Back to the Source: Molecular Identification of Methanogenic Archaea as Markers of Colonic Methane Production

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Methanogenic archaea (methanogens) are anaerobic microbes that derive their energy from metabolizing simple substrates such as the gaseous H_2 and CO_2 or the volatile methanol into methane, a unique means of survival absent in bacteria and in eukaryotes [1]. Based on their substrate usage, methanogens are further classified into three primary metabolic groups, hydrogenotrophic using H_2 and CO_2 or formate, acetoclastic feeding on acetate, and methylotrophic utilizing methanol or other methylated compounds [2]. Regardless of their specific metabolism, all methanogens share the *mcrA* gene encoding the methyl coenzyme M (CH_3 -CoM) reductase that converts CH_3 -CoM to methane. Although methanogens are frequently associated with eukaryotic hosts such as protists, plants, and animals including humans [3], their host-associated biology has scarcely been characterized. Likewise, the impact of methanogens on human health and diseases remains unknown. Nevertheless, growing evidence has suggested that intestinal overgrowth of methanogens is harmful and is associated with several diseases [4]. Prominent among them is GI dysmotility, since luminal methane slows intestinal transit in animal models [5]. Presumably, overgrowth of methanogens overproduces methane, thus contributing to adverse GI-relegated symptoms such as bloating and constipation in humans, an hypothesis supported by the observation that breath methane excretion is elevated in constipation-predominant irritable bowel syndrome (IBS-C) patients, who also have lower postprandial serotonin levels than non-methane excreting IBS patients who excrete elevated H_2 [6]. Nonetheless, diagnostic

standards for overgrowth of methanogens are ambiguous, as the understanding of this phenomenon is still in its infancy.

Overgrowth of methanogens used to be classified under the umbrella term SIBO (small intestinal bacterial overgrowth). In the latest American College of Gastroenterology (ACG) Guideline for SIBO, it is now defined separately as IMO (intestinal methanogen overgrowth) since methanogens are archaea, not bacteria, and methane (and perhaps methanogens too) exhibits unique clinical relevance [7]. Owing to the relative ease in bacterial cultivation, SIBO can be quantitatively defined as a small intestinal bacterial load $\geq 10^3$ CFUs (colony-forming units) $\cdot mL^{-1}$. This CFU diagnostic threshold is highly correlated with an increase of $H_2 \geq 20$ ppm from baseline within 90 min during a hydrogen breath test, due to rapid post-fasting metabolism of ingested carbohydrates such as glucose or lactulose by the high load of small intestinal bacteria.

Based on these observations, the semi-quantitative hydrogen breath test has been proposed as an alternative to intestinal bacterial culture for SIBO diagnosis. Although its ability to accurately diagnose SIBO in a population with nonspecific GI symptoms has been questioned, the safe and noninvasive breath test is a popular and effective tool in the clinics when used and interpreted appropriately [8]. In contrast, the criteria defining IMO are far from clear. Since methanogens are fastidious, they are not routinely cultivated in clinical laboratories and have no established CFU. Instead, empirical diagnostic recommendations using the methane breath test have been proposed. The ACG recommended the presence of breath methane ≥ 10 ppm at any point during testing after substrate ingestion for a positive IMO diagnosis, although other studies have adopted thresholds between 3 and 10 ppm. Regardless of the threshold, the clinical relevance of a positive methane breath test is not high, since breath methane excretion in health appears highly variable, ranging from below detection (< 2 ppm)–82 ppm [9]. Although the sources of this variation are not well understood, in theory methane emission from any of the ‘hot spots’ including the

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nasal, oral, and small and large intestinal mucosae, could occur. However, methanogenic activities are not well characterized within these sites (Fig. 1).

In order to improve the clinical diagnosis of IMO, additional tests will be needed. In this issue of *Digestive Diseases and Sciences*, Drs. Levi Teigen, Prince Mathai, and associates investigated whether assays of molecular markers for methanogens from stool could serve as surrogates for IMO [10]. They collected stool samples from healthy adults ($n=33$) and devised the following four tests in order to infer their methane-producing status (Fig. 1).

Fig. 1 Overview of the hot spots for methanogens and methane production in the human body (upper panel) and the four assays devised by Drs. Teigen, Mathai, and associates for colonic methanogens [10] (bottom panel). Methanogens are often detected in nasal, oral, and in the small and large intestinal sites, all of which in theory contribute to breath methane through the systemic circulation and respiration. Therefore, elevated breath methane could be due to overgrowth of methanogens at one or multiple of the four spots. Currently, quantitative contribution to breath methane from each of the four spots remains elusive. Although elevated breath methane has been linked to several diseases, its quantity varies substantially among healthy adults, compromising the utility of breath methane test for disease diagnosis. Teigen et al. developed molecular assays (16S relative abundance and targeted and untargeted *mcrA* qPCRs) for methanogens that were sensitive (33% to 100% positive rate) enough to discriminate between positive (30%) and negative methane productions measured by an in vitro fecal methane producing assay

1. A 2-h in vitro batch incubation of the samples in gas-tight septum jars aimed at measuring methane production, showing 30% of the samples were methane positive when using the ≥ 10 ppm threshold corrected to per gram of dry stool. The weight correction, not implemented in the breath test, is a practice that should facilitate future test standardization. Yet, this correction renders a direct comparison with breath methane data difficult, since their units are now different ($\text{ppm} \cdot \text{g}^{-1}$ dry stool vs. ppm). Nevertheless, the scale of variation (unitless) between the two assays should still be comparable, which is 17-fold ($83\text{--}1434 \text{ ppm} \cdot \text{g}^{-1}$) for the in vitro assay, and 8 to 27-fold ($3\text{--}82 \text{ ppm}$) depending on the positive cutoff ($3\text{--}10 \text{ ppm}$) for the breath assay from healthy adults ($n=492$) [9]. Therefore, both assays have a comparable but large variation. In order to reliably separate disease samples from healthy ones, this variation must be controlled for in future in vitro assays. Due to the ease of in vitro manipulation, at least two improvements could be reasonably implemented. First, fecal samples may be incubated for extended time and then treated with methanogenic substrates—mimicking a post-fasting measurement of methane production. This would minimize the variation caused by undigested substrates in fecal samples. Second, the incubation may be done with a continuous flow—mimicking either normal or delayed intestinal transit that would better account for the variation in transit that fluctuates considerably for the same healthy individual and is associated with methane production, an advantage over batch incubation.
2. 16S rRNA gene amplicon sequencing of the fecal community in order to estimate the relative abundance of methanogens, indicating 66% of the samples were methanogen positive ($0.002\text{--}9.23\%$ of the total community). The most prevalent and abundant methanogens belong to the hydrogenotrophic *Methanobrevibacter*, whereas occasional signatures for the methylotrophic *Methanosphaera* were also significant. At $\geq 0.097\%$, the relative abundance of 16S rRNA for *Methanobrevibacter* separated the methane-positive samples from the negative ones with perfect discrimination. Since the β (a measure of variation in microbiome composition) but not the α diversity (a measure of microbiome richness and diversity) was different between the methane-positive and methane-negative samples, a distinct but equally diverse bacterial community was also associated with the methanogens. Indeed, an enrichment of specific bacterial taxa *Muribaculaceae*, *Ruminococcaceae*, *Christensenellaceae*, and *Catenibacterium* was observed in the methane-positive samples, as opposed to an enrichment of *Bacteroides*, *Blautia*, *Bacilli*, and *Lactobacillales* in the methane-negative samples. Since the bacteria

that co-occur with methanogens may affect gut physiology in their own ways, future studies investigating the effects of these bacteria would increase understanding of the mechanism of methanogen-associated diseases.

3. An untargeted *mcrA* gene qPCR assay to quantify the absolute abundance of methanogens, delivering an impressive (and surprising) 100% methanogen positive rate (5.3×10^3 – 7.7×10^7 copies $\cdot$ g⁻¹ wet stool). This confirms *mcrA* is a superior molecular marker over 16S rRNA for methanogens, which is well documented in environmental studies. Nevertheless, very few studies have used *mcrA* to survey methanogens in human samples, suggesting an undercount of methanogens in most studies. It is therefore strongly recommended that *mcrA* assay should be considered for future studies in addition to the standard 16S assay. Sequencing all the *mcrA* genes is also recommended to exclude any false positives and better stratify the methanogen population. Like the 16S assay, the *mcrA* assay also effectively separated the methane-positive vs. -negative samples very well. The threshold for positive methane was $\geq 5.2 \times 10^5$ copies $\cdot$ g⁻¹ with near perfect discrimination.
4. A targeted *mcrA* qPCR assay to quantify four known gut methanogen species with five novel primer sets, presenting a 33% methanogen positive rate. The positives only included *Methanobrevibacter smithii* and *Methanospiraeta stadtmanae*, both already detectable by 16S. This low positive rate suggests most of the diversity revealed by the untargeted *mcrA* assay was not captured, reinforcing the recommendation to sequence all *mcrA* genes when using the untargeted assay. The reason for the extra set of primers was that two sets of primers must be developed in order to target all known *M. smithii* strains. This suggests the current classification of *M. smithii* may reflect a complex of more than one species, which has not previously been captured by the 16S phylogeny. Indeed, a very recent genome survey proposed a split of *M. smithii* into two separate species which differed considerably in their *mcrA* sequences and other genomic features [11]. If the proposal stands, the group closely related to the type strain PS (targeted by the primer set 1A in this study) will continue to represent *M. smithii*, whereas a second group represented by strain WWM1085 (targeted by this study's primer set 1B) will have a new candidate species termed *Candidatus* 'Methanobrevibacter intestini'. Since data linking *M. smithii* with diseases are often contradictory according to previous studies, it will be exciting to see if the new classification would solve some of these contradictions which will be facilitated by the targeted qPCR assays developed here.

Collectively, by focusing on the source of methane production—namely the methanogens, this study marks an important methodological advancement toward refining the diagnosis of IMO and its associated diseases. Further advancements in these methods and testing both healthy and diseased samples with a large sample size shall bring promising diagnostic solutions.

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Compliance with Ethical Standards

Conflict of interest The author has no relevant conflicts of interest to disclose.

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