Co-culture of *Methanobrevibacter smithii* with enterobacteria during urinary infection

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**Abstract**

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**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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Research in context

Evidence before this study

Generally, bacteriological research in urine samples is used for the isolation and identification of pathogenic microorganisms involved in the development of urinary tract infections. Today, with the advent of molecular biology techniques, metagenomic approaches independent of culture have shown that there is indeed a whole urinary microbiota with true bacterial diversity. All these metagenomics and culture-based studies have missed methanogens which are archaeal microorganisms otherwise detected and cultured from the gut microbiota and oral microbiota.

Added value of this study

This study is the first to have initiated the detection of methanogens by molecular approaches and especially culture in human urine samples.

Implications of all the available evidence

This is the first report of M. smithii, methanogens and archaea at large in urines as all the previous culture-based and culture-independent studies failed to detect archaea in this type of fluid. Herein detecting M. smithii indeed resulted from the engagement of laboratory tools that we specifically developed for the detection of methanogens. These specific tools could be easily implemented in other laboratories including the technique we set-up for the easy isolation of culture of methanogens enabling other laboratories to investigate the presence of methanogens in the urines.

2. Patients and methods

2.1. 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 Claux, 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.

2.2. 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 IFRS 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 an 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 (Bruker, Wissembourg, France), as previously described [16].

2.3. Methanogen DNA extraction, PCR and sequencing

The manual protocol was carried out using the NucleoSpin® Tissue Mini Kit (Macherey Nagel, Hoerd, 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′-CCGCGGTATCTAATCCGGTTC-3′ and reverse primer 5′-CTCCCAACGGTAGGTCAAA-3′ [18], and the mcrA gene forward primer 5′-GCTCTACGACCATGTTGCTTG-3′ and reverse primer 5′-CCGTAGTACCCGAAGTCAACAGCA-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/insti.fr/Blast.cgi).

Extracted DNA was also analysed by real-time PCR targeting M. smithii using the following primer pair: M. smithii-F (5′-ACCATAC yATCACGACGATTAT-3′) and M. smithii-R (5′-AGTATTGGTGAAGGATTT aCTGT-3′) (Eurogentec, Seraing, Belgium) and the M. smithii probe (6-carboxyfluorescein [FAM]-5′-ACCTTTATCATCCTTACCA TTAAyyAAGG-3′) (Applied Biosystems, Courtaboeuf, France) as described in a previous study [13].

2.4. 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 medium 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).
2.5. 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 ofloxcin (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.

2.6. Statistical analyses

Data were analysed with Prism 7.0 (GraphPad Software) by unpaired Student’s t-test and chi-square test (\( \times p < .01, * p < .05, \) 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).

3. Results

3.1. 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 (\( >10 \) leukocytes/mL) and 142 specimens yielded bacterial growth. Comparison of the data collected from laboratories 1 and 2 is shown in Table 1.

3.2. 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 mcRA 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 mcRA gene revealed 99% sequence similarity with the homologous fragment of the reference mcRA 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 1.24×10³ [3] ± 1.56×10³/mL. Detection of M. smithii in urine samples was significantly associated with leukocyte counts >10 [4]/mL, P-value = .0015 (Chi-square test).

3.3. 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 mcRA 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 ofloxcin (1 mg/L) were inactive against M. smithii, whereas metronidazole – used as a positive control – inhibited the growth of M. smithii.

3.4. 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 < 60 L/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-trimetamol, with favourable outcomes (Table 2).

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          | p = .0014 (Chi-square test) |
| Sex ratio (male/female) | 56/127       | 93/107       | p = .44 (-test)           |
| Age                     | 55 +/- 25    | 57 +/- 21    |                          |
| Number of urine samples | 183          | 200          |                          |
| Routine urine analysis  |              |              |                          |
| pH                      | 6.36 +/- 0.97| 6.31 +/- 0.97| p = .62 (-test)          |
| Urine salinity (%)      | 43.09 +/- 18.06| 42.18 +/- 16.86| p = .61 (-test)           |
| White blood cells**     | 16.1 (0.1-3505) | 8 (0-1000)  | p = .012 (-test)          |
| Red blood cells**       | 14.4 (0.1-7554) | 7 (0-1000)  | p = .06 (-test)           |
| Standard culture +      | 53/183 (29%) | 89/200 (45%) | p = .002 (Chi-square test) |
| Methanogen approach     |              |              |                          |
| Archaea 16S rRNA PCR +  | 18/183 (10%) | 16/200 (8%)  | p = .44 (Chi-square test) |
| mcRA PCR +              | 18/183 (10%) | 16/200 (8%)  | p = .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).
4. 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 in 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 laboratory 1 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 susceptibility [33]. Interestingly, the 19 patients in whose urine samples we

### 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 | Treatment (Duration) | Course of infection |
|-------------|-----------|-----------------------------------------------|------------|---------------------------|-------------------|---------------------|-------------------|
| Cystitis    | 87 (F)    | No                                            | Yes        | >1000                      | *Escherichia coli* (10⁶) | Amoxicillin-clavulanic acid (7 days) | Favourable        |
|             | 65 (F)    | No                                            | No         | 64                         | *Escherichia coli* (10⁶) and *Proteus mirabilis* (10⁶) | Fosfomycin-trometamol (1 day) | Reinfection at day 45 (*E. coli* and *P. mirabilis*) | Favourable |
|             | 74 (F)    | No                                            | No         | 5                          | *Klebsiella pneumoniae* (10⁶) | Pivmecillinam (7 days) | Favourable        |
|             | 55 (F)    | No                                            | No         | 592                        | *Escherichia coli* (10⁶) | Ofloxacin (NA) | Favourable        |
|             | 25 (F)    | No                                            | No         | 140                        | *Escherichia coli* (10⁶) | Pivmecillinam (5 days) | Favourable        |
|             | 52 (F)    | No                                            | Yes        | 10                         | *Klebsiella pneumoniae* (10⁶) | Ceftriaxone (NA) | Favourable        |
|             | 50 (F)    | No                                            | No         | 464                        | *Escherichia coli* (10⁶) | NA | NA |
|             | 83 (F)    | No                                            | No         | 23                         | *Escherichia coli* (10⁶) | Fosfomycin and Colistin (42 days) | NA |
|             | 36 (F)    | No                                            | No         | 11                         | *Escherichia coli* (10⁶) | NA | NA |
|             | 20 (F)    | No                                            | No         | 78                         | *Escherichia coli* (10⁶) | Pivmecillinam (5 days) | Favourable        |
|             | 56 (M)    | No                                            | No         | 25                         | *Escherichia coli* (10⁶) | Ciprofloxacin (3 days) | Favourable        |
|             | 59 (F)    | No                                            | No         | 4                          | *Klebsiella pneumoniae* (10⁶) | Ciprofloxacin (7 days) | Favourable        |
|             | 79 (F)    | No                                            | No         | 85                         | *Enterobacter cloacae* (10⁴) | Amoxicillin-clavulanic acid (2 days) | Favourable        |
| Pyelonephritis | 49 (F) | No                                            | No         | 150                        | *Enterobacter cloacae* (10⁴) | Ceftriaxone IV and gentamicin (NA) | Favourable        |
|             | 69 (F)    | No                                            | No         | 20                         | *Escherichia coli* (10⁶) | Ceftriaxone IV (5 days) | Favourable        |
|             | 75 (F)    | No                                            | No         | >1000                      | *Klebsiella pneumoniae* (10⁶) | Imipenem IV (10 days) | Favourable        |
|             | 83 (F)    | No                                            | No         | 53                         | *Escherichia coli* (10⁶) | Ofloxacin (1 day) and amoxicillin-clavulanic acid (7 days) | Favourable |
| Prostatitis | 69 (M)    | No                                            | No         | 221                        | *Escherichia coli* (10⁶) | Ceftriaxone and gentamicin (NA) | Favourable        |
|             | 50 (M)    | No                                            | No         | >1000                      | *Escherichia coli* (10⁶) | 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.
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 [2] in hydrogen production, and hypothesise that the inhibition of enterobacterial growth by appropriate antibiotics correlates with improved activity against human methanogenic archaea. Int J Clin Microbiol Infect 2016;35:385–91.

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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.

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