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ORIGINAL ARTICLE

Detection of *Methanobrevibacter smithii* in vaginal samples collected from women diagnosed with bacterial vaginosis

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Abstract

Vaginosis is a dysbiotic condition of the vaginal cavity that has deleterious effects during pregnancy. The role of methanogens in this disease is unknown since current methods of investigation are not appropriate for the search of methanogens. We prospectively investigated the presence of methanogens in vaginal specimens collected from 33 women thereafter diagnosed with bacterial vaginosis and 92 women thereafter diagnosed without bacterial vaginosis (control group) by direct microscopic examination and fluorescent in situ hybridization, PCR-sequencing, and real-time PCR and isolation and culture. These investigations found only one methanogen, *Methanobrevibacter smithii*, exclusively in 97% bacterial vaginosis specimens and in two intermediate microbiota specimens. *M. smithii* was detected microscopically in 2/20 specimens analyzed, by PCR-based observations in 34/125 specimens with 99% sequence similarity with the reference 16S rRNA and *mcr*A gene sequences and was cultured in 9/40 specimens. These data suggest that the detection of *M. smithii* could be used as a biomarker for the laboratory diagnosis of bacterial vaginosis.

Keywords Diagnosis · *Methanobrevibacter smithii* · Methanogens · Vaginosis

Introduction

Bacterial vaginosis (BV) is a common reason for medical consultation of women because of uncomfortable signs and symptoms [1–3]. For a long time, BV has been defined as a dysbiosis of the vaginal cavity [4, 5]. Accordingly, the diagnosis of BV is routinely based on the Nugent score and the Amsel criteria [6–11]. However, a new diagnostic rational test based on the molecular detection and quanification of *Atopobium vaginae* and *Gardnerella vaginalis* in vaginal samples was recently set up [12–15].

Previous studies investigating the BV microbiota by culture-based methods or metagenomics failed to detect methanogenic archaea [16–21]. This fact is not surprising as the detection of methanogens relies on specific methods which are not routinely implanted in clinical microbiology laboratories: methanogens are strict anaerobes cultured in the presence of hydrogen [22] and a specific extraction protocol breaking the solid cell wall is necessary for the (PCR)-based detection [23]. However, one work reported the isolation of *Methanobrevibacter smithii* in vaginal samples collected from two patients with BV [24]. *M. smithii* is the most prevalent methanogen in the digestive tract microbiota, detected in 95.7% of individuals [25, 26] and has been shown to act as a pathogen causing polymicrobial muscular and brain abscess [27, 28].

To further assess the spectrum of methanogens in BV microbiota, we prospectively searched for methanogens in vaginal samples collected from women diagnosed with BV and controls, using a polyphasic approach including fluorescence in situ hybridization (FISH), PCR-based detection, and culture.

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Patients and methods

Sample collection

We prospectively investigated the presence of methanogens in vaginal specimens collected from 125 women aged > 18 years consulting at the Gynecology Emergency Department at the Conception University Hospital, Marseille, France. The patients were recruited after they were given medical explanations, and they signed an informed consent. The study was authorized by the local Institut Fédératif de Recherche 48 Ethics Committee (Marseille, France) under agreement number 09-022. After medical examination, vaginal samples were collected in asymptomatic patients and in patients presenting with signs of BV (abnormal vaginal discharge or/and unpleasant, fishy odor) including non-pregnant women and pregnant women (pregnancy term ≤ 22 weeks of amenorrhea). After placement of a non-lubricated speculum into the vaginal vault, two sterile swabs (Sigma, Lezennes, France) were gently rotated against the vaginal wall. The first swab was placed into a Transwab tube (Sigma) containing 2 mL of Transwab medium then transferred to a sterile tube for DNA extraction and PCR-based tests. The second swab was placed into Transwab tube without Transwab medium then rolled onto a glass slide for Nugent score determination. A total of 20 sterile swabs impregnated with sterile phosphate buffered saline (PBS) (Life technologies, Courtaboeuf, France) were used as negative controls.

Diagnosis of bacterial vaginosis

On arrival at the laboratory, Nugent score was determined after microscopic examination of Gram-stained vaginal smear by two independent investigators. The samples were graded as normal microbiota, intermediate microbiota, or bacterial vaginosis according to previously reported criteria [13]. DNA was extracted from 200 μL of each vaginal sample with the QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France), as previously described [12]. Quantitative real-time PCR (qPCR) assay targeting *A. vaginae* and the human albumin gene (housekeeping gene used as a biomarker of quality of the vaginal sample) was carried out as previously reported [12, 15]. Negative controls (PCR mix) were also included in each PCR assay. The final data were reported as copies of the microorganism's DNA per 1 mL of vaginal suspension [12]. Finally, a high vaginal load of *A. vaginae* (DNA level $\geq 10^8$ copies/mL) established the molecular diagnosis of bacterial vaginosis based on a previously reported definition [12].

Isolation and culture of methanogens

Briefly, vaginal samples were inoculated into Hungate tubes (Dominique Dutscher, Brumath, France) containing 5 mL of a home-developed broth (SAB broth) as previously described [29, 30]; supplemented with ascorbic acid (1 g/L; VWR International, Leuven, Belgium), uric acid (0.1 g/L), and glutathione (0.1 g/L; Sigma-Aldrich, Saint-Quentin Fallavier, France). Sub-culturing inoculated broth on agar plates was done as previously described [31]. Growth of any methanogen was inferred from the production of CH_4 detected by gas chromatography as previously described [31, 32]. Hungate tubes inoculated with sterile PBS were used as negative control tubes (one negative control for five inoculated tubes) in culture experiments; and culture dishes containing the SAB medium inoculated with sterile PBS (deposited in the upper chamber of the double-chamber system) were used as negative control dishes in subculture experiments (one negative control for five dishes).

PCR assays

DNA extracted using a specific protocol described previously [25] was incorporated into PCR assays targeting the archaeal 16S rRNA gene and the *mcr*A gene as previously reported [33, 34]. All PCR products were sequenced, and the different fragments were assembled using ChromasPro1.34 software (Technelysium Pty. Ltd., Tewantin, Australia) and compared with sequences available in the GenBank database using the online BLAST program of NCBI (http://blast.ncbi.nlm.nih.gov.gate1.inist.fr/Blast.cgi). Then, extracted DNA was incorporated into real-time PCR assays specifically targeting the *mcr*A and the 16S rRNA genes of the methanogen *M. smithii*, as previously described [25].

Fluorescence in situ hybridization

Twenty vaginal swabs were investigated by FISH using the previously described technique [34, 35].

Results

Diagnosis of bacterial vaginosis

A total of 125 vaginal swabs were investigated for the presence of methanogens using a polyphasic approach. The qPCR targeting the albumin gene was positive for all 125 vaginal samples analyzed, showing that swabs contained mucosal material, that DNA was efficiently extracted and the absence of PCR inhibitors. Thus, all the vaginal samples were suitable for further molecular analyses. Among the 125 vaginal samples analyzed, 33 (26.4%) had an *A. vaginae* DNA level $\geq 10^8$ copies/mL. The Nugent score available for 28 of these 33 patients was considered as a diagnostic criterion of BV [12, 13] (Supplementary Table 1).

Methanogen PCR sequencing

Of the 125 vaginal samples analyzed by standard PCR, 34/125 (27.2%) were positive for both the 16S rRNA and the *mcr*A genes while 91/125 were negative in both PCR assays in the presence of 20 negative controls, which all remained negative. All 34 PCR-positive samples were collected from 32 patients diagnosed with BV and two additional patients presenting with an intermediate microbiota according to the Nugent score (*A. vaginae* qPCR of 10^3 and 10^7, respectively), whereas all the 91 negative samples were collected from patients without BV (Table 1). Sequencing the 16S rRNA gene PCR products disclosed 99% sequence identity with the homologous fragment of the reference 16S rRNA gene of *M. smithii* CNC strain (NCBI accession number LT223565). Furthermore, sequencing the PCR-amplified *mcr*A gene showed a 99% sequence similarity with the homologous fragment of the reference *mcr*A gene of *M. smithii* CNC strain (NCBI accession number LT223565) and *M. smithii* strain EG3 (accession NCBI: LN876654).

Methanogen real-time PCR assays

Real-time PCR assays targeting the 16S rRNA and *mcr*A genes confirmed the results of PCR sequencing results. Indeed, of the 125 investigated samples, 91 samples collected

Table 1 Summary of results obtained investigating 125 vaginal samples for the presence of *A. vaginae* (as a biomarker for BV) and methanogens

N° samples	Ct albumin	*A. vaginae* molecular quantification (per mL)	BV	Standard PCR (16S rRNA) methanogen detection	Standard (PCR *mcr*A) methanogen detection	RT-PCR *mcrA* methanogen detection	RT-PCR ARN 16S	RT-PCR 16S rRNA *M. smithii* quantification
1	26	105	Absence	Neg	Neg	Neg	Neg	
2	22	0	Absence	Neg	Neg	Neg	Neg	
3	24	103	Absence	Neg	Neg	Neg	Neg	
4	24	0	Absence	Neg	Neg	Neg	Neg	
5	26	0	Absence	Neg	Neg	Neg	Neg	
6	25	107	Absence	Neg	Neg	Neg	Neg	
7	28	0	Absence	Neg	Neg	Neg	Neg	
8	25	103	Absence	Neg	Neg	Neg	Neg	
9	24	0	Absence	Neg	Neg	Neg	Neg	
10	23	0	Absence	Neg	Neg	Neg	Neg	
11	23	0	Absence	Neg	Neg	Neg	Neg	
12	23	107	Absence	Neg	Neg	Neg	Neg	
13	22	0	Absence	Neg	Neg	Neg	Neg	
14	27	0	Absence	Neg	Neg	Neg	Neg	
15	24	107	Absence	Neg	Neg	Neg	Neg	
16	25	103	Absence	Positive	Positive	37.56	36.67	6,10E+01
17	26	107	Absence	Positive	Positive	37.01	37.43	1,10E+01
18	22	107	Absence	Neg	Neg	Neg	Neg	
19	22	0	Absence	Neg	Neg	Neg	Neg	
20	24	0	Absence	Neg	Neg	Neg	Neg	
21	23	109	Presence	Neg	Neg	Neg	Neg	
22	26	108	Presence	Positive	Positive	34.54	32.87	3,10E+02
23	22	108	Presence	Positive	Positive	34.19	31.10	1,74E+04
24	26	108	Presence	Positive	Positive	36.43	32.45	3,60E+02
25	24	109	Presence	Positive	Positive	32.13	29.97	6,00E+04
26	22	1010	Presence	Positive	Positive	33.21	31.04	1,70E+04
27	24	109	Presence	Positive	Positive	31.53	34.65	1,86E+02
28	22	108	Presence	Positive	Positive	30.22	30.45	6,74E+04
29	21.48	108	Presence	Positive	Positive	37.73	30.65	6,66E+04
30	20.5	109	Presence	Positive	Positive	36.34	32.56	3,41E+02
31	17.65	109	Presence	Positive	Positive	29.56	28.87	2,04E+05
32	26.22	108	Presence	Positive	Positive	28.03	29.76	1,20E+05

Table 1 (continued)

N° samples	Ct albumin	*A. vaginae* molecular quantification (per mL)	BV	Standard PCR (16S rRNA) methanogen detection	Standard (PCR *mcr*A) methanogen detection	RT-PCR *mcrA* methanogen detection	RT-PCR ARN 16S	RT-PCR 16S rRNA *M. smithii* quantification
33	31.82	109	Presence	Positive	Positive	29.87	28.03	4,30E+05
34	23.74	109	Presence	Positive	Positive	31.54	29.45	2,04E+05
35	26.33	108	Presence	Positive	Positive	27.7	24.34	9,49E+05
36	22.94	1010	Presence	Positive	Positive	29.79	27.02	6,49E+05
37	24.4	109	Presence	Positive	Positive	30.06	29.45	4,04E+04
38	25.86	108	Presence	Positive	Positive	29.98	28.09	2,04E+05
39	24.67	109	Presence	Positive	Positive	32.86	30.67	6,69E+04
40	20.66	108	Presence	Positive	Positive	29.57	28.87	2,04E+04
41	20.94	108	Presence	Positive	Positive	28.06	29.76	2,04E+05
42	30.89	108	Presence	Positive	Positive	32.78	28.93	2,04E+05
43	22.53	108	Presence	Positive	Positive	34.85	32.32	4,13E+02
44	24.4	109	Presence	Positive	Positive	26.54	26.78	8,49E+05
45	25.86	108	Presence	Positive	Positive	28.67	28.67	2,04E+05
46	24.67	108	Presence	Positive	Positive	27.36	28.68	2,04E+05
47	20.66	108	Presence	Positive	Positive	29.08	29.09	2,04E+05
48	20.94	108	Presence	Positive	Positive	26.89	26.78	8,49E+05
49	21.25	108	Presence	Positive	Positive	26.79	28.65	2,02E+05
50	26.27	108	Presence	Positive	Positive	29.33	28.54	2,08E+05
51	19.19	109	Presence	Positive	Positive	29.84	28.53	2,05E+05
52	19.67	109	Presence	Positive	Positive	27.65	27.58	6,47E+05
53	19	109	Presence	Positive	Positive	31.01	29.90	1,09E+04

BV Bacterial vaginosis, *Neg* Negative

from non-BV women were negative in the two real-time PCR assays (Ct ≥ 39) while 34 samples collected from women diagnosed with BV were positive in the two real-time PCR assays (Ct < 39) (Table 1).

Methanogen FISH testing

Of the 20 samples investigated by FISH (Fig. 1) using either a probe targeting methanogen 16S rRNA gene or a probe targeting the methanogen *mcr*A gene, two yielded diplococcobacilli morphology corresponding to the expected form of *Methanobrevibacter smithii*.

Methanogen isolation and culture

To test the viability of the archaea methanogens detected by PCR- based methods and FISH, methanogen culture was done on 40 samples in the presence of negative controls which remained negative. A total of nine samples yielded colonies within 17–20 days of incubation. All the colonies were identified as *M. smithii* on the basis of PCR-sequencing of the 16S rRNA and *mcr*A genes, which found 100% sequence similarity with the reference homologous genes 16S rRNA *M. smithii* CNC strain (NCBI accession number LT223565).

Discussion

Data herein reported indicate a significant association between BV and the presence of the methanogen *M. smithii* in the vaginal sample. Indeed, the reported sensitivity of *M. smithii* PCR-based detection was of 97%, and its specificity was of 97.2% compared with the BV biomarker *A. vaginae* [14]. The fact that the negative control introduced in every experimental step remained negative authenticates the results. Also, concordant results were obtained using three different technical approaches including PCR-based detection, FISH, and culture. Unsurprisingly, real-time PCR yielded the very same results as PCR-sequencing used as a first-line system. Interestingly, real-time PCR is well adapted to routine diagnosis. Accordingly, one previous study reported the detection of *M. smithii* in two of three vaginal samples collected from women diagnosed with BV and not in nine vaginal samples collected from controls [24].

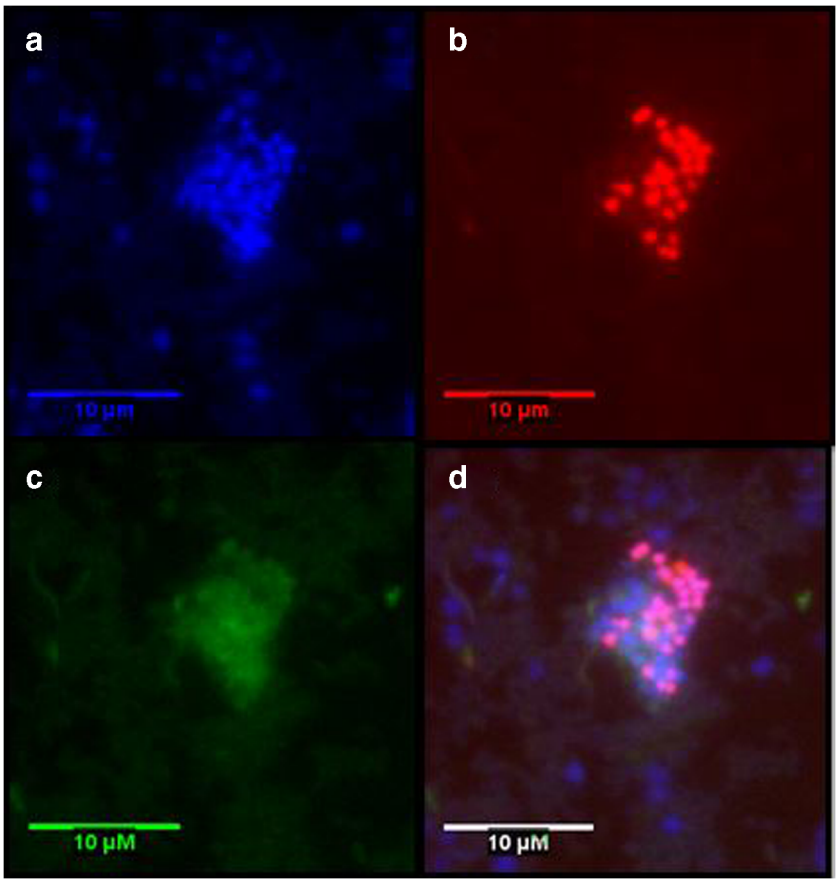

Fig. 1 Fluorescence in situ hybridization for the detection of methanogenic archaea in one vaginal sample collected in one case of BV. **a** DAPI signal (blue) and universal marker; **b** archaea ARC 915 RHOD (red) universal probe targeting the methanogen 16S rRNA. **c** Archaea *mcr*A FITC (green) probe targeting the methanogen *mcr*A gene. **d** Combined colors. Scale bar, 10 µm

We observed that only *M. smithii* was detected by PCR sequencing and culture in the vaginal samples collected from women diagnosed with BV. *M. smithii* is one of the 15 methanogens detected in humans, among which, seven are cultured [25, 35–40] (Table 2). *M. smithii* has been previously detected by molecular approaches in the respiratory microbiota and in the skin microbiota [41], in the oral cavity [38] and the intestinal microbiota [36], and has been isolated and cultured from the oral cavity and intestinal gut [35, 42].

Table 2 The methanogens currently detected in the human microbiota

Species	Detection techniques	Sources	References
Methanobrevibacter smithii	Molecular and culture	Stool, oral cavity	[25, 35, 38]
Methanobrevibacter oralis	Molecular and culture	Stool, oral cavity	[38, 39]
Methanosphaera stadtmanae	Molecular and culture	Stools	[36]
Methanomassilicoccus luminyensis	Molecular and culture	Stools	[37]
Ca. *Methanomassillicoccus intestinalis*	Molecular and culture	Oral cavity	[38]
Ca. *Methanomethylophilus alvus*	Molecular and culture	Stools	[39]
Methanobrevibacter arboriphiiclus	Molecular and culture	Stools	[36]
Methanosarcina mazei	Molecular	Oral cavity	[38]
Ca. *Methanomethylophilus* sp.	Molecular	Oral cavity	[38]
Methanobacterium congolense	Molecular	Stools	[36]
Methanoculleus chikugoensis	Molecular	Stools	[36, 39]
Methanobrevibacter millerae	Molecular	Stools	[38]
Methanobrevibacter massiliense	Molecular	Oral cavity	[40]
Candidatus Nitrososphaera evergladensis	Molecular	Oral cavity	[38]
Methanoculleus bourgensis	Molecular	Oral cavity	[38]

Metagenomic analyses of the bacterial composition of BV indicated a population including members of *Anaerococcus*, *Clostridium*, *Peptoniphilus*, and *Prevotella*, which evoked fecal populations [17]. Accordingly, the detection of *M. smithii* by molecular methods (investigating the *nif*H gene of *M. smithii*) has been previously used as a proxy for the fecal contamination of environmental sources [43, 44]. The detection of *M. smithii* in BV samples is in line with the current hypothesis that BV results from a fecal graft into the vaginal cavity [45]. Alternatively, *M. smithii* could be introduced into the vaginal cavity during oral sex, yet this hypothesis does not explain the presence of fecal bacteria into the vaginal cavity during BV.

In conclusions, this study provides evidence that among methanogens, only *M. smithii* is linked to BV. This evidence reinforces the hypothesis that BV is a dysbiosis resulting from the graft of the fecal microbiota into the vaginal cavity. We propose the detection of *M. smithii* in vaginal sample as a diagnosis biomarker of BV.

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Authors' contribution GG performed detection of methanogens, analyzed the data, and drafted the manuscript. HD took care of the patients and collected specimens. FF designed the study, analyzed the data, and drafted the manuscript. FB designed the study and took care of the patients. DR designed the study, analyzed the data, and drafted the manuscript. MD designed the study, analyzed the data, and drafted the manuscript.

All authors read and approved the final manuscript.

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Availability of data and materials The datasets used and/or analyzed during the current study are available from the corresponding author.

Compliance with ethical standards

Conflict of interest GG, FF, DR, and MD are co-inventors of a patent on the molecular detection of *M. smithii* for the diagnosis of BV.

Ethical approval The study was authorized by the local Institut Fédératif de Recherche 48 Ethics Committee (Marseille, France) under agreement number 09-022.

Informed consent All patients studied in this report have signed an informed consent.

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