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Technical note: Comparison of biomarker and molecular biological methods for estimating methanogen abundance[1]

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ABSTRACT: Quantitative real-time PCR (qPCR) has become a popular method for estimation of methanogen abundance in the ruminant digestive tract. However, there is no established method in terms of primer choice and quantification, which means that results are variable and not directly comparable between studies. Archaeol has been proposed as an alternative marker for methanogen abundance, as it is ubiquitous in methanogenic Archaea, and can be quantified by gas chromatography–mass spectrometry (GC–MS). The aim of this experiment was to compare total methanogen populations estimated using the new archaeol approach with estimates based on qPCR. Specific primer sets and probes were used to detect dominant ruminal methanogen species *Methanobrevibacter ruminantium*, *Methanobrevibacter smithii*, *Methanosphaera stadtmanae,* and total methanogen populations. There was variation in the relationships among total methanogen abundance estimates based on archaeol and qPCR. In addition, the universal methanogen primers appeared to preferentially amplify genes from *M. smithii*. Archaeol had the strongest relationship with the dominant rumen methanogen *M. ruminantium*, whereas the total methanogen primers had a comparatively weak relationship with archaeol. Archaeol analysis was a useful adjunct to molecular biology methods, but it seems that a valid specific primer for *M. ruminantium* would be more useful than a biased primer for total methanogens.

Key words: archaea, archaeol, methanogens, qPCR, ruminant digestive tract

INTRODUCTION

Quantitative real-time PCR (**qPCR**) has become the method of choice for estimating methanogen abundance in samples derived from the ruminant digestive tract. Primers to quantify these methanogens have been based on different regions of several genes, including the methanogen 16S rRNA (***rrs***) gene and methyl-coenzyme M reductase α-subunit gene (***mcr*A**). The different primers have been used with a wide range of sample types and results expressed in several ways, resulting in considerable confusion (Tymensen and McAllister, 2012; McCartney et al., 2013b).

Archaeol (2,3-diphytanyl-O-*sn*-glycerol) is a promising alternative marker for methanogen abundance, including in ruminant digesta. It is a membrane lipid that is ubiquitous in methanogenic Archaea and can be quantified by gas chromatography–mass spectrometry (**GC–MS**). Previous studies assessed archaeol as a potential molecular proxy for methanogenesis in cattle (Gill et al., 2011; McCartney et al., 2013a). However, the suitability of archaeol as a molecular proxy for methanogen abundance has not yet been assessed. The aim of this experiment was to compare total methanogen populations estimated using the new archaeol approach with estimates based on qPCR methods, targeting both total and specific methanogen populations.

MATERIALS AND METHODS

Rumen Fluid

Rumen fluid samples were collected from a previous change-over design study by McGeough et al. (2010). There were 3 samples each from 4 forage-based dietary treatments. Three treatments were based on ad libitum access to whole-crop wheat (**WCW**) silages differing in grain:straw plus chaff ratio (% DM basis: WCWI = 1:89, WCWII/III = 26:74, WCW IV = 47:53) and the fourth on a perennial ryegrass (*Lolium perenne*) silage. Four ruminally cannulated Rotbunde–Holstein steers (BW 413 ± 30.1 kg) each received a fixed allocation (2.6 kg DM/d) of concentrates. The concentrate contained (%): rolled barley (46), soybean meal (46), cane molasses (5), mineral/vitamin premix (2), and vegetable oil (1). The chemical composition of the concentrates and silages, according to methods described by McGeough et al. (2010), are provided in Table 1. Animals were adapted to diets for 20 d and then rumen fluid was sampled through the ruminal cannula at 6 h after the morning meal. This time point was chosen to ensure that the methanogens had experienced a significant period of time to proliferate in the rumen after feeding. Samples were stored frozen (–20°C).

Archaeol Analysis

Ruminal fluid was lyophilized over 2 d before archaeol analysis, according to methods outlined by McCartney et al. (2013a). Briefly, 43.4 μg of internal standard (1,2-di-*O*-hexadecyl-*rac*-glycerol) was added to 400 mg of dried sample before a monophasic extraction procedure to obtain the total lipid extract (**TLE**). Removal of polar headgroups from archaeol was then achieved by acid methanolysis. Then, TLE was separated into "apolar" and "alcohol" fractions by column chromatography. The alcohol fraction was further trimethylsilylated and then run on GC–MS. Archaeol was identified and then quantified against a calibration curve, which was constructed using an archaeol standard (1,2-di-*O*-phytanyl-*sn*-glycerol; Avanti Polar Lipids Inc., Alabaster, AL).

qPCR Study

Genomic DNA were isolated from ruminal fluid using a repeated bead beating method outlined by Yu and Morrison (2004). The yield (ng μL^{-1}) and purity of extracted DNA were assessed using a spectrophotometer (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE). Specific primer and probe sets were used to detect and quantify dominant methanogen species *Methanobrevibacter ruminantium*, *Methanobrevibacter smithii*, *Methanosphaera stadtmanae,* and total metha-

Table 1. Chemical composition of whole-crop wheat (WCW)[1] silages, grass silage (GS), and concentrate.

Chemical composition, g/kg of DM, unless otherwise stated	WCW silage[1]			GS	Concentrate
	I	II/III	IV		
DM, g/kg	462	522	584	256	868
Ash	55	48	38	103	72
ME, MJ/kg DM	9.0	10.1	11.5	11.3	12.9
CP	104	108	113	140	302
NDF	524	411	310	513	134
ADF	310	234	163	311	66
Starch	155	311	436	ND[2]	252
WSC[2]	9.3	15.6	22.7	8.0	–
pH	3.97	4.01	4.22	3.93	–
NH$_3$N, g/kg of total N	59	52	44	76	–
Acetic acid	13.9	9.45	7.4	28	–
Lactic acid	34	30.5	24	103	–
Butyric acid	ND	ND	ND	9.2	–

[1]Grain:straw plus chaff ratio: I = 11:89; II/III = 26:74; IV = 47:53.

[2]ND = not determined; WSC = water-soluble carbohydrates.

nogen populations (targeted using both *rrs* and *mcr*A genes), along with a prokaryote *rrs* reference gene (Table 2).

Quantitative real-time PCR was performed using either SYBR green chemistry (Fast SYBR green master mix; Applied Biosystems, Dublin, Ireland) or FAM dye (TaqMan; Life Technologies, Glasgow, UK) on the 7500 Fast Real-Time PCR System (Applied Biosystems). Real-time PCR amplification efficiencies (*e*) were estimated for all assays using a linear regression of the threshold cycle (Ct) for each dilution vs. the log dilution using the formula: $e = 5^{-1/\text{slope}}$ (Pfaffl, 2001) where "5" is the corresponding fold dilution.

Aliquots of 10 μL PCR products were analyzed by electrophoresis on a 2% agarose gel (wt/vol) to verify the presence and size of the amplicons. Negative controls without template DNA were included in parallel. Specificity of TaqMan assays for the quantification of *Methanobrevibacter smithii* and *Methanobrevibacter ruminantium* were verified before quantification of ruminal DNA. Each probe was validated by running a non-target clone standard as a negative control. Thermal cycling conditions applied to each assay consisted of an initial *Taq* activation step at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 60°C for 60 s, followed by an amplicon dissociation stage (95°C for 15 s, 60°C for 1 min, increasing 0.5°C/cycle until 95°C was reached), which confirmed specificity via dissociation curve analysis of PCR end products. Fluorescence detection was also performed at the end of each denaturation and extension step.

Inter-plate calibration, based on a calibrator sample included on all plates, efficiency correction of the raw cycle threshold (**Ct**) values, and results from triplicate PCR reactions for each target species, were averaged and

Table 2. Primer sequences used for relative quantification of total methanogens and individual methanogen species, using methyl coenzyme-M reductase (*mcr*A) and 16S rRNA (*rrs*) genes. The calculated efficiency of each primer set used is reported.

Target	Primer/probe name and sequence (5'-3')	Assay	Efficiency	Reference
Total methanogens (*mcr*A)	q*mcr*A-F, 5'-TTCGGTGGATCDCARAGRGC-3' q*mcr*A-R, 5'-GBARGTCCWAWCCTAGAATCC-3'	SYBR[1]	95%	Denman et al. (2007)
Total methanogens (*rrs*)	Met630F, 5'-GGATTAGATACCCSGGTAGT-3' Met803R, 5'-GTTGARTCCAATTAAACCGCA-3'	SYBR	92%	Hook et al. (2009)
Total prokaryotes (*rrs*; reference gene)	V3-F, 5'-CCTACGGGAGGCAGCAG-3' V3-R, 5'-ATTACCGCGGCTGCTGG-3'	SYBR	91%	Muyzer et al. (1993)
M. stadtmanae (*rrs*)	Stad-F, 5'-CTTAACTATAAGAATTGCTGG-3' Stad-R, 5'-TTCGTTACTCACCGTCAAGAT-3'	SYBR	98%	Zhou et al. (2009)
M. smithii (*rrs*)	Smit.16S-740F, 5'-CCGGGTATCTAATCCGGTTC-3' Smit.16S-862R, 5'-TCCCAGGGTAGAGGTGAAA-3' Smit.16S FAM, 5'CGTCAGAATCGTTCCAGTCA-3'	FAM[1]	83%	Dridi et al. (2009)
M. ruminantium (*rrs*)	Rum16S 740F, 5'-TCCCAGGGTAGAGGTGAAA-3' Rum16S 862R, 5'CGTCAGAATCGTTCCAGTCA-3' Rum16S FAM, 5'-CCGTCAGGTTCGTTCCAGTTAG-3'	FAM	85%	Carberry et al. (2011)

[1]SYBR = Fast SYBR Green I Dye assay; FAM = TaqMan probe-based assay.

the means calculated, using the software package GenEx 5.2.1.3 (MultiD Analyses AB, Gothenburg, Sweden). Abundance of methanogens (total and specific) were expressed as a proportion of total estimated ruminal bacterial 16S rDNA, as described previously (Chen et al., 2008; Guo et al., 2008, Carberry et al., 2012), according to the equation: relative quantification = $2^{-(Ct\ target–Ct\ total\ bacteria)}$, where Ct represents threshold cycle.

Relationships among the various estimates of methanogen abundance (based on qPCR or archaeol) were made using simple linear regression in the GenStat software (14th ed.; VSN International Ltd., Hemel Hempstead, UK). Initial data analysis identified results from 1 of the samples as an outlier when considering the relationships that were otherwise strongest (archaeol and *rrs M. ruminantium* gene) on the basis of Cook's test (Cook's test statistic for outlier = 3.5, cut off for exclusion is ≥3). Results from this sample were excluded from all subsequent analysis.

RESULTS AND DISCUSSION

The relationships between qPCR-based estimates of methanogen abundance (ΔCt relative units, DM basis) and archaeol (mg kg^{-1} DM) are presented as a correlation matrix in Fig. 1. The techniques examined resulted in quite different rankings of methanogen abundance and there was considerable variability in the relationships between estimates.

The strongest relationship was between estimates based on archaeol and the *rrs* gene, using primers specific for *M. ruminantium*. The comparatively weak relationship between archaeol and estimates of total methanogens using the *mcr*A and *rrs* genes was initially surprising. However, it is consistent with the observa-

tion by Tymensen and McAllister (2012) that different universal methanogen primer sets result in amplification of very different methanogen communities. There were strong relationships between estimates based on the *mcr*A gene and *rrs* gene, when using total methanogen primers or those specific to *M. smithii*.

In addition to problems with the specificity of primers, the variable relationships with estimates based on the *mcr*A gene may relate to the absence of this gene in some species. For example, *M. stadtmanae* does not appear to possess *mcr*A and, instead, has an equivalent enzyme called methyl-coenzyme M reductase II subunit A (Zhou et al., 2011). It is possible that the presence of non-methanogenic Archaea could add to variation since the *mcr*A enzyme is specific to methanogenesis. However, Shin et al. (2004) suggested that non-methanogenic Archaea represent <0.05% of all Archaea in the rumen.

This study confirmed the lack of agreement among methanogen estimates using different primer sets and possible problems with lack of primer specificity and/or the fact that some of these species represent only a very small proportion of total methanogens (Janssen and Kirs, 2008). Against this background, it is useful to have an estimate of the methanogen population based on a completely different physiological approach (i.e., archaeol). While a significant (*P* = 0.013) relationship was detected between estimates using archaeol and *rrs* total methanogen, there was no significant relationship (*P* = 0.097) between estimates based on archaeol and the *mcr*A gene, which is consistent with limitations of the *mcr*A gene as discussed above.

There has been considerable research using the *rrs* gene and specific primers have been developed for almost all known methanogen species (Narihiro and Sekiguchi, 2011). There was a comparatively weak, but

rrs^1: total methanogens	**r = 0.82** **P = 0.002**				
rrs: M. ruminantium	r = 0.31 P = 0.351	**r = 0.71** **P = 0.015**			
rrs: M. smithii	**r = 0.91** **P < 0.001**	**r = 0.83** **P = 0.001**	r = 0.44 P = 0.175		
rrs: M. stadtmanae	r = 0.36 P = 0.281	r = 0.45 P = 0.164	**r = 0.64** **P = 0.033**	r = 0.48 P = 0.138	
Archaeol	r = 0.53 P = 0.097	**r = 0.72** **P = 0.013**	**r = 0.87** **P < 0.001**	r = 0.57 P = 0.068	**r = 0.61** **P = 0.046**
	$mcrA^2$	rrs: total methanogens	rrs: M. ruminantium	rrs: M. smithii	rrs: M. stadtmanae

Fig. 1. Correlation matrix showing the relationships between archaeol concentration (mg kg^{-1} DM) and abundance of total methanogens/dominant methanogen species (ΔCt relative units, DM basis) in ruminal fluid. Values reported are: correlation coefficients (r) and corresponding P-values. [1]$rrs = 16S$ rRNA; [2]mcrA = methyl coenzyme-M reductase.

significant ($P = 0.013$), relationship among estimates based on archaeol and the rrs gene (total methanogens). *Methanobrevibacter ruminantium* had the strongest relationship ($P < 0.001$) with archaeol concentrations and is widely reported to be the dominant methanogen in the rumen. Other methanogens are from closely related species (Janssen and Kirs, 2008). The implication of this is that variation in the relationship with archaeol estimates resulting from variation in the proportion of total methanogen DNA amplified using the rrs (M. ruminantium) primers is less than variation associated with problems, such as lack of specificity, of the rrs (total methanogens) primers. The better performance of the rrs (M. ruminantium) primers in comparison with rrs (total methanogens) may be related to use of the TaqMan system (Applied Biosystems, Foster City, CA) for the former. Primers for total methanogens used in this study were developed by Hook et al. (2009) and these authors found a greater proportion of *M. smithii* (65%) than *M. ruminantium* (32%). While these primers clearly amplify DNA from *M. ruminantium*, it appears that they preferentially amplify DNA from *M. smithii* and this is further suggested by the observation that the strongest relationship between estimates based on rrs (total methanogens) was with rrs (M. smithii) ($P = 0.001$). The same situation may apply to the mcrA gene, where Denman et al. (2007) found a high proportion of clones that were not *M. ruminantium*, including many *Methanobrevibacter* that were closer to *M. smithii* than *M. ruminantium*. Indeed, the strong relationships among estimates based on the mcrA gene, rrs

(total methanogens), and rrs (M. smithii) suggest that all 3 primer sets were preferentially amplifying *M. smithii* and closely related *Methanobrevibacter*.

The weaker relationship of archaeol with estimates based on rrs primers for *M. smithii* ($P = 0.068$) and *M. stadtmanae* ($P = 0.046$) may also be in part due to a lower abundance of these methanogen species in the rumen (Janssen and Kirs, 2008). Furthermore, the *Methanosphaera* species contain hydroxyarchaeol in their membrane lipids (Koga et al., 1998), which was not detected in this study and could add further variation to the relationship.

While the discussion above has focused on problems associated with primer specificity, it seems likely that some variation is associated with the amount of archaeol per methanogen cell. Studies in other ecosystems suggest that proportions of archaeol and glycerol dialkyl glycerol tetraether (**GDGT**) content in individual methanogen cells can vary, with ~50 to 100% dialkyl glycerol ethers (e.g., archaeols and their variants) and ~0 to 50% GDGT (e.g., caldarchaeol) in the membrane lipid (Chong, 2010).

Conclusions

The relationships among total methanogen abundance estimates based on archaeol and qPCR were variable, which perhaps reflect difficulties associated with the qPCR analysis. Differing concentrations of archaeol per methanogen cell and the presence of non-methanogenic Archaea may also have contributed to the varia-

tion. The universal methanogen primers for *mcr*A and *rrs* genes appeared to preferentially amplify genes from *M. smithii*. Archaeol had the strongest relationship with the dominant ruminal methanogen *M. ruminantium*. While important in the human intestine, *M. smithii* is a minor methanogen in the rumen, where *M. ruminantium* predominates. Archaeol analysis was a useful adjunct to molecular biology methods; it seems that a reliable, specific primer and probe set for *M. ruminantium* is more useful than a biased primer for total methanogens.

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