard PID treatment may thus have ongoing upper genital tract inflammation as a result of continued infection with previously unrecognised pathogens. Such treatment failure appears to eventuate recurrent PID and infertility among some women. Replication of our results would dictate that optimal diagnosis and treatment regimens for PID be re-evaluated.

### Key messages

- ▶ Current pelvic inflammatory disease (PID) treatment regimens use broad spectrum antibiotics to target a range of pathogens, but largely directed towards eliminating *Neisseria gonorrhoeae* and/or *Chlamydia trachomatis*.
- ▶ Among women treated with cefoxitin and doxycycline, *Sneathia sanguinegenslamnionii*, bacterial vaginosis-associated bacteria 1 and *Atopobium vaginae* were associated with persistent endometritis, recurrent PID and infertility.
- ▶ Optimal antibiotic regimens for PID may require coverage for novel bacterial vaginosis-associated microbes.

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**Contributors** CLH was involved in the conception and design of the study, analysis and interpretation of data and wrote the paper. PAT was involved in the conception and design of the study, DNA extraction, PCR, analysis and interpretation of data and provided significant revisions. GT was involved in the conception and design of the study, analysis and interpretation of data, and provided significant revisions. SGA and JN were involved in the analysis and interpretation of data and samples and provided significant revisions. MJF was involved in the conception and design of the study, PCR, analysis and interpretation of data, and provided significant revisions. DCB was involved with acquisition of data, analysis of data and provided significant revisions. DHM was involved with the conception and design of the study, interpretation of data and provided significant revisions. BDT was involved in interpretation of data, drafting part of the background and provided significant revisions. RBN was involved in the conception and design of this study and the parent PEACH study, interpretation of data and provided significant revisions. All authors provided final approval.

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**Data sharing statement** All data are stored at the University of Pittsburgh, Pittsburgh, PA, under the direction of Dr Catherine Haggerty (haggerty@pitt.edu). An outside individual wishing to access the data must collaborate with Dr Haggerty to do so. A written protocol must be submitted, reviewed and approved prior to initiation of any new projects. In order to ensure integrity of the data, all analyses are conducted at the University of Pittsburgh under the direction of Dr Catherine Haggerty.

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Supplementary Table 1: Associations between selected fastidious bacterial vaginosis associated bacteria<sup>a</sup>, ureaplasma bacteria, and histologically confirmed endometritis<sup>b</sup> in a subset of women without gonorrhea and chlamydia at baseline

	Endometritis + n=115 n/N (%)	Endometritis - n=232 n/N (%)	Adjusted OR <sup>γ</sup> (95% CI)
<b>BV-associated bacteria<sup>a</sup> (1 or more +)</b>			
Cervix and/or endometrium	94/115 (81.7)	198/232 (85.3)	0.7 (0.4 – 1.3)
Endometrium	67/113 (59.3)	128/229 (55.9)	1.1 (0.7 – 1.7)
<b>BV-associated bacteria<sup>a</sup> (all +)</b>			
Cervix and/or endometrium	30/51 (58.8)	60/94 (63.8)	0.7 (0.3 – 1.5)
Endometrium	7/53 (13.2)	12/113 (10.6)	1.1 (0.4 – 3.1)
<b><i>Ureaplasma urealyticum</i></b>			
Cervix and/or endometrium	41/110 (37.3)	49/218 (22.5)	2.2 (1.3 – 3.7)
Endometrium	13/113 (11.5)	17/229 (7.4)	1.8 (0.8 – 3.8)
<b><i>Ureaplasma parvum</i></b>			
Cervix and/or endometrium	56/110 (50.9)	130/219 (59.4)	0.7 (0.5 – 1.2)
Endometrium	22/113 (19.5)	41/229 (17.9)	1.2 (0.6 – 2.1)

<sup>a</sup> *Leptotrichia sanguinegens*, *Leptotrichia amnionii*, *Atopobium vaginae*, and BVAB1. Comparison group consists of women who tested negative for all four bacteria. In model of “all positive”, women testing positive for some but not all bacteria are excluded.

<sup>b</sup>  $\geq 5$  surface epithelium neutrophils per  $\times 400$  field absent of menstrual endometrium and/or  $\geq 2$  stromal plasma cells per  $\times 120$  field

<sup>γ</sup> Adjusted for age and race

Supplementary Table 2: Associations between selected fastidious bacterial vaginosis-associated bacteria <sup>α</sup>, ureaplasma bacteria, and histologically confirmed endometritis <sup>β</sup> 30 days following treatment for pelvic inflammatory disease in a subset of women without gonorrhea and chlamydia at 30 days

	Endometritis <sup>β</sup> + n=117 n/N (%)	Endometritis <sup>β</sup> - n=175 n/N (%)	Adjusted RR <sup>γ</sup> (95% CI)
<b>BV-associated bacteria (1 or more +) <sup>α</sup></b>			
Cervix and/or endometrium	102/117 (87.2)	149/175 (85.1)	1.1 (0.5 – 2.5)
Endometrium	75/114 (65.8)	114/174 (65.5)	1.0 (0.6 – 1.8)
<b>BV-associated bacteria (all positive) <sup>α</sup></b>			
Cervix and/or endometrium	29/44 (65.9)	27/53 (50.9)	2.8 (1.0 – 7.8)
Endometrium	10/49 (20.4)	4/64 (6.3)	8.5 (1.6 – 44.6)
<b><i>Ureaplasma urealyticum</i></b>			
Cervix and/or endometrium	28/113 (24.8)	39/172 (22.7)	1.2 (0.6 – 2.3)
Endometrium	7/115 (6.1)	8/174 (4.6)	1.4 (0.4 – 4.8)
<b><i>Ureaplasma parvum</i></b>			
Cervix and/or endometrium	48/115 (41.7)	49/173 (28.3)	1.9 (1.1 – 3.5)
Endometrium	18/115 (15.7)	19/174 (10.9)	2.7 (1.2 – 6.2)

<sup>α</sup> *Leptotrichia sanguinegens*, *Leptotrichia amnionii*, *Atopobium vaginae*, and BVAB1

<sup>β</sup> ≥5 surface epithelium neutrophils per ×400 field absent of menstrual endometrium and/or ≥2 stromal plasma cells per ×120 field

<sup>γ</sup> Adjusted for age, race, and self-reported partner treatment

Supplementary Table 3: Long-term sequelae following PID with selected fastidious bacterial vaginosis-associated<sup>a</sup> and ureaplasma bacteria in a subset of women without gonorrhea and chlamydia at baseline

	Cervical and/or Endometrial BV-associated bacteria: 1 or more + N=334 n (%)	Cervical and/or Endometrial BV-associated bacteria All negative N=55 n (%)	Adjusted RR <sup>β</sup> (95% CI)	Endometrial BV-associated bacteria 1 or more + N=225 n (%)	Endometrial BV-associated bacteria All negative N=156 n (%)	Adjusted RR <sup>β</sup> (95% CI)
Recurrent PID	72 (22.9)	8 (15.1)	1.4 (0.6 – 3.2)	56 (26.0)	23 (15.9)	1.6 (0.9 – 2.9)
Infertility	70 (21.3)	5 (9.1)	2.6 (1.0 – 6.8)	51 (23.1)	25 (16.2)	1.5 (0.9 – 2.6)
Chronic Pelvic Pain	153 (47.4)	26 (49.1)	1.0 (0.6 – 1.8)	96 (44.0)	78 (51.7)	0.8 (0.5 – 1.2)
	Cervical and/or Endometrial BV-associated bacteria All positive N=107 n (%)	Cervical and/or Endometrial BV-associated bacteria All negative N=55 n (%)	Adjusted RR <sup>α</sup> (95% CI)	Endometrial BV-associated bacteria All positive N=25 n (%)	Endometrial BV-associated bacteria All negative N=156 n (%)	Adjusted RR <sup>β</sup> (95% CI)
Recurrent PID	29 (29.0)	8 (15.1)	1.9 (0.8 – 4.9)	11 (47.8)	23 (15.9)	4.7 (1.7 – 12.8)
Infertility	26 (25.0)	5 (9.1)	3.4 (1.1 – 10.4)	3 (12.0)	25 (16.2)	0.7 (0.2 – 2.8)
Chronic Pelvic Pain	42 (40.8)	26 (49.1)	0.8 (0.4 – 1.5)	13 (52.0)	78 (51.7)	1.1 (0.5 – 2.8)
	Cervical and/or Endometrial UU + N=102 n (%)	Cervical and/or Endometrial UU – N=263 n (%)	Adjusted RR <sup>α</sup> (95% CI)	Endometrial UU + N=36 n (%)	Endometrial UU – N=344 n (%)	Adjusted RR <sup>β</sup> (95% CI)
Recurrent PID	24 (25.5)	52 (20.7)	1.3 (0.7 – 2.3)	10 (30.3)	69 (21.1)	1.8 (0.8 – 4.0)
Infertility	19 (19.0)	52 (20.0)	1.0 (0.5 – 1.8)	7 (20.0)	69 (20.3)	1.0 (0.4 – 2.4)
Chronic Pelvic Pain	54 (54.5)	115 (44.9)	1.5 (1.0 – 2.5)	21 (60.0)	153 (45.8)	1.9 (0.9 – 3.8)
	Cervical and/or Endometrial UP + N=213 n (%)	Cervical and/or Endometrial UP – N=157 n (%)	Adjusted RR <sup>α</sup> (95% CI)	Endometrial UP + N=72 n (%)	Endometrial UP – N=308 n (%)	Adjusted RR <sup>β</sup> (95% CI)
Recurrent PID	44 (21.5)	32 (22.2)	1.0 (0.6 – 1.7)	18 (25.7)	61 (21.0)	1.3 (0.7 – 2.5)
Infertility	43 (20.3)	28 (18.3)	1.1 (0.6 – 1.8)	13 (18.1)	63 (20.8)	0.9 (0.5 – 1.8)
Chronic Pelvic Pain	94 (45.4)	78 (51.3)	0.8 (0.5 – 1.2)	36 (50.7)	138 (46.3)	1.2 (0.7 – 2.1)

<sup>a</sup>*Leptotrichia sanguinegens*, *Leptotrichia amnionii*, *Atopobium vaginae*, and BVAB1. Comparison group consists of women who tested negative for all four bacteria. In model of “all positive”, women testing positive for some but not all bacteria are excluded.

<sup>β</sup> All models are adjusted for age and race. Infertility model additionally adjusted for self-reported infertility at baseline.