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ORIGINAL ARTICLE *Infertility*

Abnormal vaginal microbiota may be associated with poor reproductive outcomes: a prospective study in IVF patients

T. Haahr[1], J.S. Jensen[2], L. Thomsen[1], L. Duus[3], K. Rygaard[4], and P. Humaidan[1,*]

[1]Faculty of Health, Aarhus University and the Fertility Clinic, Skive Regional Hospital, Skive, Denmark [2]Statens Serum Institute, Copenhagen, Denmark [3]Department of Clinical Microbiology, Regional Hospital Central and West Jutland, Denmark [4]Trianglen Fertility Clinic, Copenhagen, Denmark

*Correspondence address. The Fertility Clinic, Skive Regional Hospital, Faculty of Health, Aarhus University, Resenvej 25, 7800 Skive, Denmark. Tel: +4523815991; E-mail: peter.humaidan@midt.rm.dk

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STUDY QUESTION: What is the diagnostic performance of qPCR assays compared with Nugent scoring for abnormal vaginal microbiota and for predicting the success rate of IVF treatment?

SUMMARY ANSWER: The vaginal microbiota of IVF patients can be characterized with qPCR tests which may be promising tools for diagnosing abnormal vaginal microbiota and for prediction of clinical pregnancy in IVF treatment.

WHAT IS KNOWN ALREADY: Bacterial vaginosis (BV) is a common genital disorder with a prevalence of approximately 19% in the infertile population. BV is often sub-clinical with a change of the vaginal microbiota from being *Lactobacillus* spp. dominated to a more heterogeneous environment with anaerobic bacteria, such as *Gardnerella vaginalis* and *Atopobium vaginae*. Few studies have been conducted in infertile women, and some have suggested a negative impact on fecundity in the presence of BV.

STUDY DESIGN, SIZE, DURATION: A cohort of 130 infertile patients, 90% Caucasians, attending two Danish fertility clinics for *in vitro* fertilization (IVF) treatment from April 2014–December 2014 were prospectively enrolled in the trial.

PARTICIPANTS/MATERIALS, SETTING AND METHODS: Vaginal swabs from IVF patients were obtained from the posterior fornix. Gram stained slides were assessed according to Nugent's criteria. PCR primers were specific for four common *Lactobacillus* spp., *G. vaginalis* and *A. vaginae*. Threshold levels were established using ROC curve analysis.

MAIN RESULTS AND THE ROLE OF CHANCE: The prevalence of BV defined by Nugent score was 21% (27/130), whereas the prevalence of an abnormal vaginal microbiota was 28% (36/130) defined by qPCR with high concentrations of *Gardnerella vaginalis* and/or *Atopobium vaginae*. The qPCR diagnostic approach had a sensitivity and specificity of respectively 93% and 93% for Nugent-defined BV. Furthermore, qPCR enabled the stratification of Nugent intermediate flora. Eighty-four patients completed IVF treatment. The overall clinical pregnancy rate was 35% (29/84). Interestingly, only 9% (2/22) with qPCR defined abnormal vaginal microbiota obtained a clinical pregnancy ($P = 0.004$).

LIMITATIONS, REASONS FOR CAUTION: Although a total of 130 IVF patients were included in the study, a larger sample size is needed to draw firm conclusions regarding the possible adverse effect of an abnormal vaginal microbiota in relation to the clinical pregnancy rate and other reproductive outcomes.

WIDER IMPLICATIONS OF THE FINDINGS: Abnormal vaginal microbiota may negatively affect the clinical pregnancy rate in IVF patients. If a negative correlation between abnormal vaginal microbiota and the clinical pregnancy rate is corroborated, patients could be screened and subsequently treated for abnormal vaginal microbiota prior to fertility treatment.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by The AP Møller Maersk Foundation for the advancement of Medical Science and Hospital of Central Jutland Research Fund, Denmark. No competing interests.

TRIAL REGISTRATION NUMBER: The project was registered at clinicaltrials.gov (file number NCT02042352).

Key words: bacterial vaginosis / vaginal microbiota / Nugent score / IVF / *Gardnerella vaginalis* / *Atopobium vaginae* / clinical pregnancy / prevalence

Introduction

Bacterial vaginosis (BV) is the most common genital disorder in women of reproductive age (Koumans *et al.*, 2007). In the infertile population a recent meta-analysis including 12 studies, reported a BV prevalence of 19% (van Oostrum *et al.*, 2013). The disorder is characterized by a microbial dysbiosis, changing the normal acidic environment dominated by *Lactobacillus* spp. to a more heterogeneous environment with an increased number of anaerobes and facultative anaerobes such as *Gardnerella vaginalis* (Lamont *et al.*, 2011). BV may be asymptomatic in up to 50% of cases, but when present, symptoms such as a fishy odor and a grayish discharge trouble the patient (Klebanoff *et al.*, 2004; Bilardi *et al.*, 2013). Traditionally, BV has been considered an obstetrical and gynecological issue and the link between BV and preterm birth and post-surgical infections have been studied intensely (Hay *et al.*, 1994; Hillier *et al.*, 1995; Larsson *et al.*, 2000; Svare *et al.*, 2006; Thorsen *et al.*, 2006; Brocklehurst *et al.*, 2013). Only a few studies have been conducted among infertile women and, interestingly, some of these studies suggest negative implications for female fecundity (Mangot-Bertrand *et al.*, 2013; Salah *et al.*, 2013).

Traditionally, BV has been diagnosed, using either the clinical Amsel criteria which include pH > 4.5, grayish discharge, fishy odor and a positive wet smear, or the Nugent score, based on a Gram stained smear (Amsel *et al.*, 1983; Nugent *et al.*, 1991). Both methods depend on bacterial morphology and not on species identification of the bacteria involved in BV (Sha *et al.*, 2005; Lamont *et al.*, 2011). However, recent research has shed a light on the vaginal microbiota, suggesting that BV may be reframed into different molecularly defined microbial communities (Ravel *et al.*, 2011; Datcu *et al.*, 2013). The term microbiota is recommended for description of the collection of microbial taxa associated with a certain habitat and the term microbiome as the catalog of microbes and their genes and products together with those of the host (Marchesi and Ravel, 2015). Although the redundancy among species in producing lactic acid and the ethnic diversity makes it difficult to define a healthy vaginal microbiome, Ravel et al. suggested clustering the microbiotas into community groups, based on the dominating species found by 454 pyrosequencing of vaginal swabs from 396 asymptomatic reproductive age women (Ravel *et al.*, 2011). They suggested that healthy women had a vaginal microbiota dominated by one of four *Lactobacillus* spp. (*L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners*). Furthermore, they classified a group which they called the diversity group. The diversity group included the *G. vaginalis* cluster, but also other bacteria. However, as BV is asymptomatic in up to 50% of cases, some of the asymptomatic women in the diversity group did indeed have BV, and it is possible that they had the same increased risk of complications as those with symptomatic BV.

To the best of our knowledge, only one clinical study has been conducted using quantitative PCR (qPCR) for diagnosis of an abnormal vaginal microbiota (AVM) in infertile women (Mangot-Bertrand *et al.*, 2013). Thus, the primary objective of the present study was to evaluate the performance of qPCR assays compared with Nugent scoring in predicting the success rate of *in vitro* fertilization (IVF) treatment.

Materials and Methods

Study population

A total of 130 patients (90% Caucasian) undergoing IVF treatment in two fertility clinics in Denmark were included between April and December 2014. The only exclusion criterion was the prescription of antibiotics within a month before inclusion. Patients were excluded from the reproductive outcome analysis if the vaginal sample had been collected more than 2 months before embryo transfer (ET).

Sampling

During speculum examination, the clinician obtained two swabs from the posterior fornix. One swab was smeared directly onto a glass-slide and left to dry at room temperature while the second swab was collected using the Copan Eswab™ system (Cat no. 480CE, Copan Italia, Brescia, Italy) and immediately stored at −80°C. The main proportion of swabs (95%) was taken at the first consultation, usually within 2–4 weeks prior to IVF treatment. Moreover, the patient self-measured her vaginal pH at the first consultation, using the Careplan® vpH glove (Alere, Galway, Ireland) according to the instructions on the package. The pH scale on the glove was categorized as: 4.0, 4.4, 4.7, 5.0, 5.3, 5.5, 5.8, or 7.0.

Microscopy

Glass-slides were Gram stained at the Department of Clinical Microbiology, Central and West Jutland, and the Nugent score was determined (Nugent *et al.*, 1991). Nugent score is generally accepted as the gold standard for BV diagnosis (Marrazzo *et al.*, 2010). Microscopy was performed twice for each slide by trained laboratory technicians in a blinded manner in order to assess the inter-rater variability. A clinical microbiologist performed a third evaluation if the Nugent score differed between the two initial evaluations. All examiners were blinded to clinical and qPCR results. BV was diagnosed for Nugent scores of 7–10 and a score of 4–6 was considered intermediate flora (Nugent *et al.*, 1991). Furthermore, the relative abundance of leukocytes (leukocytes>epithelial cells) and candida was noted. Vaginal leukocytosis was diagnosed when more leukocytes than epithelial cells were seen, and an example is shown in Fig. 1.

QPCR and definition of an abnormal vaginal microbiota

PCR analyses for *G. vaginalis*, *A. vaginae*, *L. crispatus*, *L. jensenii*, *L. gasseri*, and *L. iners* was performed as previously described with slight modification (Datcu *et al.*, 2013, 2014). Briefly, bacterial DNA was extracted using the FastDNA™ SPIN kit for Soil (MP Biomedicals, Santa Ana, CA, USA) using 200 µl of the Eswab transport medium and elution of the DNA in 100 µl DNase free water; qPCR was performed in 50 µl total reaction volume with 5 µl of template DNA. Bacterial communities were molecularly defined based on qPCR results, following threshold determination by ROC curve analysis. Vaginal samples not dominated by any of the communities were classified as 'other'.

Reproductive outcome analysis

In the prospective analysis, the biochemical pregnancy rate (positive hCG at day 14) and the clinical pregnancy rate (ultrasound proven fetal heartbeat at 7 weeks of gestation) was investigated.

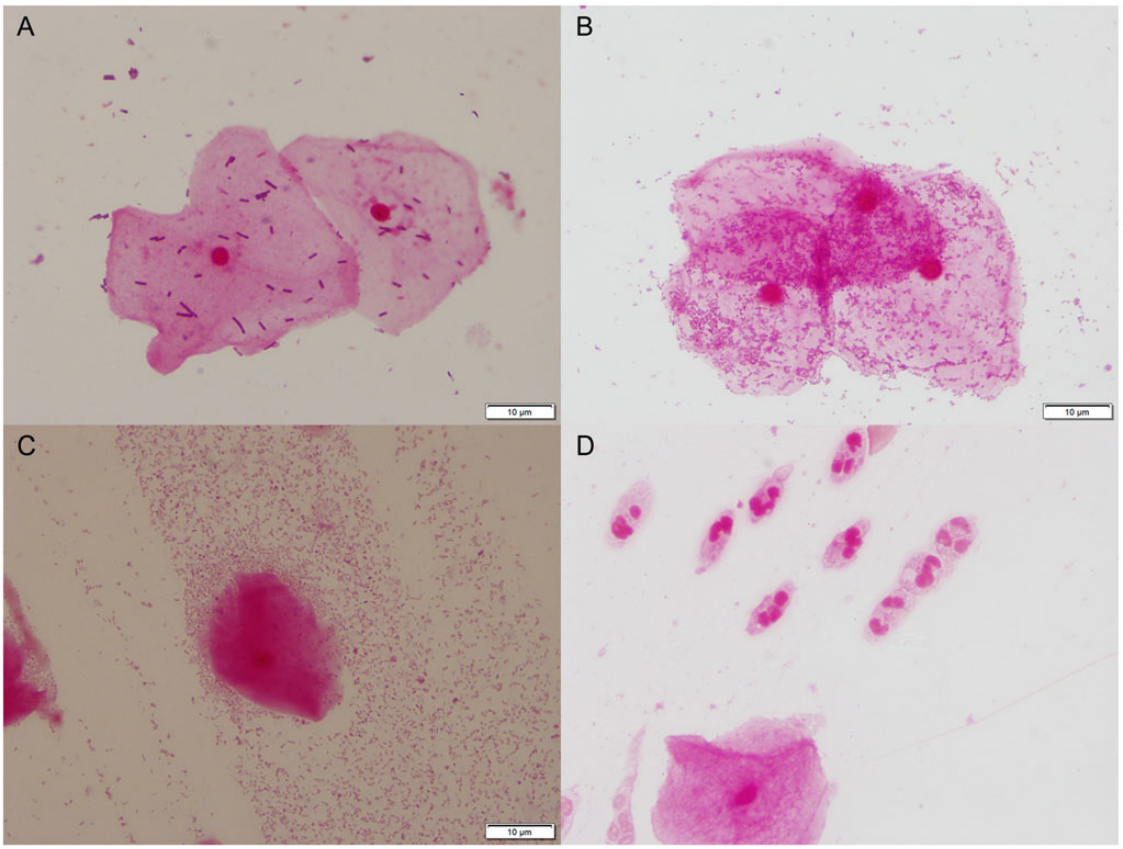

Figure 1 Gram stained vaginal smears. (**A**) Normal vaginal epithelial cells with distinct *Lactobacillus* morphotypes. (**B**) Clue cells with *Gardnerella vaginalis* morphotypes. (**C**) Gram staining showing a case where the Nugent diagnosis differed between intermediate and bacterial vaginosis between the two independent microscopists. qPCR revealed abundance of *Lactobacillus iners* subtype and absence of e.g. *Gardnerella vaginalis*. (**D**) Abundance of leukocytes in a vaginal swab from an asymptomatic patient.

Statistical analysis

Non-parametric Mann–Whitney *U*-test was used to test for differences in medians. Fisher's exact test was used to test for differences in proportions. In Table III an overall significance test was made with the Kruskal–Wallis test for medians and chi-square for proportions. Two-by-two tests were then made if significance was observed. Receiver operating characteristic (ROC) curve analysis was used to determine the optimal cutoff (threshold) for prediction of BV. The Inter-rater variability for Nugent score categories (normal, intermediate and BV) was assessed using unweighted Kappa statistics. All *P*-values were two-sided and a significance level at 0.05 was used.

An abnormal vaginal microbiota was defined when *G. vaginalis* and/or *A. vaginae* were present at concentrations above the threshold defined by ROC curve analysis using Nugent BV as the gold standard, and weighing sensitivity and specificity equal. A logistic regression model was used to adjust for confounders to the reproductive outcome. Statistical analyses were carried out using the StatsDirect software version 3.0 (StatsDirect Ltd, Cheshire, UK). However, the logistic regression analysis was carried out in Stata version 14 (Statacorp LP ©).

Ethics

This project was approved by the Danish Data Protection Agency (file number 1-16-02-26-14) and by the Regional Ethics Committee; Central Denmark Region (file number 1-10-72-325-13). The project was registered at clinicaltrials.gov (file number NCT02042352).

Results

Diagnosis of BV by qPCR

Using Nugent scores 7–10 to define BV as reference standard, threshold levels for *G. vaginalis* and *A. vaginae* were established to be 5.7×10^7 and 5.7×10^6 copies/ml respectively. The sensitivity and specificity for *G. vaginalis* cutoff were 88% and 99%. The sensitivity and specificity for *A. vaginae* cutoff were 92% and 94%. ROC curves are given in Figs 2 and 3. Using the combined criterion of either *G. vaginalis* or *A. vaginae* above threshold, the sensitivity and specificity according to the Nugent criteria was 93% and 93%. Women with vaginal loads for either of the two species were considered having abnormal vaginal microbiota (AVM). Furthermore, thresholds were established for normal microbiota bacteria against Nugent normal flora, excluding intermediate flora. Thus, *L. crispatus*, *L. jensenii*, *L. gasseri* and *L. iners* had threshold levels of 1.5×10^7, 6.1×10^4, 4.0×10^4 and 2.0×10^9 copies/ml respectively. The thresholds applied for the qPCR panel enabled the possibility to dichotomize those slides deemed intermediate by Nugent score into normal and abnormal microbiota. As shown in Table I, *G. vaginalis* and *A. vaginae* were

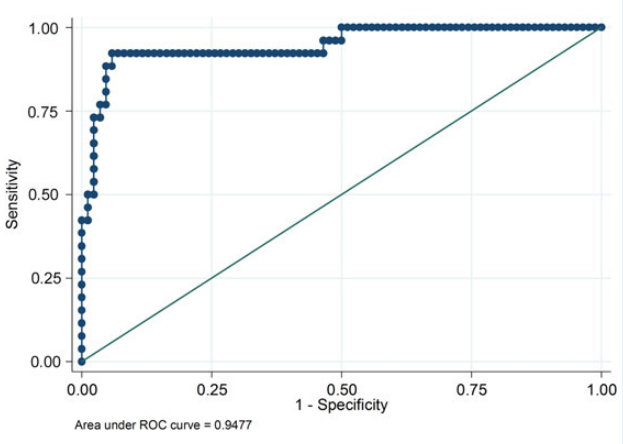

Figure 2 Receiver operating characteristic **(**ROC) curve analysis for *Atopobium vaginae* against the Nugent score as reference test. In order to apply quantitative thresholds for *A. vaginae*, Nugent scores 7–10 were used as reference test/gold standard. The sensitivity and specificity for *Atopobium vaginae* were 92% and 94% respectively. Sensitivity and specificity were weighted equally.

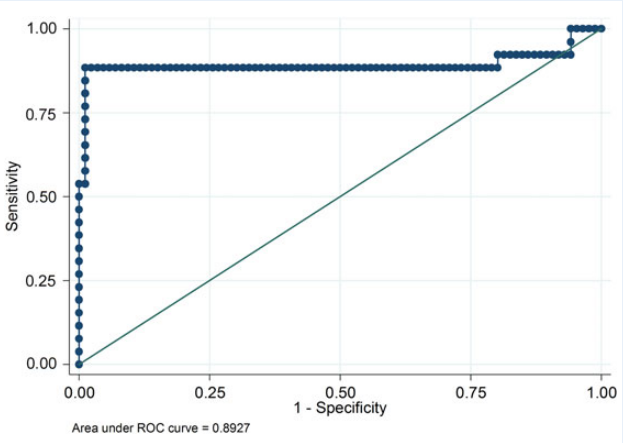

Figure 3 Receiver operating characteristic (ROC) curve analysis for *Gardnerella vaginalis* against the Nugent score as reference test. In order to apply quantitative thresholds for *G. vaginalis*, Nugent scores 7–10 were used as reference test/gold standard. The sensitivity and specificity were 88% and 99% respectively. Sensitivity and specificity were weighted equally.

associated with BV whereas *L. crispatus*, *L. jensenii* and *L. gasseri* were associated with a normal microbiota. However, the *L. iners* group differed from the other lactobacilli by not being associated with a normal microbiota. No significant association was found between the *L. iners* group and the BV group ($P = 0.08$).

BV prevalence

The prevalence of BV by the Nugent classification was 21% (27/130). The prevalence of an AVM defined by the qPCR panel was 28% (36/130). The AVM group included 93% (25/27) of the patients with Nugent BV, 7% (6/86) of patients in the Nugent normal group, and 29% (5/17) in the intermediate group. In Table II, the patient characteristics are displayed according to qPCR defined AVM. None of the investigated factors were significantly associated with AVM other than the vaginal pH. As shown in Table III, smoking was a significant risk factor for Nugent intermediate microbiota and BV. Also, there were a significantly higher proportion of women with vaginal leukocytosis in the Nugent intermediate group compared with both normal microbiota and BV.

It was observed, that the pH value was significantly lower in the normal microbiota group compared with the AVM group ($P = 0.002$).

AVM was distributed across all causes of infertility. No significant association between the AVM group and tubal infertility was observed. However, with Nugent classification the intermediate group was significantly associated with tubal infertility compared with normal flora, thus suggesting that the bacteria involved in the etiology of tubal infertility are clustered in the intermediate group (Table III). Only one of the eight women with tubal factor infertility had vaginal leukocytosis at the time of examination.

Nugent score and inter-rater variability

An in-category-agreement was observed in 66% of smears interpreted by two independent well-trained laboratory technicians (data not shown). This agreement translates into a kappa value at 0.44 which is considered fair, but not excellent. Consequently, 34% of the smears had to be diagnosed by a third and final opinion from a clinical microbiologist. *L. iners* dominated samples had a morphotype expressing some similarities with *G. vaginalis*. Figure 1 depicts how a *L. iners* qPCR classified cluster can be misinterpreted as a *G. vaginalis* morphotype in a Gram stained smear. In specimens where *L. iners* was the only bacterium exceeding the threshold in the present qPCR panel, the microscopy technicians disagreed in 38% of cases compared with 30% disagreement among all other specimens ($P = 0.4$).

Reproductive outcome analysis

Eighteen patients did not undergo fertility treatment due to patient request (15/18) or medical indication (3/18). Furthermore, 20 patients were excluded due to a vaginal swab taken more than 2 months before embryo transfer. Eight patients did not reach embryo transfer due to failed fertilization, failed cleavage, or poor embryo development. Thus, reproductive outcome data were available for a total of 84/130 (65%) patients.

The overall biochemical and clinical pregnancy rates were 45% (38/84) and 35% (29/84), respectively (Table IV). The prevalence of AVM as measured by the qPCR assay in these patients was 26% (22/84). No significant difference in the biochemical pregnancy rate was observed for the AVM group compared with the normal microbiota group with an OR of 0.36 95% CI (0.10–1.12). However, the clinical pregnancy rate was significantly lower in the AVM group compared with the normal microbiota group with a crude OR of 0.13, 95% CI (0.01–0.62). Following adjustment for number of oocytes obtained, number of previous failed cycles, number of good quality embryos available, number of embryos transferred and maternal age, both the biochemical pregnancy rate and the clinical pregnancy rate were significantly lower in the AVM group. The adjusted OR for biochemical pregnancy and clinical pregnancy were 0.22, 95% CI (0.06–0.84) and 0.06, 95% CI (0.01–0.47), respectively, when AVM was compared with normal microbiota. Nugent BV was also significantly associated with a lower clinical pregnancy rate ($P = 0.047$) as only one of 12 (8%) with BV experienced a clinical pregnancy compared with 24 (40%) of 60 with a normal microbiota (Table V). If women with

Table I Specimens above qPCR thresholds according to Nugent grading.

	Normal flora *N* = 86	Intermediate *N* = 17	Bacterial vaginosis *N* = 27
Atopobium vaginae	5 (6)	3 (18)	25 (93)
Gardnerella vaginalis	2 (2)	2 (12)	24 (89)
Lactobacillus iners	34 (40)	10 (59)	17 (63)
Lactobacillus crispatus	51 (59)	2 (12)	0 (0)
Lactobacillus jensenii	36 (42)	6 (35)	2 (7)
Lactobacillus gasseri	19 (22)	6 (35)	2 (7)
Other[1]	3 (3)	1 (6)	1 (4)

Data are *n* (percent of total number within Nugent grade).
Some patients exceeded the threshold for more than one bacterium.
[1]No abundant bacteria in the qPCR assay.

Table II Comparison of patient characteristics (*N* = 130).

	Normal vaginal microbiota	Abnormal vaginal microbiota[1]	*P*-value
Patients, *N* (%)	94 (72)	36 (28)	
Median age (quartiles)	31 (28–36)	30 (28–36)	NS
Median BMI (quartiles)	25.7 (22.3–30.4)	24.2 (21.3–29.5)	NS
Unemployed	4 (4)	2 (6)	NS
Alcohol (above WHO standard)	2 (2)	2 (6)	NS
Smoking (ever)	4 (4)	4 (11)	NS
Intercourse (within 24 h)	7 (7)	1 (3)	NS
Bleeding (within 24 h)	11 (11)	5 (14)	NS
Candida	2 (2)	2 (6)	NS
Leukocytes>epithelial cells	7 (7)	2 (6)	NS
Cycle status[2]			
Irregular cycle	11 (11)	3 (8)	NS
Day 1–15	43 (46)	22 (61)	NS
Day 16–30(±5)	35 (37)	9 (25)	NS
pH median[3] (quartiles)	4 (4–4.4)	4.7 (4–5.3)	<0.05
Cause of infertility			
Tubal factor	4 (4)	4 (11)	NS
Unknown	35 (37)	10 (28)	NS
Endometriosis	6 (6)	1 (3)	NS
Ovarian factor	9 (10)	6 (17)	NS
Male factor	35 (37)	11 (31)	NS
Single/lesbian	5 (5)	4 (11)	NS

Unless stated otherwise data are *n* (percent of patients with normal/abnormal microbiota).
[1]Patients presenting with above threshold level of *Atopobium vaginae* and/or *Gardnerella vaginalis*.
[2]Data missing for seven patients.
[3]Data missing for four patients.

intermediate flora were considered in the normal group, 28 (39%) of 72 women experienced a clinical pregnancy ($P = 0.05$). There was no difference in AVM and Nugent BV in predicting lack of clinical pregnancy (90.9 and 91.7%, respectively); absence of AVM was only marginally better in predicting success than was Nugent normal combined with intermediate flora (43.5 versus 39%, NS). Vaginal leukocytosis did not adversely affect clinical pregnancy rates ($P > 0.99$).

Discussion

In this study of infertile women attending for IVF treatment, the BV prevalence measured by Nugent score was 21% (27/130). Using qPCR diagnostics, an abnormal vaginal microbiota group defined by high loads of either *G. vaginalis* and/or *A. vaginae* was observed in 28% (36/130) of the women. The prevalence of AVM was higher than Nugent BV,

Table III Patients characteristics ($N = 130$) with respect to the Nugent classification.

	Normal flora	Intermediate flora	Bacterial vaginosis	*P*-value
Patients, *N* (%)	86 (66)	17 (13)	27 (21)	
Median age (quartiles)	31 (28–36)	37 (28–39)	30 (28–36)	<0.05*
Median BMI (quartiles)	25.6 (22.4–30.1)	27.3 (22,7–30.9)	25.6 (21.9–30.0)	NS
Unemployed	2 (2)	2 (12)	2 (7)	NS
Alcohol (above WHO standard)	1 (1)	1 (6)	2 (7)	NS
Smoking (ever)	1 (1)	2 (12)	5 (19)	<0.05**
Intercourse (within 24 h)	6 (7)	1 (6)	1 (4)	NS
Bleeding (within 24 h)	8 (9)	4 (24)	4 (15)	NS
Candida	2 (2)	1 (6)	1 (4)	NS
Leukocytes > epithelial cells	4 (5)	4 (24)	1 (4)	<0.05*
Cycle status[1]				
Irregular cycle	10 (12)	2 (12)	2 (7)	NS
Day 1–15	38 (44)	11 (65)	17 (63)	NS
Day 16–30 (±5)	28 (33)	3 (18)	6 (22)	NS
pH median[2] (quartiles)	4 (4–4.4)	4.4 (4–5.8)	5 (4–5.5)	<0.05**
Cause of infertility				
Tubal factor	2 (2)	4 (24)	2 (7)	<0.05*
Unknown	32 (37)	4 (24)	9 (33)	NS
Endometriosis	3 (3)	4 (24)	0 (0)	<0.05*
Ovarian factor	9 (10)	3 (18)	3 (11)	NS
Male factor	35 (41)	2 (12)	9 (33)	NS
Single/lesbian	5 (6)	0 (0)	4 (15)	NS

Unless stated otherwise data are *n* (percent of patients per column).
[1]Data missing for 13 patients.
[2]Data missing for four patients.
*Significance was observed only for the intermediate flora group compared with both BV and normal flora group respectively.
**Significance was observed for the BV group and the intermediate group respectively compared with the normal flora group.

Table IV qPCR classification of vaginal microbiota (VM) and reproductive outcome of IVF patients.

	Biochemical pregnancy	Clinical pregnancy
Normal VM ($N = 62$)	32 (52)	27 (44)
Abnormal VM ($N = 22$)	6 (27)	2 (9)

Data are *n* (percent of patients per row).

Table V Nugent score, reproductive outcome of IVF patients.

	Biochemical pregnancy	Clinical pregnancy
Normal flora ($N = 60$)	30 (50)	24 (40)
Intermediate ($N = 12$)	6 (50)	4 (33)
BV ($N = 12$)	2 (17)	1 (8)

Data are *n* (percent of patients per row).

primarily because Nugent intermediate flora was dichotomized. We observed inter-rater variability between laboratory technicians using the Nugent score, primarily in specimens dominated by *L. iners*. However, this was not significant. Furthermore, we observed that the Nugent intermediate group was significantly associated with tubal infertility compared with normal flora. Both methods suggested that an abnormal vaginal microbiota was associated with failure of establishing a clinical pregnancy (Tables IV and V). Taken together, we suggest that a molecular based diagnostic approach will simplify the diagnosis of AVM and may be able to diagnose IVF patients with a lower chance of obtaining clinical pregnancy. However, a clinical diagnostic qPCR panel for pathogenic bacteria to women in fertility treatment is still at a developmental stage.

The prevalence of BV in the present trial is slightly higher than the reported prevalence (14–16%) among pregnant Danish women (Svare *et al.*, 2006; Thorsen *et al.*, 2006). This observation was recently corroborated in a meta-analysis comprising 12 studies in infertile women (van Oostrum *et al.*, 2013). In this meta-analysis, the presence of an abnormal vaginal microbiota was significantly associated with tubal factor infertility. Thus, the bacteria in either BV and/or intermediate flora hold the potential to ascend to the upper genital tract and may subsequently play a role in female infertility through an infectious etiology (Swidsinski *et al.*, 2013; Mitchell *et al.*, 2015). The association between BV and tubal infertility

could also have been explained due to well-known pathogens such as *Chlamydia trachomatis* which has been described to be more prevalent in BV positive women than in women without BV (Dun and Nezhat, 2012; Tomusiak *et al.*, 2013). However, in the current study all women were negative for *C. trachomatis* as it is standard procedure to screen all Danish fertility patients for ongoing infection before initiating fertility treatment. Previous infection detected by serology was not assessed.

The Nugent score has been the laboratory diagnostic reference standard since the early 1990s. In the current study we confirmed previous observations, that Nugent BV is not a single entity (Datcu *et al.*, 2013). The Nugent score classifies bacterial communities according to morphology by Gram staining. Apparently, misclassification due to the morphological similarity between *L. iners* and *G. vaginalis* may lead to a false positive diagnosis by Nugent scoring as suggested in Fig. 1. However, not all *L. iners* dominated communities were diagnosed as Nugent BV, and this could be due to the simultaneous presence of other *Lactobacillus* spp., not detected in the current qPCR panel. Moreover, it is possible that other BV associated bacteria such as *Prevotella* spp. and *BVAB1* and *BVAB2* could have played a role in the misclassification. Thus, *L. iners* communities in this study could actually be BV subclasses dominated by e.g. *Prevotella* spp. The intermediate group is difficult to interpret clinically, and this microbiota may be an entity that should be further investigated with microbiome methods to investigate its possible pathogenicity. Interestingly, in the Nugent defined intermediate group, significantly more cases with vaginal leukocytosis were detected (Table III), suggesting that the intermediate group consists of bacterial communities which may cause vaginitis and/or cervicitis. Furthermore, three of the four women with vaginal leukocytosis were classified as normal by the qPCR, suggesting that the intermediate group contained bacterial communities with an inflammatory potential different from the BV defining species. Obviously, the intermediate microbiota would benefit from a better characterization with modern molecular techniques; however, there was no apparent association between vaginal leukocytosis and failure to obtain a clinical pregnancy, but numbers were small. Taken together, we confirmed that some of the vaginal communities are difficult to interpret with morphological methods, e.g. *L. iners* and the Nugent intermediate group. The qPCR improved the classification, although it also had difficulties classifying all vaginal microbiotas.

It is important to mention that the qPCR thresholds are arbitrary and may need to be refined according to the clinical setting. It is encouraging, however, that the thresholds for *G. vaginalis* and *A. vaginae* were in the same range as previously found in the same laboratory (Datcu *et al.*, 2013) despite the fact that the population was different and a different DNA extraction procedure was used. Furthermore, the established thresholds in the present study were in agreement with previously established thresholds from a French group in pregnant women (Menard *et al.*, 2010).

Trying to define thresholds for bacterial load based on the clinical outcomes biochemical and clinical pregnancy did not lead to meaningful associations as ROC curve analysis yielded area under the curves ranging from 0.4 to 0.51, very close to 0.5 suggesting no predictive value.

In the present study, different diagnostic approaches were tested to define an abnormal vaginal microbiota. We were able to show that both Nugent and qPCR based AVM were associated with poorer reproductive outcomes in IVF patients. Only 84/130 patients (65%) were eligible for analysis within the study period. The reason for this low figure was the study design as patients recruited at the first consultation before IVF treatment were only analyzed for the reproductive outcome if the vaginal swab was taken within 2 months before embryo transfer. A molecularly based diagnosis has the advantage of being objective and capable of classifying Nugent intermediate flora, an entity which is otherwise difficult to handle. It can be discussed whether or not it makes sense to cluster a polymicrobial disorder as BV into single species, but the results in the present study suggests that the *G. vaginalis* and *A. vaginae* cluster was significantly associated with a lower pregnancy rate. However, the weakness of the clustering in the present study is that it is limited to the two species and other bacterial species may also play an important role. Pregnancy outcomes were analyzed for each of the AVM bacteria (*A. vaginae* and *G. vaginalis*) separately and together. Furthermore, the *L. iners* group was excluded from the normal microbiota group to find a super-healthy microbiota, however, no significant findings were observed. However, the small sample number in these sub analyses should be considered.

The observation that AVM affects pregnancy rates is biologically very plausible. We hypothesized that the important factor was decreased endometrial receptivity, and, indeed, whereas biochemical pregnancy was only insignificantly affected by AVM, clinical pregnancy was much less likely in women with AVM. This could be due to upper-genital tract infection caused by *A. vaginae* and *G. vaginalis* as suggested in a recent review (Franasiak and Scott, 2015).

To our knowledge only one intervention study for BV in infertile women exists. Salah et al. allocated BV positive PCOS and unexplained infertile women to either antibiotic treatment (including the male partner) or daily standard care, reporting a significantly higher pregnancy rate in the treatment group (Salah *et al.*, 2013). However, there are important limitations to this observation especially the lack of randomization to antibiotic treatment. Furthermore, patients were not assigned to IVF procedures so the findings are difficult to compare.

Based on the findings of the present study, a molecular based test has advantages in comparison to a morphologically based test like the Nugent score. It was observed that *L. iners* is difficult to distinguish from *G. vaginalis* in Gram staining and the ability to stratify intermediate flora is a major advantage. Nugent's intermediate flora was significantly associated with vaginal leukocytosis which suggests that some intermediate communities are pathogenic due to other mechanisms than those involved in BV. AVM may negatively affect the reproductive outcome in IVF patients. If a negative correlation between AVM and the reproductive outcome is corroborated, we suggest an RCT to investigate whether or not screening and subsequent treatment for AVM prior to fertility treatment is advantageous. This minor intervention may have a significant positive impact on the pregnancy rate and ultimately the live birth rate.

A limitation of the present study is the fact that not all swabs were collected at the same time point of the fertility treatment. This might have an impact as e.g. oocyte retrieval, hormonal treatment, and cyclic fluctuations are known to affect the microbiota (Hyman *et al.*, 2012; Ravel *et al.*, 2013). However, we chose to include the small proportion of patients (5%) that did not have their swab taken at the first consultation as this study primarily was designed to evaluate the different diagnostic methods before embarking upon a larger cohort study in IVF patients. Furthermore, we could have included the Amsel criteria to better compare with other methodological studies. The reproductive outcome analysis needs to be corroborated before any firm conclusion can be made as to whether or not AVM is associated with a lower reproductive outcome. However, although the dataset was too small to allow for a detailed and conclusive adjusted analysis, there was a clear trend for a poorer biochemical as well as clinical pregnancy rate in the AVM group compared with the normal microbiota group. Next-generation-sequencing techniques would

have improved the understanding of the bacterial communities and especially the *L. iners* and 'other' groups might be better characterized. However, at present, these techniques do not have a turn-around time allowing interventions and, importantly; they do not give an exact quantitative measure of the bacterial load.

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Authors' roles

T.H., L.T. and P.H. designed the project. T.H., L.D. and J.S.J. performed the microbiological data interpretation. T.H., P.H. and J.S.J. performed the clinical data interpretation. T.H., P.H. and J.S.J. prepared the manuscript with all authors' contribution and written consent.

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Conflict of interest

None declared.

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