RESEARCH ARTICLE Indian Journal of Animal Research, Volume 54 Issue 2: 163-167 (February 2020)

Sequence analysis of Mucin 13 gene region associated with piglet diarrhoea in Indian pigs

Rebeka Sinha[1], Nihar Ranjan Sahoo[1], Kush Shrivastava[1], Pushpendra Kumar[1], Salauddin Qureshi[2], Amit Kumar[1], GVPPS Ravikumar[3], Bharat Bhushan[1]

10.18805/ijar.B-3757

ABSTRACT

The current study was undertaken for characterization and bioinformatics analysis of a region of Mucin 13 gene (MUC13) associated with piglet diarrhoea in Indian native pig (*Sus scrofa*). A total of five gene segments (three at intron 6 and two at exon 7-8 regions) were sequenced in pig population. The sequences were compared with available sequences of other pig population (Chinese pig), sheep, goat and cattle. As expected, high level of genetic similarity was seen between Indian pig and Chinese pig. Sequences of all five alleles were submitted to the NCBI with accession numbers KX686556 and KX686558-61. Reported genetic variability in MUC13 gene can be helpful in enhancing our understanding about the genetics of piglet diarrhoea resistance and susceptibility in Indian pig population.

Key words: *E.coli*, Indian native pig, Mucin 13, Piglet diarrhoea.

INTRODUCTION

Mucins are glycoproteins covering the apical surfaces of epithelial cells in gastrointestinal and respiratory tracts which form the first line of defence against pathogens entering these systems (Ringel and Lohr, 2003). They have been thought to play an important role in infectious and inflammatory diseases, cancer and metastasis (Moncada *et al*., 2003). Mucins have also become target genes in disease resistance research in piglets. Among these mucins, the mucin 13 (MUC13) gene have been proposed to be candidate gene for piglet diarrhoea resistance. Among past few years piglet diarrhoea has emerged as an important disease of piglets (Lalremruata *et al*., 2015; Pegu *et al*., 2017) causing mortality and production losses, contributing about 11.5% to 29.5% of total death (Li *et al*., 2007). The neonatal and post weaning diarrhoea in piglets is caused by *E.coli* with two main pathotypes involved *viz*., ETEC (enterotoxigenic *E.coli*) and EPEC (enteropathogenic *E.coli*). Due to its' widespread incidence (56.2% among all types of diarrhoea) researches have focussed in identification of single nucleotide variations in candidate genes that can impart resistance towards ETEC diarrhoea in piglets.

The pathogenesis of piglet diarrhoea involves attachment of ETEC fimbriae with their receptors in small intestine of piglet. Expression of these receptors is variable, among within and between breeds and adhesion of receptor with bacterial fimbriae in genetically determined and exhibits Mendelian inheritance (Bijlsma *et al*., 1982). This fact gives a window to attempt for breeding pigs for resistance towards ETEC mediated diarrhoea using putative candidate genes as markers for indirect selection. However, exact causative mutations responsible for genetic variation in susceptibility to ETEC-F4 are unknown (Schroyen *et al*., 2012), MUC13 have been proposed to be a putative candidate gene that has been extensively studied for its role in imparting resistance to piglet diarrhoea in piglets. Zhang *et al*. (2008) found out that the MUC13 is in strong linkage disequilibrium with enterotoxigenic *E.coli* (ETEC) F4ab/ac receptor locus. Ren *et al*. (2012) did genome wide mapping using 39,720 informative SNPs and revealed that the most significant markers were proximal to the MUC13 gene in the 2.3-Mb region. Association studies in other diverse outbred pig populations have suggested that MUC13 gene may be most likely to be responsible for resistance / susceptibility towards piglet diarrhoea or it may carry mutations in Linkage Disequilibrium with gene / loci affecting this trait. Owing to its importance in piglet diarrhoea resistance we characterized MUC13 gene (partial sequence) in Indian native pigs using bioinformatic tools.

[1]Division of Animal Genetics, ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, Uttar Pradesh, India.
[2]Standardization Division Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, Uttar Pradesh, India.
[3]Division of Veterinary Biotechnology, ICAR-Indian Veterinary Research Institute, Izatnagar-243 122, Uttar Pradesh, India.

Corresponding Author: Nihar Ranjan Sahoo, Division of Animal Genetics, ICAR-Indian Veterinary Research Institute, Izatnagar, Bareilly-243 122, Uttar Pradesh, India. Email: vet.nihar@gmail.com

How to cite this article: Sinha, R., Sahoo, N.R., Shrivastava, K., Kumar, P., Qureshi, S., Kumar, A., Ravikumar, GVPPS. and Bhushan, B. (2020). Sequence analysis of Mucin 13 gene region associated with piglet diarrhoea in Indian pigs. Indian Journal of Animal Research. 54(2): 163-167.

Submitted: 20-11-2018 **Accepted**: 10-01-2019 **Published**: 26-03-2019

MATERIALS AND METHODS

DNA isolation and PCR amplification

Blood samples of 80 Indian *desi* pigs, regardless of their age, sex and physiological status, from Bareilly region of

India, were used in current study. Genomic DNA was isolated from muscle tissue samples by Phenol: Chloroform: Isoamyl alcohol extraction method as described by Sambrook and Russell (2001) with minor modifications. The quality and integrity of the genomic DNA was checked by using horizontal submarine agarose gel (0.8%) electrophoresis and visualizing the gel under UV gel documentation system. Good quality DNA showed intact band and devoid of smearing.

PCR amplification and sequence analysis

PCR amplification was carried out by using primers designed for MUC13 gene as detailed in Table 1. The primers were amplified using a standard PCR mixture containing 1 pmole of each primer, 60ng genomic DNA, 12.5 µl PCR master mix (Thermo Fisher) and nuclease free water to make total reaction volume 25 µl in a thermal cycler using standard PCR programme with annealing temperatures for each primer pairs as described in Table 1. The amplified products were first checked on 2 % horizontal agarose gel electrophoresis for correct amplification and then were sequenced using automated sequencer. The sequenced products were obtained as raw and were assembled using Bioedit software. The final sequence was then subjected to BLAT/BLAST in Ensemble genome browser to find the similar genomic regions in domestic livestock. Further analysis of sequence was carried by Mega 7.0 software, heat mapper (http://www.heatmapper.ca/) and Phyre2 server to predict secondary structure of coding region (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index).

RESULTS AND DISCUSSION

Total five pairs of primers were used encompassing two major regions of MUC13 gene - intron 6 and exon 7 (partial) – intron 7 – exon 8 (partial) (Table 1). For intron 6 region, the final sequences were submitted to NCBI and accession number were assigned to them as KX686558 (allele 01), KX686559 (allele 02) and KX686560 (allele 03). The annotated sequences of intron 6 region were aligned with that of corresponding sequences of Chinese pig (*Sus scrofa*,), sheep (*Ovis aries*), goat (*Capra hircus*) and cattle (*Bos taurus*) using Ensembl BLAT server (https://asia.ensembl.org). A heat map was also prepared for pairwise comparison, after sequence comparison (Fig 1). The box 1, 2 and 3 represent the comparison of Indian pig allele 01, 02 and 03 with Chinese pig, sheep, goat and cow. As expected, high similarity was seen between both Indian and Chinese pig, whereas, the sequences of sheep and goat (except in box 2) and cow clustered separately (Fig 1). The overall mean distance between Indian pig allele 01 and other sequences was 1.486, Indian pig allele 02 and other sequences was 1.759 and Indian pig allele 03 and other sequences 6.254. This large mean distance was also expected.

For the looking to the function of gene region containing exon 7 (partial), intron 7 (complete) and exon 8 (partial), two PCR primers were used (Table 1) which amplified two

Table 1: Details of the sequenced loci of the current study.

Target gene	Region	Primer sequence (5' → 3')	Ta (°C)	Amplicon size (bp)	Genomic coverage* (bp)	Accession number assigned
Mucin 13	Intron 6 (partial)	FP: TTTCCAAGGTGGCAAGAGGG	61	202	19066-19267	KX686560
		RP: AACTCCCGATGACTGCTGTG				
		FP: AGGCAGACACACAAGTCCAC	60	275	18763-19037	KX686559
		RP: ACTGTGTGGAGCAGAGACAA				
		FP: AAAAAGCCCACAGCCAATGG	59	171	21420-21590	KX686558
		RP: CAAAGGGGAGGCAGAGTCTG				
	Exon 7 (partial) – Intron 7 – Exon 8 (partial)	FP: GATCGGTGTGATTATTATGG	59	491	22065-22555	KX686556
		RP: AGAGCATGCTGGACCCAAAG				
		FP: TGAGTGCCCCAGTGGTTTAC	61	537	22109-22645	KX686561
		RP: ATCCTCCTTGTAGCCAGGCA				

*Accession number JN613418 was taken as reference sequence.

regions, 491 bp and 537 bp. The final sequences were submitted to NCBI and accession numbers assigned to them as KX686556 (allele 01) and KX686561 (allele 02). The nucleotide sequences were analysed and compared with that of pig, sheep, goat, and cow using Ensemble BLAT and a heat map was constructed for their comparison using MEGA 7.0 (Fig 2). The box 1 represents the comparison of 01 (KX686556) allele and the box 2 represents the comparison of allele 02 (KX686561) with the related species, respectively (Fig 2). It is evident that sequences from Indian pig and Chinese pig were closely related as with the case in sequences from sheep and goat. This region of MUC13 gene is well defined as compared to the intron 6 region and hence the overall mean distance between Indian pig allele 01and related sequence was 0.149, whereas, mean distance between Indian pig allele 02 was 0.158. The exons 7 and 8 of both the sequence were then translated and joined as per their coordinates to make one single peptide chain. Among the sequences there were 21 sites of disagreement. Histidine, methionine and tryptophan were found in least amount with leucine and cysteine found in maximum amount. The homogeneity test of the sequences showed significant ($P \leq 0.05$) difference, in terms of evolution by base substitution between Indian pig, Chinese pig and cattle sequences (Fig 3). The overall mean distance between the studied amino acid sequences was 0.23. The sequences were further analysed for secondary structure prediction using phyr2 programme server. The secondary structure showed predominance of β-pleated sheet (Fig 4), as the amino acid chain was small ligand binding prediction could not be performed. Ren *et al*. (2012) has reported five SNPs in these regions (one in each segment) on MUC13 gene

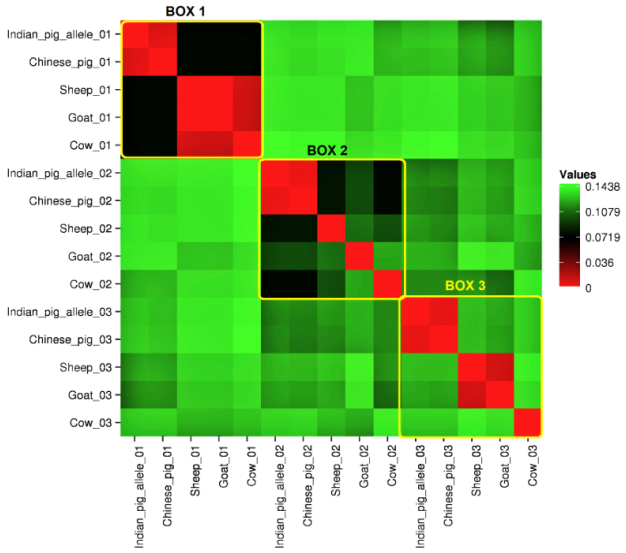

Fig 1: Heatmap of pairwise comparison of Muc13 intron 6 nucleo tide sequence between studied species.

In figure: Indian pig allele_01 is sequence no. KX686558; Indian pig allele_02 is sequence no. KX686559; Indian pig allele_03 is sequence no. KX686560; numbers 01, 02 and 03 among other sequences represents comparison between respective sequences with sequenced alleles.

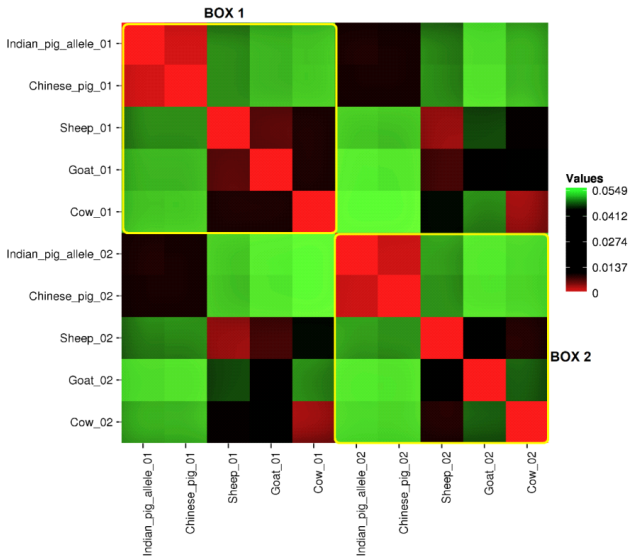

Fig 2: Heatmap of pairwise comparison of Muc13, exon 7 (partial)– intron 7 – exon 8 (partial) nucleotide sequence between studied species.

In figure: Indian pig allele_01 is sequence no. KX686556; Indian pig allele_02 is sequence no. KX686561; numbers 01 and 02 among other sequences represents comparison between respective sequences with sequenced alleles.

	1	2	3	4	5
1. Indian pig		0.00	0.02	0.02	0.26
2. Chinese pig	1.00		0.02	0.02	0.26
3. Sheep	0.42	0.40		0.00	0.00
4. Goat	0.37	0.42	1.00		0.02
5. Cow	0.02	0.02	1.00	0.41	

Fig 3: Test of the homogeneity of substitution patterns between sequences.

[*The probability of rejecting the null hypothesis that sequences have evolved with the same pattern of substitution, as judged from the extent of differences in base composition biases between sequences (Disparity Index test). P-values smaller than 0.05 are considered significant (marked with yellow highlights) The estimates of the disparity index per site are shown for each sequence pair above the diagonal.*]

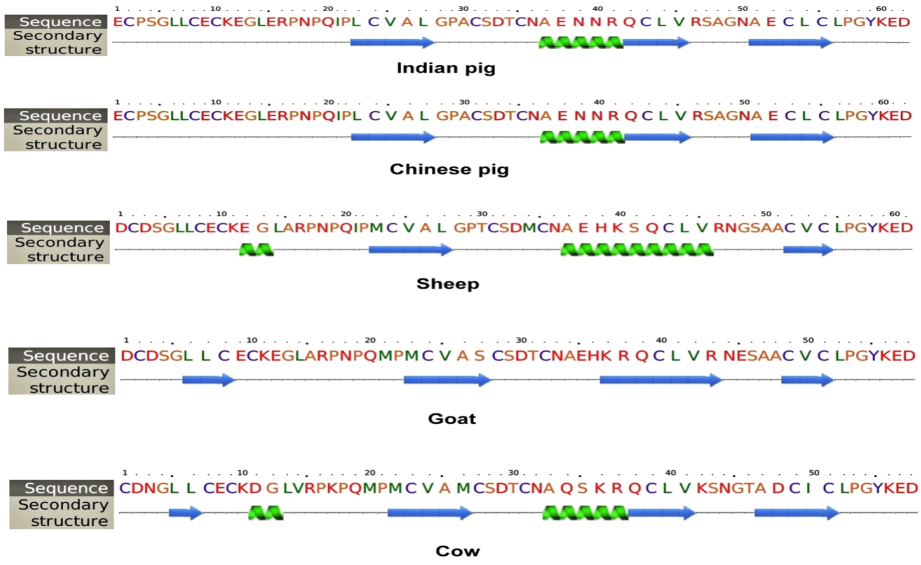

Fig 4: Secondary structure of amino acid sequence of studied species, as predicted by Phyre 2.

and they were reported to be associating with resistance to piglet diarrhoea. Sinha *et al.* (2018a) genotyped these SNPs in Indian native pig population of same region (Bareilly, India) by using PCR-RFLP protocol and found all SNPs to be polymorphic One of these SNP (g.22304A>G) situated in sequence KX686561 was showing a significant effect of genotypes on *E.coli* F4ab/ac mediated diarrhoea (Sinha *et al*., 2018a). Earlier, Sinha *et al*. (2018b) have shown that MUC13 is differentially expressed in jejunum tissue of Indian native pig which is differentially adhesive to diarrhoeagenic *E.coli*. This is indicative that MUC13 polymorphism may be involved in resistance/ susceptibility to *E.coli* F4ab/ac mediated diarrhoea (Sinha *et al*., 2018a, b).

CONCLUSION

The current study characterized the regions of MUC13 gene in Indian native pig by sequencing. The only regions reported to be harbouring SNPs that may have significant effect on piglet diarrhoea were studied. Indian native and Chinese pigs showed maximum sequence similarity at nucleotide as well as on amino acid level. As the amino acid sequence was short in length, ligand binding prediction could not be performed. Predicted secondary structure of amino acid chain showed the predominance of beta sheet structure in pig and other species.

ACKNOWLEDGEMENT

The authors would like to thank the Director, ICAR-IVRI for providing necessary facilities to carry out this research work in form of an institute project. The first author is also thankful to Indian Council of Agricultural Research, India for providing financial assistance in the form of institutional fellowship.

Conflict of Interest

Authors declare that they have no conflict of interest between them.

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