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Standardization and Improved Protocols for Evaluation of Relative Abundance of Methanogens in Rumen of Dairy Cows

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ABSTRACT: Methanogens are obligate anaerobes. Ruminal methanogens are still difficult to be cultured in laboratories, owing to their tough cell wall. The conventional methods of DNA extraction would not be feasible as they are found to be qualitative rather than quantitative. Hence, an improved methodology was attempted by quantitative real-time PCR (qRT-PCR) technique for determining the abundance of methanogen in the rumen of cattle. The relative abundance of all methanogens as well as key species such as *Methanobrevibacter* sp., and *Methanosphaera stadtmanae* were determined in the rumen liquor of cattle fed with 60:40 roughage and concentrate diet in the farm with standard managemental conditions in the Cauvery Delta Zone of Tamilnadu. DNA extraction was followed in three processing methods. Gradient PCR and RT-PCR has been carried out by use of published primers. *Methanosphera standmanae* was observed as the more abundant species than *Methanobrevibacter* sp. in this study. The parameters for standardisation of RT-PCR such as annealing temperature, melting curve can be used in future as standard to conduct are search protocol. The area and feeding system specific standardised protocols can be widely adopted in the rumen analytical studies to assess relative abundance of methanogens.

Keywords: Methanogens, RT-PCR, Gradient PCR, Methane, *Methanosphera standmanae*, Rumen.

INTRODUCTION

Methane, carbon dioxide and nitrous oxide are called as greenhouse gases (GHGs), which are the main gases responsible for global warming. A little under 25% of all human-induced GHGs are produced by agriculture, which is one of the main sources of GHGs. Methane (52%) and nitrous oxide (84%) are particularly common in agricultural emissions. The need for effective sustainable agriculture practices that may increase production while reducing agricultural greenhouse gas (GHG) emission are on great demand (Dar *et al*., 2019). Methane emission through mouth is significantly higher than through manure. Cattle manure could be used for biogas production for efficient utilization of energy. Biogas from cattle manure contains 50 - 60 percent methane (Singh *et al*, 2017). Enteric fermentation, or the digestion of organic matter by animals, mostly beef cattle, is the main source of methane emissions (Moraditochaee, 2015). Cattle with crop residue based feeding system were main contributor of methane emission from livestock. Paddy straw based feeding system is widely adopted in the dairy farms of Cauvery delta zone of Tamilnadu. Host animal phenotype post a potential effect on rumen core microbiome composition of bacteria, protozoa, fungi, virus and including methanogens and methane production. It was also explored by many studies, that there exists a natural diversity among microbial population not only solely depending on the different feed stuffs consumed by the animals but also significant variation depending on the other factors like living geographical area (Agarwal *et al.,* 2015), managemental practices, seasons and other intrinsic features of animal breed (Henderson *et al.,* 2015) or phenotype (Mizrahi and Jami 2018). The phenotypic performances of the host like quantity and quality of milk production have been significantly shown to have the impact by this bidirectional interaction between the animal and microbiota.

Methanogens are peculiar that they produce methane gas as a byproduct of their metabolic processes. In the

rumen, they play a crucial role in breaking down complex carbohydrates present in the ruminants diet, such as cellulose and hemicellulose, through a process called anaerobic fermentation. Carberry *et al.* (2014) found that *Methanobrevibacter* sp. were dominant among the rumen methanogens across the contrasting diets, with *Methanobrevibacter smithii* being the most abundant species followed by *Methanobrevibacter ruminantium* and *Methanosphaera stadtmanae*. Nearly eighty percent of the emissions from agriculture are generated by enteric methane emissions from ruminants (Steinfeld *et al.*, 2006). Methanogens, a particular class of bacteria known as archaea, are responsible for the formation of methane (CH_4) during the microbial fermentation of feed in the rumen. Eructation releases most of the CH_4 that the rumen produces into the atmosphere. Additionally, enteric CH_4 generation results in a sizable (2–15%) reduction in dietary gross energy consumption (Van Nevel *et al.*, 1996). One way to reduce the global methane emission is to check emission of methane by livestock (Wright *et al*., 2007). Therefore, any decrease in intestinal CH_4 emissions could potentially have positive effects on the environment and the economy (Reynolds *et al.*, 2010).

Methanogens are strict anaerobes and are difficult to be cultured in laboratory and also owing to tough cell wall of ruminal bacteria, conventional methods of DNA extraction wouldn't be feasible. Moreover, clone sequencing and next generation methanogen specific tag-encoded pyrosequencing were the methods used to show the specific species of methanogen (Carberry *et al.*, 2014). However, these methods aren't precise enough to properly measure distinct methanogens, especially when species are scarcely present.

Flora of methanogens fluctuates based on the feeding cycle and diet. Assessing the relative abundance of methanogen is essential in methane ameliorating studies. Hence, standardization of protocol to assess the relative abundance of methanogen to area specific feeding practices is inevitable. The influence of existing feeding conditions especially in the paddy straw feeding areas, the diversity of the ruminal microbial community in Cauvery delta zone of Tamil Nadu, remains unexplored. Hence, before planning for any methane emission reduction studies with dietary modification in the study area, it was felt important to study the adoptability and suitability of the relative methanogen quantification techniques. Therefore, this study is envisaged to standardize the protocol in the farming conditions of Cauvery delta zone of Tamil Nadu. The findings from the study will be useful for understanding the nutritional status, core methanogenic population and suitable laboratory protocols to be followed in future studies.

MATERIALS AND METHODS

Animal management. Standardization of protocol in this experiment is aimed specifically to the feeding practices in the Cauvery delta zone of Tamilnadu to identify the relative abundance of methanogens in lactating cows. Six Jersey cross cows in early lactation were selected. Cumbunapier CO_4, paddy straw and concentrates were fed (60% roughage and 40% concentrate). Clean drinking water was offered *ad libitum*. All cows were dewormed periodically with fenbendazole @7.5mg per kg body weight. Cows were maintained under standard managemental conditions.

Sampling. Six rumen liquor samples were taken from six cows after 3 hours of feeding. Rumen liquor collection site, left paralumbar fossa area has been cleaned aseptically. 20ml syringe with 18G needle has been used to collect rumen liquor at the time of ruminal contraction cycle from the aseptically prepared site. A 10 ml sample of rumen liquor was transferred into a sterilised and labelled container using a sterilised pipette and tip. The sample was promptly frozen in the liquid nitrogen. Then the sample was stored in -80°C until processing for DNA extraction (Carberry *et al.*, 2012).

Primers. Table: 1 has the polymerase chain reaction (PCR) primer sets used in this study for amplification of total methanogens, *Methanobrevibacter* sp. and *Methanosphaera stadtmanae* with the size of the product and primer size and sequence were the same as those referenced by Zhou *et al.* (2009).

Removal of RNA, protein, and DNA purification

Total genomic DNA extraction was done from rumen liquor collected by following three sample processing methods. In the first method, rumen fluids were sonicated, the resultant supernatant was column purified and used for DNA extraction (Method 1). The obtained DNA was found positive by gradient PCR. In second method, sonicated supernatant was directly used as template without column purification and was found to be negative for gradient PCR (Method 2). In third method, the rumen liquors were first centrifuged at slower speed of 1000 rpm for 3 minutes. Then 2 ml supernatant was collected in Eppendorftube and again centrifuged at 10000 rpm for five minute to get pellet, which is washed twice with normal saline (Method 3). Clear pellets were used for total genomic DNA extraction by column purification method as described below. Total genomic DNA obtained in this protocol was found to be positive for gradient PCR.

DNA was extracted at room temperature of 20° to 25°C, by taking equal quantity of samples or (pelleted rumen liquor of method 3) and lysis buffer mixed thoroughly and incubated in water bath at 56°C for 30 mins followed by purification using Qiagen DNA purification kit (QIAamp DNA Stool Mini Kit, Germany). Then ethanol was added, vortexed before loading in DNA QIAamp column and again centrifuged for one minute at 10000 rpm for 5 minutes. Both the flow through as well as collection tube were discarded. 500µl of buffer AW1 (Qiagen) was added and centrifuged for 1 minute at 6000 rpm for 3 minutes. The flow through content was discarded and again 500µl of buffer AW2 (Qiagen) was added and again centrifuged and flow through was removed. Then an empty run was given. Finally, 50 µl elute buffer was added to extract the DNA. Using a Nanodrop ND-1000 spectrophotometer, the DNA's quantity and quality

were evaluated (Nano Drop Technologies). The DNA was kept at -40° C. Gradient PCR run with denaturation step set for 5 minutes at 95°C then 35 cycles performed for 30 seconds at 95°C, followed by 30 seconds with varying annealing temperature and finally 30 seconds at 72°C (S1000 thermal cycler, Bio-Rad). The product was run on agarose gel (1%), with 5µl sample using ethidium bromide dye and visualized in Gel documentation (Bio Rad Inc., USA).

Real Time PCR Protocol. Total genomic DNA extracted from method 3 was utilized for qRT-PCR protocol.Two level of reaction mixture has been prepared at 10 microlitres and 20 microlitres. Three pairs of primers used to detect total methanogens, *Methanosphaera stadtmanae* and *Methanobrevibacter sp*. strain AbM4 in rumen liquor sample for qRT-PCR was given in Table 1. In 20 microlitre reaction, 10µl of SYBR green master mix, 1 µl of forward primer, 1 µl of reverse primer, 4 µl of nuclease free water, 4 µl of sample DNA were added. In 10 microlitre reaction, 5µl of SYBR green master mix, 0.5 µl of forward primer, 0.5 µl of reverse primer, 2 µl of nuclease free water, 2 µl of sample DNA were added. qRT-PCR was performed with SYBR green chemistry (TB Green Premix Ex Tad Cat: RR820A, Takara), using the Hi Media real-time PCR system with an initial denaturation at 95°C for 5min followed by 40 cycles, with annealing temperature of 55°C for 30 seconds, third cycle at 72°C for 30 seconds with sampling stage and a melting curve section at 95°C for 15 seconds. The amplification and melting curves were analysed for primers to determine the conditions for RT-PCR of the methanogens at the laboratory of Department of Veterinary Microbiology, Veterinary College and Research Institute, Orathanadu, Tamilnadu.

RESULT AND DISCUSSION

In this study, the primers referenced by Zhou *et al.* (2009) selected for amplification of *Methanobrevibacter, Methanosphaera standtmanae* and total methanogens. The attempt was found successful for standardization of PCR Protocol for estimating the relative abundance of methanogen in rumen of cattle reared under paddy straw based feeding system in Cauvery delta zone of Tamilnadu. A sensitive technology is needed to detect microorganisms using their DNA, and PCR is frequently utilized (Goudarzi *et al*., 2015).

Gradient Polymerase Chain Reaction. The product obtained in gradient PCR has been run in 0.8% agarose gel with 100 base pair DNA ladder (Fig. 1). The sample contained primer specific DNA *viz. Methanobrevibacter* sp. strain *AbM4*, *Methanosphaera stadtmanae* and total methanogens were documented approximately160 bp, 150 bp and 160 bp level, respectively (Fig. 1). Hence, it was decided to continue further for standardization of the protocol for measuring the relative abundance of methanogens in the rumen liquor samples.

The melting curve (Fig. 2 and Table 2) for total methanogens, *Methanobrevibacter* sp., and *Methanosphaera standtmanae* were obtained at 81-82°C, 85°C and 81-82°C, respectively in RT-PCR. Hence, the primers referenced by Zhou *et al.* (2009) can be used to estimate the relative abundance of methanogens in rumen of cattle reared under paddy straw based feeding system in Cauvery delta zone of Tamilnaduthrough RT-PCR study.

Selection of Primers. Most previous research exclusively looked at phylogenetic alterations and failed to relate such alterations to genes involved in methanogenesis processes. However, Shi *et al.* (2014) as well as Wallace *et al.* (2015) attempted to link the production of CH_4 with microbial genomes. Metagenomics as well as metatranscriptomics applications could potentially be utilised to link the rumen microbiome information with emissions of CH_4 across a large number of cattle with the objective to distinguish less CH_4 generating ruminant microbiomes from lower CH_4-yield phenotype ruminants and to understand energy-effective routes of metabolism in the rumen (Pitta, *et al.*, 2022).

The most common methanogen found in rumen of cattle was *Methanobrevibacter* sp (King *et al*., 2011; St-Pierre and Wright 2012 and 2013). *Methanobrevibacter* species were poorly to completely mobile, short rods or lancet-shaped cocci, and prefer temperatures from 37 to 39 °C (Sirohi*et al.* 2010). *Methanobrevibacter* exists in a variety of species, such as *Methanobrevibacter smithii, Methanobrevibacter gottschalkii, Methanobrevibacter thaueri, Methanobrevibacter ruminantium, Methanobrevibacter olleyae, Methanobrevibacter millerae, Methanobrevibacter wolinii, Methanobrevibacter woesei,* and *Methanobrevibacter arboriphilus* (Son *et al*., 2023).

For the generation of methane, *Methanobrevibacter* species typically use carbon dioxide and hydrogen, while certain species can also use formate and carbon dioxide. The ability to use formate is also shown by the following species: *Methanobrevibacter olleyae, Methanobrevibacter millerae, Methanobrevibacter smithii, Methanobrevibacter ruminantium, Methanospirillum hungatei,* and *Methanobrevibacter woesei*are among the methanobacteria that may produce methane (Hook *et al*., 2010).

The genus *Methanosphaera* has been observed in the rumen of cattle (King *et al.* 2011; St-Pierre and Wright 2012). *Methanosphaera stadtmanae,* a non-motile methanogen, exhibits cell wall properties that were similar to those of species in the *Methanobacteriaceae* family. *Methanosphaera stadtmanae* (order *Methanobacteriales*) uses hydrogen to convert methanol to methane rather than being able to convert carbon dioxide to methane (Miller and Wolin, 1985). Less than 2% of the total clones of both Jersey and Holstein dairy cattle, Western Australian sheep, Norwegian and Svalbard reindeer, and alpacas typically had *Methanosphaera stadtmanae* phylotypes. (King *et al*.2011; St-Pierre and Wright 2013; Sundset *et al.* 2009a, b). Nevertheless, it was recognised as the predominant methanogen discovered in faeces from ten Sumatran orangutans kept in captivity and fed a diet

predominantly consisting of fruit (Facey *et al.*, 2012) and has also been identified in the human intestine. Facey *et al.* (2012) *suggested* that *Methanosphaera stadtmanae* thrived because anaerobic bacteria converted pectin contained in fruit into methanol, but pectin is typically absent from ruminant diets, which significantly reduces methanol levels and the predominance of this methanogen.

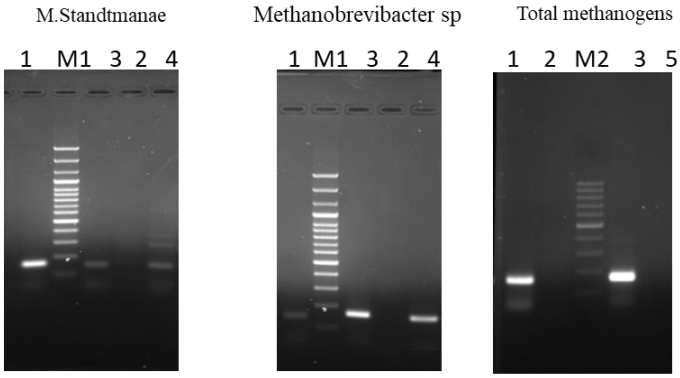
Rumen of adult cattle contain other methanogenesis routes, such as the methylotrophic pathway used by the recently identified *Methanomassiliicoccaceae* family (Oren and Garrity 2016; Nkamga and Drancourt 2015). *Methanosarcinales* members use less common acetoclastic routes pathways (Lambie *et al.*, 2015; Patra *et al.*, 2017). The relative abundance of *M. stadtmanae* and *M. ruminantium* was higher when animals were offered the low fiber compared to the high fiber diet (Carberry *et al.*, 2014). Thus, in order to efficiently limit CH_4 generation without disrupting the ruminal environment, it is crucial to understand what methane producing bacteria and their interaction exist in the rumen, who the key players in methane production.

In this study, total methanogens level was found higher than the *Methanobrevibacter* sp. and *Methanosphaera standtmanae*. During initial fermentation process large quantity of hydrogen were released by the rumen bacterial population. Methanogens thriving on 0.1–50 µM of dissolved hydrogen concentration (Janssen, 2010), with more than 5.0 Pa threshold are hydrogenotrophes like *Methanobrevibacter*, *Methanomassiliicoccales* The majority of the rumen methanogens are hydrogenotrophic, they convert carbon dioxide (CO_2) and formate into methane by utilising the hydrogen (H_2) produced from bacterial fermentation. As the methanogens work as an electron sink to lower the partial pressure of H_2 in the rumen, which at high concentrations inhibits bacterial fermentation. This role is essential for maintaining the directionality of the rumen metabolism (Morgavi *et al.*, 2010). Among all ruminant animal species and geographical areas studied, *Methanobrevibacter* was consistently found to be the most commonly encountered genus (Morgavi *et al.*, 2010; Henderson *et al.*, 2015). Hence, a positive correlation exists between hydrogenotrophs and CH_4 emissions. The biological diversity of methanogenic archaea seen in anoxic habitats does not detract from their shared effort to produce CH_4.

In contrary, the present study has higher relative abundance of *Methanosphaera standtmanae* than the *Methanobrevibacter* sp (Fig. 2). *Methanosphaera* are methylotrophic methanogens, they utilize methylamines and methanols. They thrive at low H_2 thresholds concentration with 1.0 Pa. Notably, in recent studies with using RNA-based methods, such as metatranscriptomics (Sollinger *et al.*, 2018) and 16S rRNA gene sequencing techniques (Pitta *et al.*, 2022), with higher influence of methylotrophs, suggests that *Methanosphaera* group might be more important for CH_4 production than previously thought.

The sampling for this study was done through left paralumbar fossa from the left dorso -caudal ruminal sac after 3 hours of feeding during ruminal contractions. The primary fermentation has taken place in the cranial ruminal sacs, which might release more hydrogen and methane, whereas in the caudal sacs the end products of the initial fermentation process were accumulated with low hydrogen concentration. This might be the reason for higher abundance of *Methanosphaera standtmanae* than *Methanobrevibacter* sp. Higher abundance of *Methanosphaera standtmanae* was also reported earlier in calves (Dong *et al.*, 2019).

The melting curve (Fig. 3) for total methanogens, *Methanobrevibacter* sp., and *Methanosphaera standtmanae* were obtained at 81-82°C, 85°C and 81-82°C respectively in RT-PCR. Hence, the primers referenced by Zhou *et al.*, 2009 can be used to estimate the relative abundance of methanogens in the rumen of cattle through RT-PCR study.



Lane 1- Sample 1, Lane 2- Sample 2, Lane 3- Sample 3, Lane 4- Positive control, Lane 5- Negative control, M1- 100bp plus, M2- 100 bp marker

Fig. 1. Gel Documentation System.

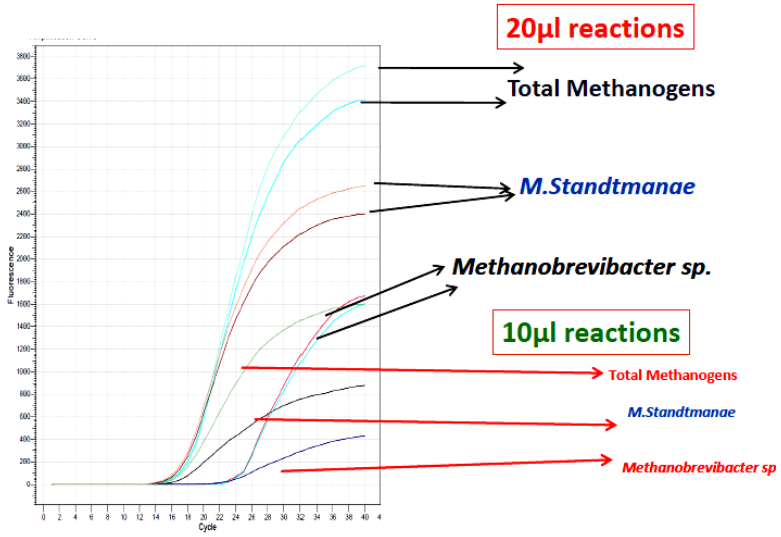


Fig. 2. RT-PCR amplification curve of Methanogen genes.

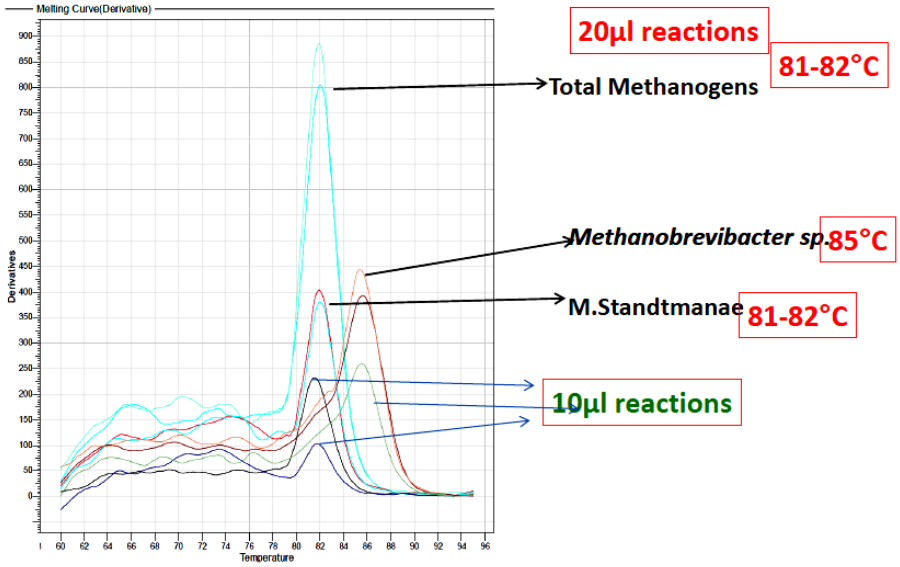


Fig. 3. RT-PCR Melting curve of Methanogen genes.

Table 1: Primers used in the protocol to study the Methanogens in the rumen liquor of cows.

Sr. No.	Genes	Size of the Product (bp)	Forward and Reverse Primers	Primer Sequence	Size of the Primer Product
1.	*Methanobrevibacter* sp. strain	160	AbM4-F	TTTAATAAGTCTCTGGTGAAATC	23
			AbM4-R	AGATTCGTTCTAGTTAGACGC	21
2.	*Methanosphaera standtmanae*	150	Stad-F	CTTAACTATAAGAATTGCTGGAG	23
			Stad-R	TTCGTTACTCACCGTCAAGATC	22
3.	Total methanogens	160	uniMet1-F	CCGGAGATGGAACCTGAGAC	20
			uniMet1-R	CGGTCTTGCCCAGCTCTTATTC	22

Table 2: Determination of Melting Temperature in RT-PCR protocol.

Sr. No.	Genes	Size of the Product (bp)	Annealing Temperature
1.	*Methanobrevibacter* sp. *strain AbM4*	160	53°C
2.	*Methanosphaera standtmanae*	150	52.8°C
3.	Total methanogens	160	60°C

CONCLUSIONS

Methanosphaera standmanae was higher in this present study than *Methanobrevibacter* sp. Hence, sampling can be done in the left paralumbar fossa site for specific study on *Methanosphaera standmanae*. Otherwise, the sampling of rumen liquor can be made through stomach tube from cranial sac of rumen where initial active fermentation takes place for routine studies. This site will be more representative for all rumen microbes than

the site at left paralumbar fossa. The primers used in the study can be effectively used owing to the prudentiality and estimation of relative abundance of methanogens in Jersey cross bred dairy cows of this region with the existing managemental conditions. In DNA extraction protocol, column purification should be carried out. The procedure without column purification will not be useful to extract DNA to observe the abundance and quantification of the methanogens. The selection and designing of novel primers for different area specific feeding practices will be useful in study of kinetics of the methanogens in rumen. Hence, the above mentioned improved protocols of this study can be followed for estimating the relative abundance of methanogens in the rumen in paddy straw based feeding practices adopted areas.

FUTURE SCOPE

The standardized and improved protocol will be followed in future for the estimation of relative abundance of the methanogens in rumen.

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Conflict of Interest. None.

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