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Quantification and Microbial Diversity Analysis of Ruminal Methanogenic Populations in Indian Gir Native Cattle

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ABSTRACT

Background: Methane emission from the ruminants is receiving global attention due to its global warming potential. It imposes the development of cost-effective and easily adoptable energy to reduce methane emissions from ruminants. Apart from this, the information available on the methanogenic microflora and their populations in the Indian native cattle is also very limited. In this study, the normal methanogenic microflora of Indian native Gir cattle was studied using molecular methods and compared with cross breed cattles.

Methods: The rumen fluid was collected and DNA isolation was carried out from Gir, Gir cross and Kangayam crossbred cattle. The partial 16S rRNA and mcrA gene amplification were carried out by PCR and further subjected to sequence analysis. Further, Methanogenic population in the ruminal fluid were analyzed by SYBR Green-based real-time PCR.

Result: This study provides a basic understanding of the normal methanogenic microfloral diversity and their population in the Gir native cattle of Indian origin compared with cross breed cattle.

Key words: 16S rRNA gene, mcrA gene, Methane, Methanogens, Phylogenetic analysis, qRT-PCR analysis, Ruminal fluid.

INTRODUCTION

There are various microbial communities that exist in the rumen and it forms a complex system that includes bacteria, protozoa, archaea and fungi. In this microbial community, bacterial populations are highest and diverse in nature. Rumen microorganisms play an important role in the emission of a greenhouse gas called methane. Methane occupies the second-largest gas that contributes to the greenhouse gas which is primarily produced by ruminants. Globally, about 80 million tonnes of methane are produced annually from enteric fermentation mainly from ruminants out of which Indian livestock contributes about 15.1% of total global enteric methane emission (Patra *et al.*, 2014). As the demand for meat and milk continues to grow worldwide especially in developing countries, methane emission from ruminants will likely continue to increase in the years to come unless effective and practical methane mitigation strategies are implemented in ruminant feeding. Over the past decade, intensive research has been carried out all over the world to identify and develop effective and practical means to decrease the methane emission from ruminants (Hristov *et al.*, 2013).

In India there are 37 different indigenous breeds of cattle and 13 different indigenous breeds of buffalo are available. Among cattle breed, the Gir cattle is known for its milk producing quality which has one of the principle zebu breeds originating in India. Kangayam cattle breeds are known for drought which is found in Tamil Nadu. These two cattle breeds are very much suitable for milk and drought in India which has also been cross bred with different cattle bred to increase the milk production and for other purposes. When compared to exotic cattle and their cross bred, native cattle produce 5-8% less methane by enteric fermentation.

The present study was undertaken to ascertain the methanogenic archaeal diversity and their population in the rumen of Gir, Gir cross and Kangayam cross bred by16S rRNA and mcrA gene sequencing and their population quantification by quantitative real time PCR.

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MATERIALS AND METHODS

Cattle, diet and rumen sample collection

A 100-200 ml of rumen fluid was collected from Gir, Gir cross and Kangayam cross bred cattle maintained at Livestock Farm Complex (LFC), Madhavaram milk colony, Chennai, Tamil Nadu by rumen fluid extraction pump. The rumen fluid was collected in an anaerobic container and then transferred to a separate sterile container, labelled and transported to laboratory and stored at -20°C until further analysis. All the animals were fed with standard milch cattle ration.

DNA extraction

Total bacterial DNA from the rumen fluid of all the gir, gir cross and kangayam cross bred were extracted using qiagen DNA stool kit as per the manufacturer instructions.

The quantification and purity of the extracted DNA was measured at A260/280 using nanodrop spectro photometer.

PCR amplification of 16S rRNA and mcrA gene

The PCR amplification of partial 16S rRNA gene was carried out with an initial denaturation for 5 min at 94°C, 30 cycles at 94°C for 30 s, 57°C for 1 min, 72°C for 1 min and final elongation for 7 min at 72°C and PCR amplification of mcrA gene was carried out with an initial denaturation for 3 min at 95°C, 30 cycles at 95°C for 30 s, 55°C for 30 s, 72°C for 1 min and final elongation for 5 min at 72°C.The reaction was performed for the total volume of 25 µl which consist of 1 µl of forward and reverse primer (20 pmol concentration), 12.5 µl of master mix, 3 µl of DNA template and 7.5 µl of nuclease free water (NFW). The amplified PCR product were analysed in 1.2% agarose gel containing ethidium bromide under gel documentation unit. The primers used for PCR and real time PCR details were given in Table 1.

Sequencing and phylogenetic analysis

The PCR amplified products of partial 16S rRNA and mcrA gene were purified using Qiagen PCR gel purification kit and sequencing was performed at Eurofins genomics sequencing Ltd, Bengaluru, India. The nucleotide sequence data was subjected to BLAST analysis (www.ncbi.nlm.nih.gov), assembled are analyzed and using seq man and mega align programs of lasergene package version 7.1.0. Nucleotide sequence alignment was performed by Clustal W method with Mega Align program and predicted amino acid sequence was analysed by proteon program of DNA Lasergene (DNA star Inc). Phylogenetic analysis of 16S rRNA and mcrA gene sequence was performed using maximum likelihood method with 1000 bootstrap replication in the MEGA software Version 10. The reference sequences used for construction of phylogenetic analysis are given in Table 2.

qRT-PCR analysis of methanogenic bacterial populations

The selected methanogenic bacterial populations were determined by calculating the copy number of 16S rRNA genes. Three pair of primers was used to detect *Methanobrevibactersp*, *Methanosphaera stadtmane* and total methanogens from rumen samples.

qRT- PCR was performed using SYBR green master mix with the total reaction volume of 10 µl in each well in triplicate. The reaction volume consists of 5 µl of SYBR green master mix, 0.5 µl (5 pmol) of forward and reverse primer, 3 µl of nuclease free water (NFW) and 1 µl of DNA template. Copy number of a DNA was calculated using serially diluted DNA and used as standard in the quantification. The qRT-PCR was performed using the real time PCR system (Applied Biosystems) with the initial denaturation 95°C for 10 min, which is followed by 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 35 s. For melt curve analysis, the temperature was increased 0.3°C every 20 s from 60°C and 95°C. The standard DNA curve were constructed by methanogenic species specific primers based on the serial dilution of standard DNA. The copy number of the standard DNA was calculated using the following formula:

$$\frac{(X(ng) \times 6.022 \times 10^{23} \text{ molecules/mole}}{(N \times 660 \text{ g/mole} \times 1 \times 10^{9} ng/g)}$$

Where,

X is amount of DNA in nanograms (ng).

N is the length of DNA in basepairs (bp).

6.022×10^{23} is Avogadro constant and 660 g/mole is average mass of 1 bp dsDNA.

This research was carried out in Department of Animal Biotechnology, Madras veterinary college for period of 1year (2021).

RESULTS AND DISCUSSION

The cattle population in India has been increasing from 190.90 million in 2012 to 192.50 million in 2019 (Agricultural Research Data Book, 2019). Basic understanding about rumen methanogens microbial diversity in indigenous and exotic cattle is important to formulate the strategies for methane mitigation from Indian livestock. In the present study, diversity of methanogens is explored in the Gir native cattle along with crossbred cattle of Gir and Kangayam bred fed with standard milch animal diet using molecular approaches based on 16S rRNA and mcrA gene. To the best of our knowledge, it is the first report on the methanogens microbial diversity analysis in Indian Gir native cattle along with crossbred cattle of Gir and Kangayam.

PCR amplication and sequence analysis

The partial 16S rRNA gene of methanogenic bacteria of ruminal fluid from Gir, Gir cross and Kangayam cross bred was amplified using the methanogenic specific 16S rRNA primers with product size of 800 bp and sequenced (MW916668- MW916670) (Fig 1). There are several reports about the use of 16S rRNA gene for the identification of methanogens from environmental samples. The 16S rRNA gene sequence of all the three strains of methanogenic producing bacteria from Gir, Gir cross and Kangayam cross revealed 99-100% identity with other KX787709, KX787608 and HQ616028, respectively. The phylogenetic analysis also revealed that, all these three strains of 16S rRNA from Gir, Gir cross and Kangayam Cross were claded with EU330421 and M59142, which are methanogenic bacteria earlier reported from India which indicates our all the three strains belongs to *Methanobacteriales* (Fig 3). The phylogeny of methanogens determined using mcrA sequences in accordance with those determined using 16S rRNA gene sequences (Friedrich, 2005).

However, the use of 16S rRNA is imposes the risk of amplification of other bacteria along with Methanogenic bacteria. It is essential to detect the methanogens on the basis of functional genes that are found to be unique in methanogenesis. The Methyl coenzyme M. reductase (mcr) is the terminal enzyme involved in methanogenesis, which reduces the methyl group bond of methyl coenzyme M with the release of methane (Friedrich, 2005). Because the α-

Table 1: Primers used in this study for PCR and qRT-PCR analysis.

Organism targeted	Primer	Sequence (5′-3′)	Product size	Reference
16S rRNA	Met 86f	GCTCAGTAACACGTGG	800 bp	Zhou *et al.*, (2009)
	Met 915r	GTGCTCCCCCGCCAATTCCT		
mcrA gene	MLFP	GGTGGTGTMGGATTCACACARTAYGCWACAGC	470 bp	Sirohi *et al.*, (2013)
	MLRP	TTCATTGCRTAGTTWGGRTAGTT		
Methanobrevibacter sp.	AbM4-F	TTTAATAAGTCTCTGGTGAAATC	160 bp	Zhou *et al.*, (2009)
	AbM4-R	AGATTCGTTCTAGTTAGACGC		
M. stadtmanae	Stad-F	CTTAACTATAAGAATTGCTGGAG	150 bp	Zhou *et al.*, (2009)
	Stad-R	TTCGTTACTCACCGTCAAGATC		
Total methanogens	uniMet1-F	CCGGAGATGGAACCTGAGAC	160 bp	Zhou *et al.*, (2009)
	uniMet1-R	CGGTCTTGCCCAGCTCTTATTC		

Table 2: Reference sequence used in this study for phylogenetic analysis.

Reference methanogenic bacteria used for construction of phylogenetic tree based on 16S rRNA gene	Accession number	Reference methanogenic bacteria used for construction of phylogenetic tree based for mcrA gene	Accession number
Methanococcus igneus	M59125	*Methanoculleus bourgensis* MS2	AB300787
Methanococcus vannielii	AY196675	*M. bourgensis* RC/ER	AB300785
M. vannielii	M36507	*M. bourgensis* CB1	AB300786
Methanococcus voltae	U38488	*Methanoculleus*. sp. T14	AB288291
Methanococcus jannaschii	M59126	*Methanoculleussp*. HC-1	AB288267
M. thermolithotrophicus DSM 2095	M59128	*Methanoculleus methanosarcina* sp.	AB288266
Methanosarcina mazei	AY196685	*Methanobrevibacter* sp.	EU919429
Methanosarcina barkeri str. CM1	AJ002476	*M. gottschalkii*	EU919431
M. mobile BP strain DSM 1539	M59142	*M. mobile*	AF414044
Methanobrevibacter thaueri strain CW	U55236	*Methanobrevibacter smithii*	GU385700
Methanobrevibacter sp. SM9	AJ009958	*M. thermophilus* DSM 2624	AF313804
Methanobrevibacter sp. 1Y	DQ135988	*Methanoculleus thermophilus*	AB300783
Methanobrevibacter gottschalk ii strain HO	U55238	*Methanospirillum hungatei* JF-1	AF313805
Methanobrevibacter gottschalk ii strain PG	U55239	*Methanospirillum hungatei* DSM 864	AF414038
Methanobrevibacter smithii strain PS	AY196669	*Methanogenium boonei*	DQ229161
Methanobrevibacter woesei strain GS	U55237	*Methanogenium frigidum*	DQ229158
Methanobrevibacter sp. AbM4	AJ550156	*Methanosarcina mazei*	AB300782
Methanobrevibacterwolinii strain SH	U55240		
Methanobrevibacterar boriphilus strain A2	AY196662		
Methanobrevibacterar boriphilus strain DH-1	AY196665		
Methanobrevibacter sp. ATM	AF242652		
Methanobrevibacter sp. 30Y	AY615205		
Methanobrevibacter sp. OCP	AY615203		
Methanobrevibacter olleyae strain KM1H5-1P	AY615201		
Methanobrevibacter sp. FM1	AJ550157		
Methanobrevibacter sp. AK-87	AY615202		
Methanobrevibacter sp. Z8	AY196672		
Methanobrevibacter sp. NT7	AJ009959		
Methanosphaera stadtmanae	AY196684		
Methanobacterium bryantii	M59124		
Methanobacterium bryantii strain MOHG	AY196658		
Methanobacterium formicicum	AY196659		
M. thermautotrophicus strain delta H	AY196660		
Methanothermobacter marburgensis	X15364		

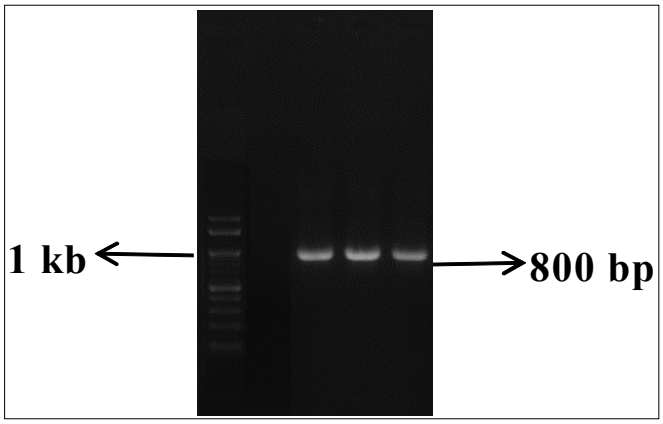

Fig 1: PCR amplification of 16s rRNA gene of Gir, Gir Cross and Kangayam cross.
M-100 bp DNA ladder, L1-Negative control, L2-L4-PCR amplification of 16s rRNA of Gir, Gir cross and Kangayam cross breed.

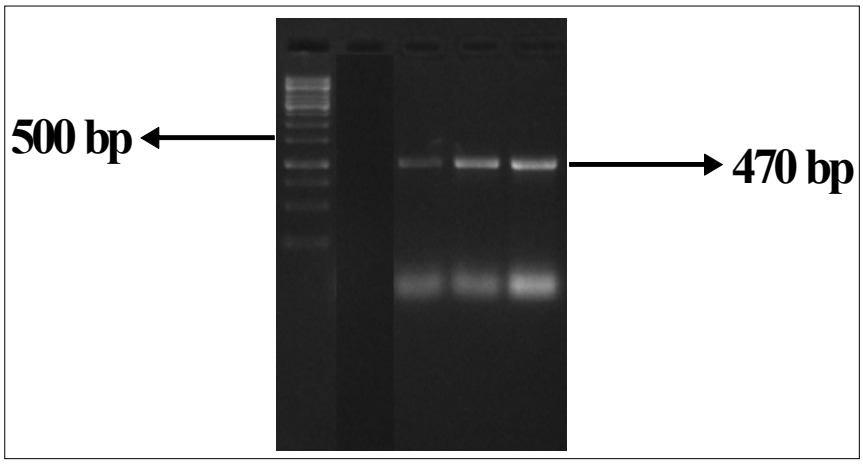

Fig 2: PCR amplification of mcrA gene of Gir, Gir Cross and Kangayam cross.
M-100 bp DNA ladder, L1-Negative control, L2-L4-PCR amplification of mcrA of Gir, Gir cross and Kangayam cross breed.

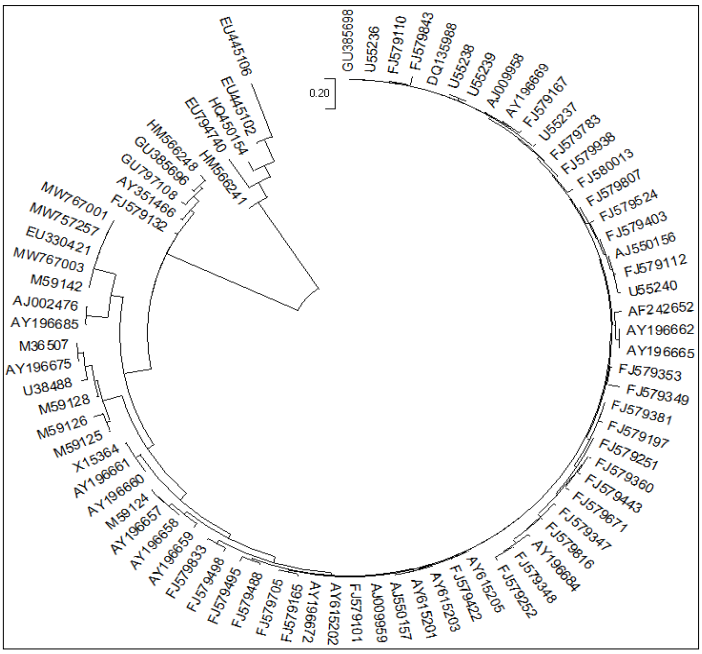

Fig 3: Phylogenetic analysis of 16S rRNA gene sequence of Gir, Gir cross and Kangayam cross bred cattle.

subunit of mcr(mcrA) and its isoenzyme gene (mrtA) are highly conserved among methanogens and that these genes are almost exclusively found in methanogens, mcrA/mrtA-based detection of methanogens has been used.

Further in this study, mcrA gene of methanogenic specific bacteria of ruminal fluid from Gir, Gir cross and Kangayam cross bred was amplified using specific primers with the product size of 470 bp (Fig 2) and sequenced (MW757257, MW767001 and MW767003).The sequence analysis revealed that the all the sequence of mcrA gene has more than 99 percentage of identity with AB928466, AB615893 and AB617118 for Gir, Gir cross bred and Kangayam cross bred. The phylogenetic analysis of mcrA gene forms three different clade in which all the three sequences belongs to *Methanomicrobiales* along with AF414044 (Wiltshire SP40JG, UK), HQ450171 (Haryana, India) and HQ450167 (Haryana, India) (Fig 4). Comparative studies between the 16S rRNA and mcrA gene revealed mcrA gene is more efficient for classifying the diverse group of methanogens in phylogenetically (Juottonen *et al*., 2006, Jerman *et al*., 2009).

Our findings revealed the dominance of *Methanobrevibacter*-related sequences on the basis of 16S rRNA and mcrA gene sequence homology. The presence of *Methanobrevibacter* as major phylotype in the Gir cattle, Gir cross bred and Kangayam cross bred is in agreement with earlier reports of Denman *et al*., (2007) who reported *Methanobrevibacter sp*. are the dominant methanogens in Brahman crossbred steers (*Bosindicus*). In another study, Jeyanathan *et al*., (2011) revealed that *Methanobrevibacter sp.* are dominant in cattle from New Zealand. Franzolin *et al.* (2012) have also identified *Methanobrevibacter* as the dominant genus in Holstein cattle, water buffaloes and crossbred buffalo, respectively. Dominance of genus *Methanobrevibacter* observed in our study is in accordance to Janssen and Kirs, (2008) who surveyed that 61.6% of the rumen archaea belongs to this genus. Skillman *et al.* (2006) and Wright *et al.* (2007) have reported the presence of *M. stadtmanae* in bovine rumen. The majority of the sequences retrieved from bovine rumens and cattle dung are belongs to *Methanomicrobiales* and *Methanobacteriales* (Rastogi *et al*., 2008). On the other hand, the same group observed that in Surti rumen 16S rRNA library, 97% clones were related to *Methanomicrobiales* and *Methano bacteriales*. Many studies are also carried out based on the 16s rRNA gene sequences due to their highly conserved nature and availability of the sequences from databases. However, based on the 16S rRNA sequence analysis it is very difficult to differentiate closely related taxons. Recently, studies were being carried out with mcrA gene instead of 16s rRNA gene since the mcrA has higher evolutionary rate, less conserved sequences and can also afford information related to the functional diversity of methanogens (Sheppard *et al*., 2005). For these properties of mcrA gene it's used as a marker for phylogenetic analysis in conjunction with or independent of, 16S rRNA genes (Steinberg and Regan, 2008).

Montoya *et al.* (2011) reported the number of differences per site in the mcrA gene fragment is 2-3 times higher than that in the full length 16S rRNA and therefore, mcrA sequences offers conclusive resolution and assignment of genera than 16S rRNA gene sequences (Meyer and Kuever, 2007). Overall, the findings from the present study indicate that methanogenic populations of Gir cattle and the crossbred of Gir and Kangayam cattle are predominant with *Methanobrevibacter* related phylotypes. The diversity analysis using mcrA gene has provided better insight of

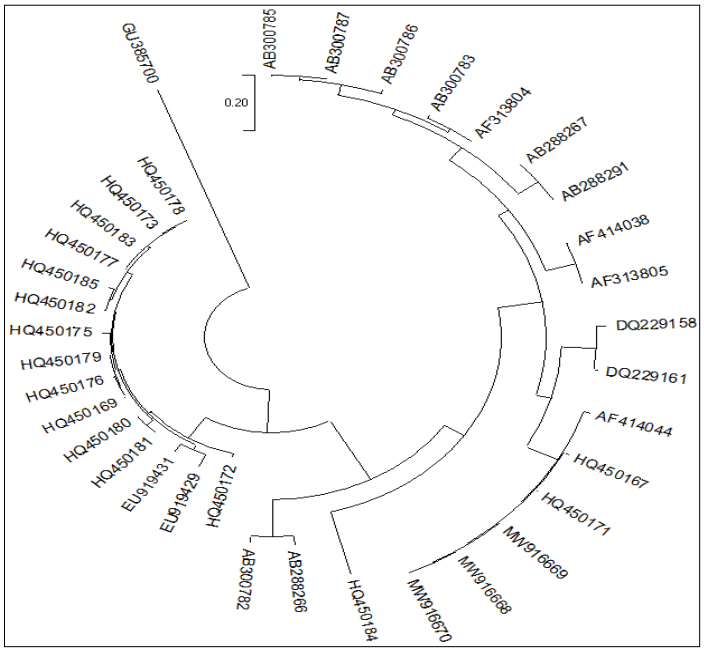

Fig 4: Phylogenetic analysis of mcrA gene sequence of Gir, Gir cross and Kangayam cross bred cattle.

Table 3: Per cent identity of mcrA gene of Gir, Gir cross and Kangayam cross bred.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
1		70.7	71.0	73.5	73.0	72.3	73.1	73.4	64.8	63.2	67.8	68.3	4.5	62.8	68.2	1	**mcrA AB288266**
2	37.1		99.7	91.5	88.7	88.2	89.0	91.0	76.5	72.5	76.4	76.9	3.1	56.4	78.4	2	**mcrA AB288267**
3	36.8	0.1		91.8	89.0	88.4	89.2	91.3	76.7	72.7	76.6	77.2	3.1	56.6	78.6	3	**mcrA AB288291**
4	32.7	9.6	9.5		93.4	92.6	93.5	98.4	78.8	75.4	81.5	81.4	3.1	55.7	81.4	4	**mcrA AB300783**
5	33.1	12.8	12.6	6.9		96.8	99.6	93.4	79.0	75.8	81.5	81.6	4.0	55.1	81.4	5	**mcrA AB300785**
6	34.2	13.9	13.7	7.8	3.3		97.2	92.9	79.0	75.8	81.5	81.6	4.0	54.7	81.2	6	**mcrA AB300786**
7	32.9	12.4	12.3	6.8	0.4	2.9		93.5	79.2	75.6	81.7	81.8	4.0	55.1	81.6	7	**mcrA AB300787**
8	33.1	10.2	10.1	1.6	6.9	7.5	6.8		79.0	76.8	81.9	81.8	3.1	56.0	81.8	8	**mcrA AF313804**
9	44.8	28.0	27.7	23.8	23.5	23.4	23.2	23.5		79.2	92.5	92.0	5.7	58.9	95.9	9	**mcrA AF414044**
10	45.3	30.7	30.4	26.0	26.0	25.7	25.7	25.1	24.5		79.7	80.0	6.6	64.5	79.7	10	**mcrA DQ229158**
11	40.6	25.9	25.6	22.6	22.9	22.5	22.6	22.0	2.9	22.8		99.8	5.7	59.6	100.0	11	**mcrA MW916668**
12	39.7	25.4	25.1	22.4	22.4	22.1	22.1	21.8	3.0	22.3	0.2		5.7	59.6	99.8	12	**mcrA MW916669**
13	150.5	141.8	141.8	136.7	143.3	144.1	143.3	136.0	129.9	121.5	131.9	132.5		7.8	5.7	13	**mcrA GU385700**
14	50.4	59.9	59.4	57.0	59.0	60.3	59.0	56.4	56.6	55.5	54.4	54.3	151.3		57.7	14	**mcrA HQ450181**
15	40.3	26.4	26.1	22.6	23.2	22.9	22.9	22.0	2.8	22.8	0.0	0.2	127.1	53.9		15	**mcrA MW916670**
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		

Table 4: Deduced amino acid variations of mcrA protein.

Amino acid position	1	2	3	16	17	20	26	29	30	35	36	37	38	39	41	49	50	51	52	54
Gir mcrA gene	G	G	V	N	I	E	M	I	K	V	D	Y	K	N	S	T	Q	E	V	N
Gie cross mcrA gene	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Kangayan cross mcrA gene	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BAF46705	.	.	.	D	.	N	V	.	N	-	G	A	A	.	G	.	L	.	.	K
BAF46706	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	Y	D	I	.
BAF46717	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	Y	D	I	.
BAF56662	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	.	D	I	.
BAF56664	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	.	D	.	.
BAF56665	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	.	D	.	.
BAF56666	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	.	D	.	.
AAK16834	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	.	D	I	.
AAL29293	-	-	-	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ADC54991	V	V	F	D	V	D	K	V	E	G	L	T	E	A	N	N	M	D	T	L
ADR66506	.	.	.	.	V	D	K	V	E	D	L	C	A	A	N	N	M	D	T	L

Amino acid position	**56**	**57**	**58**	**61**	**62**	**63**	**64**	**65**	**70**	**73**	**74**	**75**	**77**	**88**	**89**	**91**	**92**	**94**	**95**	**97**	**98**
G	I	A	T	N	L	N	G	M	Q	T	M	M	D	L	A	S	C	I	T	S	I
GC	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
KC	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BAF46705	.	.	.	T	.	Y	.	I	K	.	A	L	.	.	.	A	A	V	A	A	L
BAF46706	.	S	.	A	.	.	.	.	.	.	.	.	.	.	.	A	S	L	S	.	.
BAF46717	.	S	.	A	.	.	.	.	.	.	.	.	.	.	.	A	S	L	S	.	.
BAF56662	.	.	.	T	.	.	A	.	.	.	.	.	.	I	.	A	S	L	S	.	.
BAF56664	M	.	.	T	.	.	A	.	.	.	.	.	G	I	.	A	S	L	.	A	.
BAF56665	M	.	.	T	.	.	A	.	.	.	.	.	.	I	.	A	S	L	.	A	.
BAF56666	M	.	.	T	.	.	A	.	.	.	.	.	.	I	.	A	S	L	.	A	.
AAK16834	.	.	.	T	.	.	A	.	.	.	.	.	.	I	.	A	S	L	S	.	.
AAL29293	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
ADC54991	V	G	S	S	F	Y	A	L	E	A	L	L	T	I	S	A	A	C	S	A	F
ADR66506	V	G	.	A	F	Y	A	L	E	A	L	L	T	I	S	A	A	C	S	A	F

Amino acid position	**103**	**104**	**105**	**108**	**113**	**115**	**116**	**120**	**121**	**127**	**137**	**140**	**141**	**143**	**144**	**145**
Gir mcrA	S	N	A	N	S	F	M	G	W	F	S	S	L	M	E	P
Gir cross mcrA	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Kangayan cross mcrA gene	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
BAF46705	A	.	.	S	.	Y	L	A	.	.	A	V	.	Y	Q	G
BAF46706	.	.	.	.	C	L	L	.	.	.	.	.	.	I	R	G
BAF46717	.	.	.	.	C	L	L	.	.	.	.	.	.	I	R	G
BAF56662	.	.	.	.	.	L	L	.	.	S	.	.	.	.	.	S

Table 4: Continue...

Table 4: Continue...

BAF56664	.	.	.	.	.	L	L	.	.	.	.	.	.	.	.	.
BAF56665	.	.	.	.	.	L	L	.	.	.	.	.	.	.	.	.
BAF56666	.	.	.	.	.	L	L	.	.	.	.	.	.	.	.	.
AAK16834	.	.	.	.	.	L	L	.	.	.	.	.	.	.	.	S
AAL29293	.	.	.	.	.	L	.	.	.	.	.	.	.	.	.	.
ADC54991	A	Q	T	S	G	Y	L	Q	H	Y	A	V	F	I	R	N
ADR66506	A	Q	T	S	.	Y	L	Q	H	Y	A	V	F	I	R	N

Table 5: Absoulte quantificaton and statistical analysis of total methanogens, *M. stadtmanae* and *Methanobrevibacter* sp from Gir, Gir corss and Kangayam cross cattle.

Organism group	No. of copies/ml			*p*	F	Proportion of total methanogens (%)			*p*
	Gir cattle	Gir cross cattle	Kangayam cross cattle			Gir cattle	Gir cross cattle	Kangayam cross cattle	
Total methanogens	$0.20\pm0.11 \times 10^8$	$0.32\pm0.06 \times 10^8$	$0.38\pm0.09 \times 10^8$	0.375	1.16	100	100	100	0.05
M. stadtmanae	$5.26\pm0.27 \times 10^8$	$6.16\pm0.65 \times 10^8$	$6.33\pm0.44 \times 10^8$	0.306	1.45	5.3	6.2	6.3	0.05
Methanobrevibacter sp.	$1.82\pm0.33 \times 10^8$	$0.84\pm0.55 \times 10^8$	$0.68\pm0.41 \times 10^8$	0.169	2.43	1.8	0.8	0.7	0.05

N>- Non significant ($p \geq 0.05$).

rumen methanogens than 16S rRNA gene.

Per cent identity of mcr agene

The mcrA gene reveals 92%, 92.5% and 95.9% percent identity to Gir, Gir cross and Kangayam cross bred with AF414044 and AF414044 respectively. Among the three mcrA gene sequences, Gir cattle reveals 100% identity with Kangayam cross and 99.8% identity with Gir cross bred (Table 3).

Deduced amino acids variations

The deduced amino acid variations of mcrA gene reveals, there are 57 amino acid variations while comparing with GU385700. The amino acid changes are conserved across Gir, Gir cross and Kangayam cross bred, however it varies for all other mcrA gene of methanogenic bacteria. The deduced amino acid variation was shown in Table 4.

Absolute quantification of methanogenic population of ruminal fluid by qRT-PCR

The total methanogen population, *Methanobrevibacter sp.* and *Methanosphaera stadtmanae* from the ruminal fluid of three cattle from each bred of Gir, Gir cross and Kangayam cross were analyzed using absolute quantification by real time PCR. This study reveals that the total methanogen populations of all the three Gir cattle is 0.26 mg/ml, 1.95 mg/ml and 0.31mg/ml, Gir cross is 0.23 mg/ml, 0.26 mg/ml and 0.44 mg/ml and for Kangayam cross is 4.91mg/ml, 5.08 mg/ml and 5.78mg/ml respectively. The absolute copy number of partial 16S rRNA gene of *Methanosphaera stadtmanae* of all the three Gir cattle is 0.63 mg/ml, 1.12 mg/ml and 0.29 mg/ml, Gir cross is 0.17 mg/ml, 0.02 mg/ml and 0.38 mg/mlandfor Kangayam cross is 5.15 mg/ml, 5.94 mg/ml and 7.36 mg/ml respectively. This confirming more or less similar quantity of methanogen in the ruminal fluid. The absolute copy number of *Methanobrevibacter sp*. of partial 16S rRNA gene of all the three gir cattle is 2.12 mg/ml, 2.18 mg/ml and 1.17 mg/ml, gir cross cattle is 0.29 mg/ml, 0.31 mg/ml and 0.55 mg/ml and for kangayam cross cattle are 5.66 mg/ml, 6.16 mg/ml and 7.17 mg/ml respectively (Table 5). This confirming more or less similar quantity of methanogens in the ruminal fluid.

The proportions of 16S rRNA genes of *Methanobrevibacter sp.* and *Methanosphaera stadtmanae*, the later reveals higher significance difference between these two methanogenic bacteria in the ruminal fluid of Gir, Gir cross and Kangayam cross.

Our results of qRT-PCR analysis revealed that the *Methanobrevibactersp* is the most dominant gene in rumen and *Methanosphaera stadtmanae* copy numbers are very low level in the rumen total methanogens. The unique combination of ruminal microbiota in each animal may have important roles in the host's nutrient uptake and energy metabolism, phenotypes that are usually regulated by the genetics, diet and environment of the host.

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Conflict of interest

The authors declare that they have no conflict of interest.

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