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1 **REVISED VERSION**

2 **Detection of methanogens in peri-appendicular abscesses: Report of four cases**

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19 **Abstract**

20 The etiology of appendicular abscess is predominantly microbial with aerobic and anaerobic
21 bacteria from gut flora origin. In this study, using specific laboratory tools, we co-detected
22 *Methanobrevibacter oralis* and *Methanobrevibacter smithii* among a mixture of
23 enterobacteria including *Escherichia coli*, *Enterococcus faecium* and *Enterococcus avium* in
24 four unrelated cases of postoperative appendiceal abscesses. These unprecedented
25 observations raise a question on the role of methanogens in peri-appendicular abscesses,
26 supporting antibiotics as an alternative therapeutic option for appendicitis, including
27 antibiotics active against methanogens such as metronidazole or fusidic acid.

28 **Keywords:** *Methanobrevibacter smithii*, *Methanobrevibacter oralis*, methanogens, bacteria,
29 appendicular abscesses.

30 1. Introduction

31 Methanogens are strictly aero-intolerant archaea with a specific metabolism
32 characterised by the production of methane [1]. They are part of the stable human intestinal
33 microbiota as early as the first weeks of life [2-4]. To date, the most cultured species from the
34 intestinal tract are *Methanobrevibacter smithii*, *Methanobrevibacter stadtmanae* and
35 *Methanomassiliicoccus luminyensis* with respective prevalences of 95%, 30% and 4% [5-7].
36 Furthermore, methanogens have been associated with gastrointestinal dysbiosis in pathologies
37 such as inflammatory bowel disease (IBD), colorectal cancer and diverticulosis [8-10]. More
38 recently, methanogens have been described as opportunistic pathogens after their co-detection
39 with bacteria in infectious samples such as the blood of febrile patients, as well as in pus
40 samples from musculoskeletal and brain abscesses [11-13]. These studies open a new field in
41 clinical microbiology and contribute to a better knowledge of these microorganisms.
42 Nevertheless, methanogens remain neglected in human infections and it would be interesting
43 to determine their potential role as pathogens in other infectious settings.

44 Appendicitis is an acute inflammation of the appendix and considered as the most common
45 abdominal surgical emergency [14]. **It is classified into two categories: uncomplicated and**
46 **complicated forms [15]. The complicated appendicitis occurs in 15% and 30% of cases and**
47 **characterised by the occurrence of intra-abdominal abscesses requiring surgical drainage**
48 **associated with antibiotic treatment and/or an appendectomy [16, 17].**

49 Moreover, luminal obstruction is a frequent cause of appendicitis and the most isolated
50 microbes in ruptured appendices are *Bacteroides fragilis*, *Escherichia coli*, *Pseudomonas*
51 *aeruginosa* as well as *Peptostreptococcus* species and *Fusobacterium* species [18].

52 Additionally, the etiological spectrum of inflamed appendices include fungi, viruses, and
53 parasites [19-24]. Since methanogens are ubiquitous in the gut microbiota, they could also

54 play an unsuspected role in the pathogenesis of appendicitis. To our knowledge, no studies
55 have been conducted on the possible implication of methanogens in this pathological
56 situation.

57 Here, we used methanogen-targeted methods, including molecular and microscopic
58 methods to investigate the presence of methanogens in four cases of appendicular abscesses.

59 **2. Material and methods**

60 **2.1. Sample collection**

61 As a part of the routine analyses of the diagnostic laboratory of IHU Méditerranée
62 Infection, four drained appendicular abscess samples were directly inoculated into anaerobic
63 blood culture bottles (BacT/ALERT FN Plus, bioMérieux, Lyon, France) and incubated inside
64 the BACT/ALERT VIRTUO apparatus (bioMérieux) for bacterial growth monitoring. These
65 four samples were subsequently investigated **retrospectively** for the presence of methanogens
66 as a part of a study aiming at investigating the occurrence of methanogens in infectious
67 samples. This work was approved by the IHU Méditerranée Infection Ethics Committee (No.
68 2021-010) and no sample were taken specifically for this study.

69 **2.2. Methanogen detection**

70 **2.2.1. PCR based detection**

71 Total DNA was extracted using the automat extractor EZ1 advanced XL with EZ1
72 DNA Tissue kit (Qiagen, Courtaboeuf, France), after sonication for 20 minutes with the
73 Branson 2510 ultrasound sonicator (Branson, Rungis, France) as previously described [13].
74 These samples were then screened using standard polymerase-chain-reaction (PCR) targeting
75 16S rRNA **archaeal** gene with the primers SDArch0333aS15 (5'-TCCAGGCCCTACGGG-
76 3') and SDArch0958aA19 (5'-YCCGGCGTTGAMTCCAATT-3') (Eurogentec, Seraing,

77 Belgium) as previously described [25]. PCR products were purified and sequenced using a
78 Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).
79 The resulting sequences were assembled using the ChromasPro 1.34 software (Technelysium
80 Pty. Ltd., South Brisbane, Australia). The sequences were then blasted against sequences
81 available in GenBank database using the online NCBI BLAST program
82 (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>).

83 Real-time PCR assays specifically targeting the 16S rRNA **methanogens** gene were
84 also performed as confirmatory tests using the primers and probes Metha_16S_2_MBF (5'-
85 CGAACCGGATTAGATACCCG - 3'); Metha_16S_2_MBR (5'-
86 CCCGCCAATTCCTTTAAGTT- 3'); Metha_16S_2_MBP 6FAM-
87 CCTGGGAAGTACGGTCGCAAG. Master mix and RNase-free water were used as negative
88 control. **Real-time PCR was considered positive for any cycle threshold (Ct) value \leq 38, and**
89 **the results were validated by the PCR detection of human β -actin gene as an internal control**
90 **in each sample. To estimate the methanogens inoculum in each sample by extrapolation of the**
91 **Ct values, a calibration curve was generated from an *M. smithii* and *M. oralis* DNA sample**
92 **calibrated at 10^9 colony-forming units (CFU)/mL, followed by 10-fold cascade dilutions up to**
93 **10^1 CFU/mL.**

94 **2.2.2. Microscopic examination**

95 PCR- positive samples were observed for the presence of methanogens using scanning
96 electron microscopy. Briefly, 100 μ L of the sample were fixed with 2.5% glutaraldehyde,
97 spotted onto a glass slide by cytocentrifugation (Shandon cytopspin4, Thermo Scientific,
98 Waltham, MA, USA), stained using 1% phosphotungstic acid (Sigma Aldrich, Saint-Louis,
99 MO, USA) for 2 minutes at room temperature and observed using a TM4000 Plus scanning
100 electron microscope (Hitachi, Tokyo, Japan).

101 **3. Case presentation**

102 **Case 1.**

103 A 63-year-old Caucasian man with a medical history of psychosis treated by lithium
104 and risperidone. He presented with a left iliac pain and fever and was diagnosed with
105 appendicitis and peritonitis confirmed by the CT-scan which disclosed a mixed aeric left iliac
106 collection without lymph nodes and without thrombosis. **Percutaneous drainage** of abscess
107 was performed and was directly collected into an anaerobic blood culture bottle (bioMérieux).
108 Microbiological investigations of this abscess pus yielded amoxicillin-susceptible *E. coli* in
109 culture along with *M. oralis* detected by specific PCR-based methods. The patient was
110 administered intravenous piperacillin/tazobactam 4 gr three times a day plus ciprofloxacin
111 500 mg twice a day. On day one, the evolution was unfavourable with septic shock. **The**
112 **biological profile revealed an inflammatory syndrome with a hyperleukocytosis of 18 G/L and**
113 **a CRP of 84 mg/L.** The patient underwent an appendectomy with peritoneal debridement. To
114 limit perfusion time and prevent the risks of infection, antibiotic treatment was switched for
115 oral administration of ciprofloxacin 500 mg three times a day plus metronidazole 500 mg
116 three times a day for one week. The patient was discharged on day 21 and the one-month
117 follow-up examination confirmed that the patient was completely cured.

118 **Case 2.**

119 A 39-year-old woman underwent laparoscopic surgery with an appendectomy and
120 caecum repair, for peritonitis with perforation of the caecum.
121 The patient was treated with amoxicillin-clavulanate 1gr three times a day plus gentamicin
122 160 mg one time a day for 24 hours, which was then replaced by Piperacillin/tazobactam 4 gr
123 three times a day for nine days and finally switched for oral administration of amoxicillin-
124 clavulanate 1gr three times a day for additional week. On day 2 post-surgery, the patient was
125 febrile (38.5°C) and had abdominal pain. **The biological profile revealed an inflammatory**
126 **syndrome with a hyperleukocytosis of 11 G/L and a CRP of 350 mg/L.** A CT-scan diagnosed

127 a right iliac abscess treated on day 4 post-surgery **by radiological drainage**. Microbiological
128 investigations of this abscess pus yielded *E. coli* in culture along with *M. smithii* detected by
129 specific PCR-based methods. The evolution was favourable at the six-week postoperative
130 medical consultation.

131 **Case 3.**

132 A 14-year-old boy presented with acute appendicitis with peritonitis and peritoneal
133 abscess, which was treated medically with multiple antibiotic therapy consisting of
134 Cefotaxime 2 gr three times daily for three days plus metronidazole 500 mg three times daily
135 for 20 days, gentamicin 160 mg once daily for 6 days and piperacillin/tazobactam 2 gr three
136 times daily for 10 days as well as by **percutaneous puncture of the abscess**. Microbiological
137 examinations of this abscess pus revealed the presence of *E. coli* and *E. faecium* in culture as
138 well as *M. smithii* detected by specific PCR-based methods. **Six month later**, the patient was
139 febrile (38.8°C) **and the biological profile revealed an inflammatory syndrome with a**
140 **hyperleukocytosis of 14 G/L and a CRP of 268 mg/L**. A laparoscopic appendectomy was
141 performed. Final antibiotic therapy consisted of a combination of ciprofloxacin 500 mg three
142 times daily and metronidazole 500 mg three times daily for one week. The evolution was
143 favourable one month after surgery.

144 **Case 4.**

145 A 19-year-old male with a medical history of autism, treated with Valproic acid and
146 Amitriptyline, underwent a laparoscopic appendectomy. Histological examination of
147 specimen confirmed the diagnosis of acute and suppurative appendicitis and peritonitis. On
148 Day 6 post-surgery, the patient presented with a fever of 38.1°C and abdominal pain. **The**
149 **biological profile revealed an inflammatory syndrome with a hyperleukocytosis of 14 G/L and**
150 **a CRP of 177 mg/L**. A 25-mm pericaecal abscess was diagnosed by CT-scan and treated by
151 **percutaneous drainage** and medically using ceftriaxone 2 gr/day plus metronidazole 500

152 mg/day for 24H, which was then replaced by piperacillin/tazobactam 2 gr three times a day
153 for 12 days, followed by amoxicillin-clavulanate 1gr three times a day for one week.
154 Microbiological investigations of this **drained abscess** pus yielded *E. coli* plus *E. avium* in
155 culture along with *M. smithii* detected by specific PCR-based methods. The evolution was
156 favourable at the five-week postoperative medical consultation.

157 **4. Results**

158 **4.1. Molecular detection of methanogens**

159 A total of four intra-abdominal peri-appendicular abscess samples were positive for
160 methanogens. Sequencing of archaea 16S rRNA PCR products yielded, for one sample, 100%
161 sequence similarity with the 16S rRNA gene of *Methanobrevibacter oralis* strain VD9^T
162 (accession number LN898260) and, for three samples, 100% sequence similarity with the 16S
163 rRNA gene of *Methanobrevibacter smithii* strain ATCC 35061^T (accession number
164 GCA_000016525.1). Quantitative PCR analyses targeting the **methanogens** 16S rRNA gene
165 confirmed the results of PCR sequencing with a median Ct of 30.98 (Interquartile range, IQR,
166 32.94-23.87), **indicative of a methanogens load between of 4.16×10^4 /mL and 2.48×10^8**
167 **/mL (Table 1).**

168 All four samples were also positive for *Escherichia coli*. Additionally, *Enterococcus faecium*
169 and *Enterococcus avium* were found associated with both methanogens' species in two
170 samples (Table 1).

171 **4.2. Microscopic observation**

172 Scanning electron microscopy revealed two morphological forms in the samples
173 collected from Case No. 1 and Case No. 2. In each case, one of the observed morphologies
174 matched that of a methanogen, a diplococcoid morphology with a diameter ranging from 0.5
175 to 1 μm similar to that of *M. smithii* as well as a bacilli shape with a diameter ranging from
176 0.7 to 1.2 μm characteristic of *M. oralis*. The other shape observed in the samples, - bacilli

177 with lengths of 0.5 to 4 μm with a diameter of 0.5-1.2 μm , similar to that of the co-detected *E.*
178 *coli* (Figure 1)

179 **4. Discussion**

180 Here, we are reporting the first detection of the methanogenic archaeal species, *M.*
181 *smithii* and *M. oralis*, in four patients suffering from appendicular abscesses using molecular-
182 based and electron microscopic-based observations. Despite the depth bias associated to
183 molecular methods, these methanogens were detected using two PCR-based methods,
184 quantitative PCR and standard PCR. The latter was further validated by sanger sequencing of
185 the PCR product leading to a definite identification of the detected methanogen. Moreover,
186 using electron microscopy, cells with methanogen-like shaped were observed in each culture.
187 Bacteriological analysis of the abscess samples led the isolation of facultative anaerobic
188 bacteria including *E. coli*, *E. avium* and *E. faecium*, consistently with prior analyses of
189 appendicular abscesses [26, 27].

190 To our knowledge, this is the first study reporting the detection of methanogens in
191 appendicular abscesses. To date, the presence of methanogens in such a clinical situation has
192 remained unknown because they are not easily detected in clinical microbiology using routine
193 analysis methods such as microscopic observation after Gram staining, culture-based and
194 PCR-based methods. In fact, microscopic examination after Gram staining of methanogens is
195 unreliable and non-specific. Furthermore, the culture of methanogens requires an unusual
196 atmosphere consisting of hydrogen and carbone dioxide whereas and PCR-based detection
197 requires specific DNA extraction including sonication as a method of mechanical lysis [28].
198 Routine diagnostic laboratory is based on timely result report to quickly provide a diagnosis
199 and therapeutic plan for the patient. Therefore, slow growing organisms such as methanogens,
200 can easily escape routine detection. Although this is a first report, the detection of
201 methanogens is not surprising, given the anatomical location of the appendix in the

202 gastrointestinal tract. In fact, *M. smithii* is ubiquitous in healthy adult and is stable over the
203 life course with a prevalence increasing with age [29, 30]. Additionally, methanogens are
204 hydrogens-consuming microorganisms that interact preferentially with hydrogens-producing
205 bacteria through syntrophic process to reduce CO₂ into methane [31]. Here, all methanogens-
206 positive samples were co-detected with *E. coli* which is a known hydrogen producer [32].

207 The relationship between bacteria and methanogens are paramount for intestinal
208 homeostasis. Thus, any fluctuation in the level of methanogens could be involved in the
209 physiopathology of some diseases [33-38]. In the context of appendicular abscess, we suggest
210 two plausible mechanisms by which methanogenesis could be involved in the occurrence of
211 this situation or their implication as potential risk factor, either through the promotion of the
212 growth of pathogenic bacteria such as enterobacteria by maintaining a low partial pressure of
213 hydrogen, which is a very important condition for the fermentation process [39, 40], or by
214 increasing methane excretion due to high methanogens concentrations, leading to a toxic
215 atmosphere. The possible implication of methanogens in appendicitis is corroborated by the
216 recent study reporting an association between appendectomy and decreased methanogen
217 levels, suggesting that the appendix may serve as a reservoir for the intestinal microbiome,
218 including methanogens [41].

219 To date, the gold standard treatment of complicated appendicitis is based on invasive
220 and costly methods, including percutaneous drainage of appendicular abscesses or
221 laparoscopic appendectomy [16]. In some cases, a nonoperative management of complicated
222 appendicitis is adopted, but this is associated with a risk of recurrence [42, 43].

223 Methanogens are naturally resistant to beta-lactams which are the most used antibiotics in
224 appendicular abscess. It would be interesting to prescribe antibiotics active against

225 methanogens such as metronidazole or fusidic acid [44], as was done for three patients
226 reported ins this study.

227 Based on these preliminary results, largest cohort studies are needed for investigating
228 specifically the prevalence of methanogens and confirm their possible implication in the
229 occurrence of appendicular abscesses. In fact, if the implication of methanogens is
230 demonstrated, they should be taken into consideration in the diagnosis of this infection to
231 adapt medical treatment.

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239 **Conflict of interest:**

240 The authors declare no conflicts of interest.

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388 **Table 1.** Patient information along with PCR sequencing, RT-PCR and bacterial culture
 389 results.

Case	Sex	Age	PCR-Sequencing	RT-PCR targeting β -actin	RT-PCR targeting methanogens 16S gene	Methanogens load	Bacterial culture
1	M	63	<i>M. oralis</i>	24,82	23.87	2.48×10^8 / mL	<i>E. coli</i>
2	F	39	<i>M. smithii</i>	26,08	32.94	4.16×10^4 / mL	<i>E. coli</i>
3	M	14	<i>M. smithii</i>	25,24	30.25	5.48×10^5 / mL	<i>E. coli</i> + <i>E. faecium</i>
4	M	19	<i>M. smithii</i>	24,73	31.71	1.35×10^5 / mL	<i>E. coli</i> + <i>E. avium</i>

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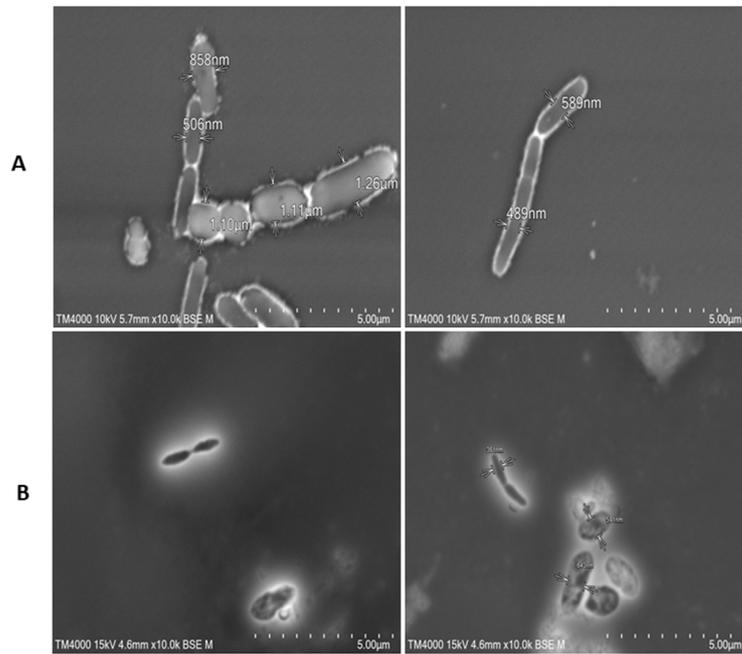
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401 **Figure 1.** Scanning electron microscopy imaging of peri-appendicular abscess pus sample in
 402 one patient diagnosed with mixed *M. oralis* and *E. coli* infection (Panel A) and in one patient
 403 diagnosed with mixed *M. smithii* and *E. coli* infection (Panel B). The scale bars indicate 5 µm.