Co-culture of Methanobrevibacter smithii with enterobacteria during urinary infection

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Summary

Background. Urinary tract infections are known to be caused by bacteria, but the potential implications of archaea have never been studied in this context.

Methods. In two different university hospital centres we used specific laboratory methods for the detection and culture of archaeal methanogens in 383 urine specimens prospectively collected for diagnosing urinary tract infection (UTI).

Findings. *Methanobrevibacter smithii* was detected by quantitative PCR and sequencing in 34 (9%) of the specimens collected from 34 patients. *Escherichia coli, Klebsiella pneumoniae, Enterobacter* sp., *Enterococcus faecium* and mixed cultures were detected along with *M. smithii* in eighteen, six, three, one and six urine samples, respectively. Interestingly, using our specific culture method for methanogens, we also isolated *M. smithii* in 31 (91%) of the 34 PCR positive urine samples. Genotyping the 31 isolates using multispacer sequence typing revealed three different genotypes which have been previously reported in intestinal microbiota. Antibiotic susceptibility testing found the 31 isolates to be *in vitro* susceptible to metronidazole (MIC: 1 mg/L) but resistant to fosfomycin, sulfamethoxazole-trimethoprim, amoxicillin-clavulanate and ofloxacin, commonly used to treat bacterial UTI. Finally, 19 (54%) of the 34 patients in whose urine samples *M. smithii* was detected were diagnosed with UTIs, including cystitis, pyelonephritis and prostatitis.

Interpretation. Our results show that *M. smithii* is part of the urinary microbiota of some individuals and could play a role in community-acquired UTI in association with enteric bacteria.

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Introduction

Forty-eight-hour culturing of a large volume of urine samples collected from women by transurethral catheter in parallel with suprapubic aspiration has shown that urine hosts a resident microbiota, which includes bacteria missed by routine procedures[1]. This resident urinary microbiota has been further explored using an expanded quantitative urine culture (EQUC) method[2,3]. Metagenomic analyses have confirmed that urine is not sterile in healthy individuals[4-8] .

An interesting concept currently emerging is that cases of urinary tract infection (UTI) may result from disequilibrium in the repertoire of the urinary microbiota rather than from the introduction of an exogenous pathogenic organism[8,9]. Therefore, efforts to characterise the repertoire of organisms residing in the urinary microbiota are important for clinical microbiology and medicine.

Current culture-dependant and culture-independent studies of the urinary microbiota rely on methods that are unable to detect archaea. These micro-organisms form a living domain distinct from that of bacteria and have been detected and cultured from the oral and gut microbiota[10-12]. Moreover, the specific archaea group of methanogens, characterised by the ability to produce methane from hydrogen, are recognised as emerging pathogens in a few clinical situations, including life-threatening brain abscess [12-15].

In this study, we aimed to characterise the repertoire of human urine microbiota using laboratory protocols specifically designed for the detection and culture of methanogens in urines. Surprisingly, we found that among the six previously-described methanogens in non-urine human microbiota, only *Methanobrevibacter smithii* can be detected and cultured from urines.

Patients and methods

Sample collection. The retrospective study was approved by the Ethics Committee of the IHU Méditerranée Infection under n° 2016-01. A total of 383 urine specimens taken for microbiological diagnosis of UTI in 383 patients without urinary catheter were prospectively collected at the IHU Méditerranée Infection Laboratory, Marseille, France (henceforth referred to as laboratory 1) (183 urines) and at the Laboratory of Bacteriology at the University Centre of Nice (henceforth referred to as laboratory 2) (200 urines) using a BD Vacutainer (Becton Dickinson, Le Pont de Claix, France). In this study, clean catch midstream urine specimen were collected by from September 2017 to November 2017 at laboratory 1 and from January 2018 to March 2018 at laboratory 2. A total of 50 BD vacutainer tubes filled with sterile phosphate buffered saline (PBS) (Thermo Fisher Scientific, Villebon-sur-Yvette, France) were intercalated in every 8 urine specimens to serve as negative controls in all further laboratory steps. The following data on each patient included in the study were anonymously collected: age, sex, underlying disease of the urinary tract, immune status, symptoms of urinary tract infection, antibiotic treatment (molecule and duration) and clinical outcome.

Routine investigations. Urine leukocyte and erythrocyte counts were obtained with a UF-1000i® analyser (Sysmex, bioMérieux, Craponne, France) in laboratory 1 and with an IQ 200 IRIS analyser (Beckman Coulter, Villepinte, France) in laboratory 2. Gram staining was carried out with the automated PREVI Color Gram (bioMérieux) at both laboratories in order to stain bacteria. Indeed, methanogens are not usually coloured by using Gram staining. A 200-µL urine volume was cultured on a COLUMBIA COS medium (bioMérieux) (laboratory 1) or on Uriselect medium (Bio-Rad, Marnes-la-Coquette, France) (laboratory 2) and incubated at 37 °C for 24 h (both laboratories), while visible colonies were identified by

matrix-assisted laser desorption ionisation–time of flight mass spectrometry (Brucker, Wissembourg, France), as previously described[16].

Methanogen DNA extraction, PCR and sequencing. The manual protocol was carried out using the NucleoSpin® Tissue Mini Kit (Macherey Nagel, Hoerdt, France) as previously described[11,17]. Extraction of 250 µL of sterile PBS was included in each DNA extraction series as a negative control.

Methanogen PCR and sequencing was performed as described in previous articles[11,17] using the 16S rRNA broad range archaeal forward primer 5'-CCGGGTATCTAATCCGGTTC- 3' and reverse primer 5'-CTCCCAGGGTAGAGGTGAAA-3'[18], and the *mcr*A gene forward primer 5'-GCTCTACGACCAGATMTGGCTTGG-3' and reverse primer 5'-CCGTAGTACGTGAAGTCATCCAGCA-3'[19]. All PCR products were sequenced and the various fragments were assembled using the ChromasPro1.34 software (Technelysium Pty. Ltd., Tewantin, Australia) and compared with sequences available in the GenBank database using the online NCBI BLAST program (http://blast.ncbi.nlm.nih.gov.gate1.inist.fr/Blast.cgi). Extracted DNA was also analysed by real-time PCR targeting *M. smithii* using the following primer pair: *M. smithii*-F (5'-ACCATAACyATCAGCAGCATTAT-3') and *M. smithii*-R (5'-AGTATTGGTGAAGGATTTaCTGT-3') (Eurogentec, Seraing, Belgium) and the *M. smithii* probe (6-carboxyfluorescein [FAM]-5'ACCyTTATCAGCTTTACCA TTAATyAAAG-3') (Applied Biosystems, Courtaboeuf, France) as described in a previous study[13].

Methanogen isolation and culture. A 250 µL volume of urine sample was seeded in ambient air in a sterile Hungate tube[20] (Dominique Dutscher, Brumath, France). The Hungate culture tube was developed specifically for growing and storing strictly anaerobic bacteria and archaea. It features an autoclavable screw cap with a 9-mm opening, a non-toxic, gas-impermeable butyl rubber stopper and a disposable screw cap. We placed 5 mL of SAB broth[21] into each Hungate tube, seeded it with urine then inoculated it with *Bacteroides*

thetaiotaomicron (10^5 cells/mL) for hydrogen production[22]. The mixture in the Hungate tube was then incubated at 37 °C with agitation for seven days. Methanogen growth was inferred from the production of CH_4 detected by gas chromatography, as previously described[11] . The subculture was seeded on a Petri dish containing SAB medium supplemented with 15 g/L agar and placed in the upper chamber of a double-chamber box. Tubes inoculated with sterile PBS were used as negative controls in the archaea isolation experiments (one negative control every five tubes); culture dishes containing the SAB medium inoculated with sterile PBS were used as negative controls in the sub-culture experiments (one negative control every five dishes).

Characterisation of isolates. Isolates were further genotyped using the Multi Spacer Typing method, as previously described[23]. In addition, we determined the antibiotic susceptibility profile of the isolates, as previously described, by incorporating the following antibiotics: fosfomycin (100 mg/L), sulfamethoxazole-trimethoprim (50 mg/L), amoxicillin-clavulanate (100 mg/L) and ofloxacin (1 mg/L) (BIOGARAN, Colombes, France)[21]. *M. smithii* culture with metronidazole (1 mg/L) was used as the positive control and *M. smithii* culture without metronidazole as the negative control.

Statistical analyses. Data were analysed with Prism 7.0 (GraphPad Software) by unpaired Student's t-test and chi-square test (** $p < 0.01$, * $p < 0.05$, ns: non-significant). We used the former to compare the quantitative variables from the two centres (pH, urine salinity, white and red blood cells), and the latter to compare the categorical variables (sex ratio, % of standard cultures positive, % of urine samples positive for methanogens by molecular or culture methods).

Results

Population and routine investigations. A total of 383 urine specimens from 383 patients were collected and analysed prospectively, 183 specimens in laboratory 1 and 200 specimens in laboratory 2. Mean patient age was 56 years (0-95 years) and 61% of the patients were women. Leukocyturia was significant in 205 of the 383 specimens ($> 10^4$ leukocytes/mL) and 142 specimens yielded bacterial growth. Comparison of the data collected from laboratory 1 and from laboratory 2 is shown in Table 1.

Methanogen quantitative PCR and sequencing. Of the 383 urine samples analysed by standard PCR sequencing, 18/183 (10%) from laboratory 1 and 16/200 (8%) from laboratory 2 were positive for both archaeal 16S rRNA PCR and the *mcr*A PCR, while 165/183 (90%) and 184/200 (92%), respectively, were negative in both PCR assays in the presence of 50 negative controls, all of which remained negative. Sequencing the 16S rRNA PCR products revealed 99% sequence similarity with the homologous fragment of the reference 16S rRNA gene of *M. smithii* strain NVD (accession NCBI: LT223565). Further sequencing of the PCR-amplified *mcr*A gene revealed 99% sequence similarity with the homologous fragment of the reference *mcr*A gene of *M. smithii* strain NVD (accession NCBI: LT223565) and *M. smithii* ATCC 35061 (accession NCBI: NR_074235). Quantitative PCR analyses targeting the *M. smithii* 16S rRNA gene yielded a median Ct of 33.7 ± 2.47, indicative of an *M. smithii* load of $1.24*10^3 \pm 1.56*10^3$ / mL. Detection of *M. smithii* in urine samples was significantly associated with leukocyte counts greater than 10^4 /mL, P-value = 0.0015 (Chi-square test).

Isolation and culture of *M. smithii*. To test the viability of the *M. smithii* organisms detected by the PCR-based methods, methanogen was cultured on 18 samples in laboratory 1 and 16 samples in laboratory 2. Fifteen of the 18 cultured samples in laboratory 1 and all 16 cultured samples in laboratory 2 yielded colonies, which were obtained after 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 revealed 100% sequence similarity with the reference homologous genes in *M. smithii* strain NVD (accession NCBI: LT223565) (Table 1). MST genotyping indicated that the *M. smithii* isolates belonged to genotype 2 (n=13), genotype 3 (n=10) and genotype 1 (n=8) (Supplementary Table 1). Antibiotic susceptibility testing indicated that fosfomycin (100 mg/L), sulfamethoxazole-trimethoprim (50 mg/L), amoxicillin-clavulanate (100 mg/L) and ofloxacin (1 mg/L) were inactive against *M. smithii*, whereas metronidazole - used as a positive control - inhibited the growth of *M. smithii*.

Microbiological and clinical analyses. *M. smithii* was detected in 34 (9%) of the 383 urine samples analysed in the study. *Escherichia coli*, *Klebsiella pneumoniae*, *Enterobacter* sp. and *Enterococcus faecium* were detected along with *M. smithii* in 18, 6, 3 and 1 urine samples, respectively. The other six samples in which *M. smithii* was detected yielded mixed cultures including *Enterobacteriaceae*. Of the 34 patients with *M. smithii* in their urine samples, 63.4% were women, and mean patient age was 63 years (20-95). Ten patients (30%) had an underlying urinary tract disease, 14 (40%) were immunosuppressed and 19 (44%) had chronic renal failure (GFR < 60L/min). It is worth noting that *M. smithii* was associated with UTI in 19 (56%) patients and with colonisation in 15 (44%). Of the 19 patients with UTIs, 17 (88%) were community acquired, 13 presented with cystitis, while four were diagnosed with pyelonephritis and two with prostatitis. All 19 patients received antibiotic treatments targeting the isolated bacteria and commonly used to treat UTIs, including beta-lactams, fluoroquinolones and fosfomycin-trometamol, with favourable outcomes (Table 2).

Discussion

M. smithii was the only methanogen prospectively detected in the urines using a polyphasic approach, including molecular detection by PCR sequencing of two unrelated genes, and isolation and culture methods specifically developed for methanogenic archaea. Given that the negative controls included in every experimental procedure remained negative, that *M. smithii* was detected by different unrelated technical approaches, and that similar data were obtained in two unrelated laboratories, we can exclude the possibility of in-laboratory contamination and centre bias in the study. These pieces of experimental evidence, therefore, validate the data reported here.

M. smithii was first detected as a normal organism in the gut microbiota of the vast majority of individuals[11,24], although it is depleted in children diagnosed with severe malnutrition and kwashiorkor[25]. It was then shown to be a component of the oral cavity microbiota in the saliva[17] and the dental plaque, including periodontitis[26,27] and peri-implantitis lesions[10,28]. More recently, the pathogenic potential of *M. smithii* was illustrated by its isolation and culture from a muscular abscess[14] and by its detection in one case of brain abscess[13]. Our study is of interest because it reports for the first time *M. smithii*, methanogens and archaea at large in urines, as all previous culture-based and culture-independent studies have failed to detect archaea in this type of fluid. Our success in detecting *M. smithii* was down to the use of laboratory tools that we developed specifically for detecting methanogens[22, 29-31]. These tools, including the technique we set up for easy isolation and culture of methanogens, could be easily implemented in other clinical microbiology laboratories enabling them to investigate the presence of methanogens in urines[30].

We detected *M. smithii* in 9% of urine samples, and, furthermore, in the same proportions of patients in two unrelated laboratories (10% in laboratory1 and 8% in laboratory 2). This supports the idea that *M. smithii* may be part of "the urinary microbiota", a new

concept that has emerged since the expansion of urine metagenomic analyses and EQUC approaches. It is worth noting that all the urine samples which cultured *M. smithii* also cultured enterobacteria, such as *E. coli*, *K. pneumoniae*, *Enterobacter sp.*,which are known to produce hydrogen as a substrate for methanogenesis[32]. The fact that *M. smithii* was isolated along with enterobacteria and never detected in a urine sample without bacterial growth suggests that it could play a role in or even induce dysbiosis, which facilitates the growth of enterobacteria, acknowledged agents of UTI. In our study, 19 (54%) of the 34 patients with *M. smithii* in their urine samples presented with UTIs, were most of them community acquired. These preliminary results need to be confirmed by further studies, including epidemiological, clinical and fundamental approaches, in order to determine the role of *M. smithii* in UTIs. Finally, our study indicates that the antibiotics commonly used to treat bacterial urinary tract infections are inactive against *M. smithii*, consistent with a previous report of its narrow spectrum of *in vitro* susceptibility[33]. Interestingly, the 19 patients in whose urine samples we detected *M. smithii* and who presented with UTI symptoms showed clinical improvement after receiving antimicrobial treatment active against enterobacteria but inactive against methanogens. We hypothesise that the inhibition of enterobacterial growth by appropriate antibiotic treatment induces a decrease ²in hydrogen production, a major substrate for methanogenesis, and therefore destroys the metabolic cooperation between these two microorganisms.

In conclusion, our data show that *M. smithii* is part of the urinary microbiota in some patients and suggest that it plays a role in community-acquired UTIs in association with enterobacteria. Therefore, we encourage colleagues to use the above-described methods to investigate methanogens in urines, including *M. smithii*, in order to determine its potential contribution to the pathogenesis of UTIs.

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Author contributions

GG and RL wrote the manuscript. GG, RL cultivated the methanogens and carried out the PCR. DC, RL and AC performed the clinical analyses. MD, DR and RR supervised the study.

Conflicts of interests

GG, RL, DC, AC, DR, MD, RR declare no potential conflict of interest.

Table 1. Comparison of the data collected in laboratory 1 and in laboratory 2.

	Laboratory 1	**Laboratory 2**	**Statistical analysis (+)**
Number of patients	183	200	
Sex ratio (male/female)	56/127	93/107	p=0.0014 (Chi-square test)
Age	55 +/- 25	57 +/- 21	p=0.44 (t-test)
Number of urine samples	183	200	
Routine urine analysis			
pH*	6.36 +/- 0.97	6.31 +/- 0.97	p=0.62 (t-test)
Urine salinity ‰*	43.09 +/-18.06	42.18 +/- 16.86	p=0.61 (t-test)
White blood cells**	16.1 (0.1-3505)	8 (0-1000)	p=0.012 (t-test)
Red blood cells**	14.4 (0.1-7554)	7 (0-1000)	p=0.06 (t-test)
Standard culture +	53/183 (29%)	89/200 (45%)	p=0.002(Chi-square test)
Methanogen approach			
Archaea *16S rDNA* PCR +	18/183 (10%)	16/200 (8%)	p=0.44(Chi-square test)
mcrA PCR +	18/183 (10%)	16/200 (8%)	p=0.44(Chi-square test)
Sequencing	*M. smithii* (n=18)	*M. smithii* (n=16)	
Methanogen culture +	15/183 (8.2%)	16/200 (8%)	p=0,83(Chi-square test)
Strain identification	*M. smithii* (n=15)	*M. smithii* (n=16)	

* pH and Urine salinity values are expressed as mean +/- SD.**White and red blood cells are expressed as median (range).

Table 2. Clinical and microbiological features of 19 patients presenting with UTIs and in whose urines *M. smithii* was detected

Type of UTI	Age (sex)	Underlying diseases of the genitourinary tract	Nosocomial	Leukocytes in urine (/µL) **	Associated bacteria (CFU/mL)	Treatment (Duration)	Course of infection
Cystitis	87 (F)	No	Yes	>1000	*Escherichia coli* (10^6)	Amoxicillin-clavulanic acid (7 days)	Favourable
	65 (F)	No	No	64	*Escherichia coli* (10^6) and *Proteus mirabilis* (10^6)	Fosfomycin-trometamol (1 day)	Reinfection at day 45 (*E. coli* and *P. mirabilis*)
	74 (F)	No	No	5	*Klebsiella pneumoniae* (10^4)	Pivmecillinam (7 days)	Favourable
	55 (F)	No	No	592	*Escherichia coli* (10^3)	Ofloxacin (NA)	Favourable
	25 (F)	No	No	140	*Escherichia coli* (10^6)	Pivmecillinam (5days)	Favourable
	52 (F)	Renal transplant	Yes	10	*Klebsiella pneumoniae* (10^6)	Ceftriaxone (NA)	Favourable
	50 (F)	No	No	464	*Escherichia coli* (10^6)	NA	NA
	83* (F)	No	No	23	*Escherichia coli* (10^6)	Fosfomycin and Colistin (42 days)	NA
	36 (F)	No	No	11	*Escherichia coli* (10^6)	NA	NA
	20 (F)	No	No	78	*Escherichia coli* (10^6)	Pivemecillinam (5days)	Favourable
	56* (M)	No	No	25	*Escherichia coli* (10^6)	Ciprofloxacin (3 days)	Favourable

	59 (F)	Renal transplant	No	4	*Klebsiella pneumoniae* (10^6)	Ciprofloxacin (7 days)	Favourable
	79* (F)	Chronic renal failure	No	85	*Enterobacter cloacae* (10^6)	Amoxicillin-clavulanic acid (2 days)	Favourable
Pyelonephritis	49 (F)	Renal lithiasis	No	150	*Enterobacter cloacae* (10^3)	Ceftriaxone IV and gentamicin (NA)	Favourable
	69 (M)	Renal lithiasis	No	20	*Escherichia coli* (10^6)	Ceftriaxone IV (5days)	Favourable
	75 (F)	Vesico-vaginal fistula	No	>1000	*Klebsiella pneumoniae* (10^6)	Imipenem IV (10 days)	Favourable
	83 (F)	No	No	53	*Escherichia coli (*10^6)	Ofloxacin (1day) and amoxicillin-clavulanic acid (7days)	Favourable
Prostatitis	69 (M)	No	No	221	*Escherichia coli (*10^5)	Ceftriaxone and gentamicin (NA)	Favourable
	50 (M)	No	No	>1000	*Escherichia coli* (10^4)	Pivmecillinam (15 days)	Favourable

(*) *M. smithii* was detected in the urine of this patient using molecular methods, but not isolated in culture using the approach developed specifically for archaea.
(**) Significant threshold was ≥10/µL for leukocyturia. NA: not available. Favourable: patient with favourable outcome based on clinical evidence.

Supplementary Table. Multispacer sequence typing (MST) genotyping of the 31 strains of *M. smithii* isolated during the study.

Sample	spacer 1	spacer 2	spacer 3	spacer 4	spacer Type
13	X	X	X		1
14	X	X	X	X	2
41		X	X		3
42		X	X		3
52	X	X	X		1
58		X	X		3
63	X	X	X	X	2
75	X	X	X	X	2
99		X	X		3
106	X	X	X		1
112		X	X		3
126	X	X	X	X	2
128	X	X	X	X	2
133	X	X	X		1
149	X	X	X	X	2
167		X	X		3
4	X	X	X	X	2
6	X	X	X		1
11	X	X	X	X	2
13		X	X		3
49	X	X	X		1
67	X	X	X	X	2
81	X	X	X	X	2
87		X	X		3
108	X	X	X	X	2
114		X	X		3
115	X	X	X		1
125		X	X		3
173	X	X	X	X	2
177	X	X	X		1
188	X	X	X	X	2