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Expression profiling of mucin 20 gene in pigs differentially adhesive to diarrhoeagenic *Escherichia coli*

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Abstract
The *Escherichia coli* mediated piglet diarrhea is the major problem of the piggery industry. *Escherichia coli* bind to the brush border of the epithelial cells of the intestine on this basis of receptors and fimbriae adhesion pattern. Exploring the genetics behind this trait will immensely benefit pig welfare as well as the pig breeding industry by catering an opportunity to select against genetically susceptible animals. Among the putative candidate genes associated with adhesion pattern, mucin20 gene was localized on the targeted region of SSC13 and considered as a putative candidate gene due to its biochemical property as well as physical location. The present investigation was conducted to study mucin20 expression profile in different adhesion phenotypes. The relative expression of mucin20 mRNA was significantly up-regulated in weak adhesive, adhesive and strongly adhesive phenotypes suggesting its important role in attachment of *E coli*, thereby influencing the diarrhoea occurrence.

Keywords: Mucin 20, Jejunum expression profiling, Desi Pig

1. Introduction
Indian agriculture economy primarily depends on the marginal farmers where pig rearing is the alternative for their livelihood which gives them sustainable income source. Among the meat producing animals, pig occupies a unique position in the several parts of India [1]. Though pig farming is a sustainable farming but the population of the pig was started declining after 2003 in our country which is because of diseases, social taboos on pig rearing and lack of breeder farmers. Pigs are mostly prone to various diseases. Among all diseases, most important disease after swine fever is piglet diarrhoea. *Escherichia coli* are the major pathogenic bacteria causing diarrhoea in swine, which accounts for 56.2% of the incidence of piglet diarrhoea and 24.7% of the mortality from diarrhoea [2]. There are several reports of incidences of piglet diarrhoea in various farms of India (AICRP Reports 2008-14) resulting in huge loss of piglets**.** This organism can adhere and colonize at the brush border membrane of the epithelial cells of a piglet's small intestine through its fimbriae and secrete enterotoxins [3]. An enterotoxin stimulates the small intestine for secreting massive fluid and electrolyte into the gut lumen resulting diarrhoea. Therefore, adhesion to the epithelial cells of the small intestine is an essential prerequisite for the bacteria to cause diarrhoea among piglets. However, not all piglets are equally susceptible to *E. coli*. Certain piglets are innately resistant, as they can prevent the adhesion of *E. coli* to the epithelial cells of small intestine. The adhesion difference happens because of the presence or absence of specific bacterial adhesion receptors in the small intestine epithelial cells of the host. These receptors are not present in each and every pig and their absence can cause resistance to *E. coli* induced diarrhoea [3]. An enterotoxin stimulates the small intestine for secreting massive fluid and electrolyte into the gut lumen resulting diarrhoea. Therefore, adhesion to the epithelial cells of the small intestine is an essential prerequisite for the bacteria to cause diarrhoea among piglets. The adhesion was found to be genetically controlled and inherited in a dominant fashion [4]. However, not all piglets are equally susceptible to *E. coli*. The adhesion difference happens because of the presence or absence of specific bacterial adhesion receptors in the small intestine epithelial cells of the host. However, the exact/specific genes that encode for the receptor/susceptibility are not yet known. In the past few decades, linkage analyses have shown that the loci encoding for the receptor(s) for the two most frequent variants F4ab and F4ac were mapped to 13th

chromosome (SSC13) of the pig [5]. Mucin20 is one among the putative positional candidate genes found in this region. In India, although there are several reports of incidences of piglet diarrhea in various organized farms of different parts of India [6]. Hence, the study was designed with the objectives to evaluate the Indian desi pigs in terms of *E. coli* adhesion pattern (using Indian isolate of diarrheagenic *E. coli*) and to study the jejunal expression profile of mucin20 in different adhesive phenotypes.

2. Materials and Methods

2.1 Sample collection and Microscopic Adhesion Test

A total of 150 desi pigs slaughtered in different places of Bareilly, U.P. were screened for *E. coli* adhesion pattern. Jejunum tissue samples were collected within 30 min of slaughter and brought to the laboratory in ice maintaining sterile condition. After cleaning it properly with cold PBS, a small part (250 mg) of the sample was stored in 1 ml RNA later solution at -20^{0}C for RNA isolation. The part of the jejunum (approximately 2 cm) was kept at 4^{o}C for the Microscopic Adhesion Test (MAT) on the same day. Samples were screened for adhesion pattern of the porcine brush border epithelial cells with Indian isolate of diarrheagenic *E. coli* through MAT as described by Li *et al.* [7].

2.2 Bacterial strain and preparation of suspension

The *E. coli* strains were isolated from diarrhoeic piglets of All India Co-ordinated Research Project (AICRP) on Pig, Indian Veterinary Research Institute (IVRI) unit, Bareilly, U.P., India and characterized biochemically and sequencing of partial 16S ribosomal RNA gene (KJ810542). The isolate was cultured in BHI agar plate for overnight at 37^{o}C and a single colony was picked up from BHI agar plate for inoculation in LB broth medium (Trypton, Yeast extraction, NaCl, pH 7.0−7.2) at 37^{o}C for 16-18 hours at 180 rpm. The optical density was checked for 1.0 at 520 nm. The culture was preserved at 4^{0}C for use at the same day. Animals were classified as strongly adhesive, adhesive, non-adhesive and weakly adhesive as per Li *et al*. [7]

2.3 Isolation of total RNA and first strand cDNA synthesis

The animals with different adhesion patterns (non-adhesive, weak adhesive, adhesive and strong adhesive) 6 samples each were subjected to jejunal expression profiling of mucin20 gene. Total RNA was isolated from jejunum tissue using Trizol reagent (Thermo Scientific, USA) and chloroform according to manufacturer's protocol [8]. The quality and quantity were checked by nanodrop spectrophotometer, RNA samples showing the OD 260:280 values more than 1.8 were used. For the synthesis of first strand cDNA, reverse transcription was carried out in 20 µl reaction mixtures using Revert Aid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, USA) as per manufacturer's instruction. The integrity of the cDNA was checked by PCR with porcine GAPDH primers to yield 90 bp amplicon [9].

2.4 Real-time PCR

The resulting cDNAs were used for quantitative RT-PCR reactions with two sets of primers one from mucin20 gene and other from GAPDH (as housekeeping gene) (Table 1). Quantitative Real-time PCR was performed with SSO Fast Eva Green ® qPCR kit (Biorad) using Agilent Mx3005P QPCR System (USA) operated by MxPro QPCR software. The Master Mix was prepared using 8.0 µl of nuclease free water, 10 µM of forward and reverse primers each and 10 µl of Eva green mix (BioRad) and 1 µl of cDNA was added. A qPCR amplification programme was used (One cycle of Hot start PCR at 95° C for 20 sec followed by 40 cycles of denaturation at 95° C for 3 sec and annealing/ extension at 58°C - 60°C for 30 sec).

Table 1: Primer sequences used for relative quantification of Mucin20 gene using real time qPCR

Target gene	Primer name	Sequence of nucleotide (5'-3')	Frag. size (bp)
MUCIN20	RT_MUC20_F RT_ MUC20_R	F: CACCCCTACCACTGTTCCAA R: TGGGGTCAGTGAGGTCTTCT	107
GAPDH	GAPDH_F GAPDH_R	F:ACACTCACTCTTCTACCTTTG R:CAAATTCATTGTCGTACCAG	90

2.5 Quantification of candidate gene expression

Once the C_T value is collected for each reaction, it can be used to generate a relative expression level. In our experiment, there were four conditions (Non-adhesive, Weak-adhesive, Adhesive and Strong adhesive), where we measured the expression levels of mucin20 genes compared to an endogenous control gene (GAPDH) using the method described by Livak *et al.* [10]. Non-adhesive samples were taken as control. The statistical significance of differences ($P<0.05$) in mRNA expression was assessed by using one way ANOVA with Tukey's Multiple Comparison Test as post hoc was performed to determine the significant differences between dCTs of the analyzed groups.

3. Results and Discussion

The real-time quantitative amplification was performed for mucin20 with GAPDH gene (as housekeeping gene) and the results of the qPCR allowed us to compare gene expression differences between non-adhesive group with adhesive groups (which includes weak adhesive, adhesive and strong adhesive). RT-PCR analysis revealed that porcine mucin20 mRNA expression was different across adhesive phenotypes (Fig. 1) with the highest level in the *strongly adhesive* followed by *adhesive*; moderate levels in the *weakly adhesive* and low levels in the *non-adhesive* type. The mRNA expression of mucin20 gene was found to be 2.54, 4.21 and 5.72 $\log_2$ fold difference of in weak adhesive, adhesive and strong adhesive groups respectively, as compared to the non-adhesive group which was found to be statistically significant ($P<0.05$) (Fig. 1). Schroyen *et al.* [11] also study on mucin20 mRNA expression but they found mucin20 gene showed no expression differences between F4ac receptor positive and F4ac receptor negative animals. Francis *et al.* [12] and Jacobsen *et al.* [13] did not find any correlation between the expression of the mucin-type sialoglycoprotein receptor and adherence of bacteria to the brush border by studying expression levels of the five genes in enterocytes from jejunum. We observed different results. In our study, we observed significant expression differences of mucin20 gene between non-adhesive, weak adhesive, adhesive and strong adhesive groups.

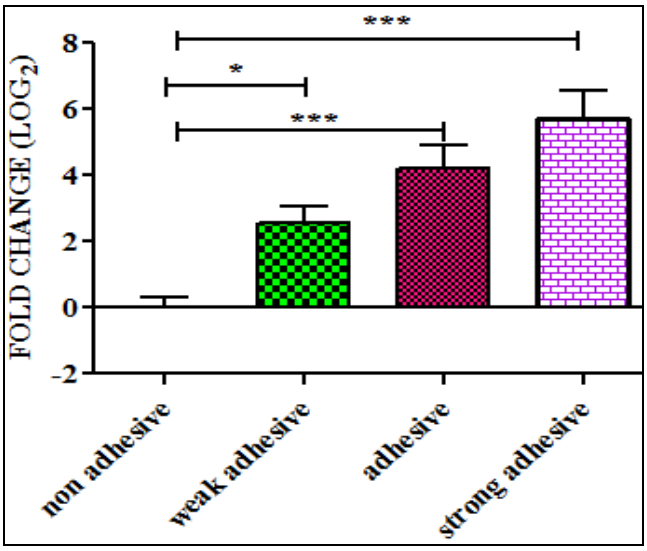

Fig 1: Expression profiling of Mucin 20 gene

4. Conclusion

The increased level of mucin20 expression along with the degree of adhesion as well as the chemical nature of the gene product indicates its role in influencing adhesion pattern of *E. coli* which causes diarrhoea. These genes although may not be directly affecting resistant/ susceptibility towards diarrhea due to *E. coli* in pigs. However, it certainly could form a positional genetic maker owing to their mapping location.

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