Comparative jejunal expression of MUC 13 in Indian native pigs differentially adhesive to diarrhoeagenic E. coli

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1. Introduction

Diarrhoea in neonatal and weaned piglets is a serious welfare problem and financial burden in the pig production system, which was consistently identified as a primary infectious disease resulting in poor health and death losses. Worldwide, this disease accounts for 11.5%–29.5% of death in piglets (Li et al. 2007). Among the bacterial causes of diarrhoea, Escherichia coli are important in swine, which account for 56.2% of the incidence of piglet diarrhoea and 24.7% of the mortality from diarrhoea (Shi 2003). 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 (Sellwood et al. 1975). An enterotoxin stimulates the small intestine for secreting massive fluid and electrolyte into the gut lumen resulting in 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 the small intestine. The adhesion difference happens because of the presence or absence of specific bacterial adhesion receptors in the small intestine’s 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 (Sellwood et al. 1975). The adhesion of ETECF4ab/ac fimbriae was found to be genetically controlled and inherited in a dominant fashion (Bijlsma et al. 1982). However, the exact/specific genes that encode for the receptor/susceptibility to ETECF4ab/ac are not yet known. The genetically determined differential adhesion pattern or resistance can help in identifying the genetic cause for susceptibility which can potentially be explored for an effective selection programme. The locus encoding the intestinal receptor responsible for adhesion was mapped to the q41 region on pig chromosome 13 (SSC13) by different linkage analyses. Among the putative positional candidate genes found in this region (MUC4, MUC13, TFRC and MUC20), the association studies in a collection of diverse outbred populations strongly supported that MUC13 is the most likely responsible gene (Zhang et al. 2008; Ren et al. 2012). Zhou et al. (2012) described the downregulation of MUC 13 after infection with F4ac ETEC. They found this gene influenced the inflammatory response of cells induced by ETEC using a MUC 13 knockdown (Zhou et al. 2013) approach and suggested it might be an important signalling molecule in response to ETEC.

In India, neonatal diarrhoea is one of the most common causes of morbidity and mortality among piglets causing heavy economic losses in the pork industry (Pathak et al. 2004; Shome et al. 2005). E. coli were found to be associated with severe diarrhoea with heavy mortality, particularly during the first week of life of piglets (Goswami et al. 1993; Dutta et al. 2001; Kumar & Soman 2001). There are several
reports of incidences of piglet diarrhoea in various organized farms of different parts of India (Anonymous 2014). However, the research has been mostly confined to characterization of E. coli based on serotyping (O' antigen). Yet there is scarcity of literature on the existence as well as inheritance of the receptors controlling the adhesion pattern in Indian pig population which if explored could be utilized to enhance genetic resistance against piglet diarrhoea. Hence, the study was designed with the objectives to evaluate the Indian desi pigs in terms of E. coli adhesion diarrhoeagenic E. coli and to study the jejunal expression profile of Mucin 13 in different adhesive phenotypes.

2. Materials and methods

2.1 Experimental animals and tissue collection

A total of 80 desi pigs slaughtered in different places of Bareilly, Uttar Pradesh, were screened for E. coli adhesion pattern. Animals of different ages and sexes were included in the investigation. 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°C for RNA isolation. The remaining part of the jejunum (approximately 2 cm) was kept at 4°C for the Microscopic Adhesion Test (MAT) on the same day.

2.2 Determination of phenotype by MAT

Samples were screened for adhesion pattern of the porcine brush border epithelial cells with Indian isolate of diarrhoeagenic E. coli through MAT as described by Li et al. (2007).

2.2.1 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, Uttar Pradesh, India, and characterized biochemically and sequencing of partial 16S ribosomal RNA gene (KJ810542). However, when subjected to PCR-based fimbrial typing for ETECF4 using primers and protocol as per Li et al. (2007), they were found to be negative (Figure 1). The isolate was cultured in BHI agar plate overnight at 37°C and a single colony was picked up from the BHI agar plate for inoculation in LB broth medium (Trypton, Yeast extraction, NaCl, pH 7.0–7.2) at 37°C for 16–18 h at 1800 rpm. The optical density was checked for 1.0 at 520 nm. The culture was preserved at 4°C for use on the same day.

2.2.2 Preparation of brush border epithelial cells

The small intestine was separated from the mesentery, and for isolation of intestinal brush borders, a piece of the mid-jejunum (approx. 2 cm) about 1 m proximal to the ileocaecal valve was excised from the small intestine within 30 min of slaughter. It was cut open along the longitudinal axis and washed with cold PBS (pH 7.4) containing 0.1 M EDTA to make free of contents and placed on ice until processing. Subsequently, the sample was immersed in a cold hypotonic EDTA solution (5 mM EDTA, adjusted to pH 7.4 with Na2CO3) for 20 min and rinsed gently to eliminate debris. Epithelial cells were removed by scraping the mucosal surface of the jejunum with the edge of a glass microscopic slide and immersed in cold hypotonic EDTA solution for 30 min. Then the enterocytes were homogenized with tissue homogenizer (Star Micronic Devices) till an even suspension of the tissue was made and filtered through a clean muslin cloth. The filtrate was centrifuged at 3500 rpm for 10 min to pellet and cells were re-suspended in 5–6 ml cold PBS. The process of re-suspension and centrifugation was repeated twice. Brush border suspension was added with 100 µl gentamicin sulphate (1 mg per ml) and sodium azide (3 mM) to make a final concentration of 1 × 10^6 cells per ml and stored at 4°C for use on the same day.

2.2.3 Adhesion test

Equal volumes of brush border cell suspension and fresh bacterial suspension (100 µl each) were mixed in 6 ml tissue culture plate and incubated for 30 min at 37°C at 200 rpm. A drop of the mixed suspension was examined for the adhesion pattern using a light microscope under 40× objectives. A single epithelial cell was considered adhesive when more than five bacterial adhered to the brush border membrane (Figure 2). Animals were classified as strongly adhesive, adhesive, non-adhesive and weakly adhesive as per Li et al. (2007). Twenty well-separated and intact enterocytes were checked from the epithelial cell specimen of each animal. The effects of different non-genetic factors (sex and age) on adhesion pattern were determined.

2.3 Expression profiling of MUC 13

2.3.1 Isolation of total RNA and first-strand cDNA synthesis

The animals with different adhesion patterns (5 samples each) were subjected to jejunal expression profiling of the Mucin 13
gene. Total RNA was isolated from jejunum tissue using Trizol reagent (Thermo Scientific, USA) and chloroform (Sambrook & Russell 2001) according to the manufacturer’s protocol and was precipitated using isopropanol, washed twice with 70% ethanol and stored at −80°C. The quality and quantity were checked by nanodrop spectrophotometer against nuclease-free water as blank and RNA samples showing that OD 260:OD 280 values more than 1.8 were used. The quality and integrity were also checked using denaturing agarose gel electrophoresis to reveal two intact bands of 28S and 18S with smearing in between. For synthesis of the 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 the manufacturer’s instruction. Total RNA of 1 μg for each reaction was dissolved in nuclease-free water and oligo (dT)18 primer was added to make the final volume of 12 μl. The components (5× reaction buffer; Ribolock RNase Inhibitor; 10 mM dNTP Mix and RevertAid H Minus M-MuLV Reverse Transcriptase) were added to the tube for reverse transcription. The reaction mixture was incubated at 42°C and 70°C, for 60 and 5 min, respectively, and finally at 4°C to hold. The integrity of the cDNA was checked by PCR with porcine GAPDH primers (Nygard et al. 2007) to yield 90 bp amplicon.

2.3.2. Real-time PCR
The resulting cDNAs were used for quantitative RT–PCR reactions with two sets of primers (Table 1) one from the Mucin 13 gene and another from GAPDH (as housekeeping gene). Quantitative real-time PCR was performed with SSO Fast Eva Green® qPCR kit (Bio-Rad) using the Agilent Mx3005P QPCR System (USA) operated by MxPro QPCR software. The primer efficiencies were determined by running a standard curve for each assay prior to processing experimental samples. A standard curve was obtained by levels of six serial dilutions of cDNA as template and a regression equation in relation to the threshold values (‘Ct’) was formulated. The primer efficiency within the range of 90–115% was considered to be good. A no template control (Master Mix and primers) was put for gene quantification for checking contamination in the reaction components. 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 (Bio-Rad) and 1 μl of cDNA was added. A segmented qPCR amplification programme was used (One cycle of Hot start PCR at 95°C for 15 s followed by 35cycles of denaturation at 95°C for 10 s and annealing/extension at 60°C for 12 s). The dissociation curve analysis was performed for one cycle at 95°C for 1 min, 65°C for 30 s, 65–95°C (2°C per min) and 95°C for 30 s. The amplification and denaturation data were acquired and cycle threshold (Ct) values as well as amplification plots for all determined factors were estimated using the ‘EvaGreen® Dissociation Curve’ method of the real-time machine.

2.3.3. Statistical analysis of qPCR data
Optical data were collected at the end of each extension step and relative expression of PCR product was determined by the equation; Ratio = (E_{target})^{ΔCt \text{ target (control-sample)}}/(E_{ref})^{ΔCt \text{ ref (control-sample)}} (Pfaffl 2001), where the ratio was the relative expression, E_{target} was the real-time efficiency of the target gene transcript and E_{ref} was the real-time efficiency of the housekeeping gene transcript. The statistical significance of differences in mRNA expressions of the examined factors was assessed by using one-way analysis of variance (ANOVA) using SPSS 17.0 software. Differences were considered significant if P < .05.

3. Results and discussion

3.1 Microscopic adhesion test
The present study was undertaken to explore E. coli adhesion patterns and their frequencies. A total of 27 animals (33.75%) were found to be negative for adhesion (i.e. non-adhesive), 46 animals (57.5%) were adhesive and 7 (8.75%) were weakly adhesive (Table 2). About one-third of the samples under the present investigation had shown a non-adhesive phenotype. The result indicates that enough starting material was available within the desi pig to start with breeding programmes against diarrhoea. Three different E. coli adhesion patterns were observed in Indian pigs, but the category strongly adhesive
(reported by Li et al. 2007) was not observed in the present sample which might be due to smaller sample size or may indicate these pigs to be relatively less susceptible to E. coli-mediated diarrhoea. Yan et al. (2009) reported the existence of eight previously reported F4 adhesion patterns. They further reported that all the Tibet and Lantang pigs and a majority of the Erhualian and Rongchang pigs were resistant (non-adherent) to ETEC F4, whereas all the Loiwu pigs and most of the Jiangquhai and Tongcheng pigs were susceptible (adhesive) to at least one of the F4 strains. Yushan Black pigs were uniformly resistant to F4ab, and Jinhua pigs were predominantly resistant to F4ac. Susceptible and resistant animals were observed in the other breeds, indicating that diarrhoea caused by ETEC F4 could be prevalent in these breeds. Bijlsma et al. (1982) revealed five different porcine phenotypes with the three serological variants of the K88 antigen in the brush border adhesion test. Li et al. (2007) obtained four types of adhesion pattern in three pig breeds (Landrace, Large white and Sangilia Black). Zhang et al. (2008) also reported three types of adhesion pattern in White Duroc × Erhualian inter-cross. However, Baker et al. (1997) classified differently with a pig as adhesive (susceptible) to ETEC F4ab/ac if at least 2 of 20 brush borders bound more than 2 bacteria. Pigs with all brush borders bound by fewer than two bacteria were judged as non-adhesive (resistant) animals. Other pigs were considered as weakly adhesive.

### 3.1.1 Effect of other non-genetic factors

Effect of sex and age was investigated for its association with E. coli adhesion pattern and it was found that the effect of sex was significantly \((P < .05)\) associated. However, considering the smaller number of females in the present study, it would be too early to conclude about the effect of sex. But the effect of age was found not to be significantly \((P < .05)\) associated with any kind of adhesion pattern. This shows the receptor may be present even at adult age in the brush border. The enterocyte F4R is expressed in pigs throughout their entire lifetime. However, Nagy and Fekete (2005) reported their expression decreases with age making older pigs less susceptible to disease. However, the presence of F4R in the mucus was reported to cease by 6 months and declines significantly at the time of weaning (Willemsen & de Graaf 1992; Chandler et al. 1994).

#### 3.2 Jejunal expression of MUC 13 mRNA

RT–PCR analysis revealed that porcine MUC13 mRNA expression was different across adhesive phenotypes (Figure 3) with the highest level in the adhesive, moderate levels in the weakly adhesive and low levels in the non-adhesive type. The mRNA expression of MUC13 in weakly adhesive and adhesive samples was found to be 1.22 ± 0.35 and 2.67 ± 0.69 times higher as compared to the calibrator, respectively. The mRNA expression level was found to be statistically non-significant \((P < .05)\) in both the weakly and adhesive samples using one-way ANOVA. Schroyen et al. (2012) also reported that MUC13 showed a significant difference in expression \((P < .05)\) between F4ac receptor-positive and F4ac receptor-negative animals. However, they believed this difference to be a consequence of lower epithelial tissue levels due to possibly other diarrhoea-causing agents. Ren et al. (2012) looked at MUC 13 but did not find significant expression differences between susceptible and resistant animals. He suggested that one of the MUC 13 variants was the causative gene for ETEC susceptibility after performing

![Figure 3. Jejunal expression of mucin 13 mRNA using quantitative RT–PCR.](image)

#### Table 1. Primer sequences used for relative quantification of Mucin 13 gene using real-time qPCR.

| Target gene | Primer name | Sequence of nucleotide \((5'-3')\) | Fragment size (bp) | Genbank/EMBL/Reference |
|-------------|-------------|------------------------------------|--------------------|------------------------|
| MUC13       | MUC13_RTF   | GGAGTTGGCCTGTAAGGCC              | 87                 | JN613417               |
|             | MUC13_RTR   | TGGCAATCCACGGCTGAGGA             |                    |                        |
| GAPDH       | GAPDH_RTF   | ACACCTACTTTACTCTTTG             | 90                 | Nygard et al. (2007)   |
|             | GAPDH_RTR   | CAAATTCAATGGCTGACCAG            |                    |                        |

#### Table 2. Non-genetic factors affecting E. coli adhesion phenotypes.

| Factor | Variants | Adhesive | Weakly adhesive | Non-adhesive | Total | \(P\)-value | Odds ratio (95% CI) |
|--------|----------|----------|-----------------|--------------|-------|-------------|---------------------|
| Sex*   | Male     | 42       | 4               | 26           | 72    | .043        | 6.66 (1.06–41.64)   |
|        | Female   | 4        | 3               | 1            | 8     | 1.00        |                     |
| Age    | ≤1 years | 18       | 3               | 11           | 32    | .996        | 1.00 (0.37–2.68)    |
|        | >1 years | 28       | 4               | 16           | 48    |             | 1.00                |
| Total  |          | 46       | 7               | 27           | 80    |             |                     |

*Indicates statistical significance at \(P \leq .05\).
adhesion tests between the MUC 13 protein and the fimbriae F4ab/ac. However, Goetsstouwers et al. (2014) rejected that hypothesis. Rampoldi et al. (2014) examined four different small intestinal sites instead of one for adhesion of the fimbrial variants but did not find any relations to the strong receptors for variants F4ab and F4ac. Previous studies on RT–PCR analysis reported that the porcine MUC13 mRNA expression was markedly different in diverse tissues, with the highest level in the jejunum; moderate levels in the trachea, stomach and liver; and low levels (or no expression) in the other 14 tissues (Zhang et al. 2008).

In conclusion, there is an abundance of non-adhesive phenotypes (about one-third) among Indian desi pigs which implicates that sufficient input material in terms of genetic resources are available to work on the breeding programme against diarrhoea. Three different E. coli adhesion patterns were found in Indian pigs, but the category strongly adhesive was not observed which might be due to smaller sample size or it may indicate these desi pigs might be relatively less susceptible to E. coli–mediated diarrhoea. Although, non-significant differences in expression were observed among different phenotypes, the descending expression of MUC 13 in adhesive and weakly adhesive samples speculates an important role of this gene in adhesion of E. coli.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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