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Research Article

Differential expression analysis of *HEG1* and *MUC20* genes in multiple tissues of Large White piglets with resistance and susceptible phenotypes to ETEC-F4ac diarrhea

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Abstract

Diarrhea represents one of the most frequent major problems during piglets' neonatal and post-weaning periods leading to tremendous economic losses in the swine industry. This study focused on the expression of candidate genes *HEG1* and *MUC20* in the porcine small intestine and immune-related tissues (thymus, spleen and lymph) of piglets with emphasis on the porcine small intestine as an organ that is responsible for piglets' diarrhea. Enterotoxigenic *Escherichia coli* strain possessing F4 fimbriae adheres to specific receptors on the enterocytes of the small intestine. These receptors interact with F4ab and F4ac adhesins, and the expression of this receptor is inherited following Mendelian inheritance. The involvement of *HEG1* and *MUC20* in piglets' susceptibility to ETEC diarrhea remained equivocally understood. Our study aimed at mainly investigating the expression of *HEG1* and *MUC20* as well as inflammatory genes in the small intestine and their involvement in piglets' susceptibility to ETEC diarrhea. The piglets were divided into two groups (adhesive and non-adhesive) of four animals each, and tissues of the aforementioned organs were collected from each animal. Subsequently, tissues were subjected to total RNA extraction, cDNA and RT-qPCR experiments respectively. Our finding showed that both *HEG1* and *MUC20* genes were significantly differentially expressed in the small intestine ($P < 0.05$) suggesting that both *HEG1* and *MUC20* could be involved in piglets' susceptibility to ETEC diarrhea. Furthermore, *MUC20* was also found significantly differentially expressed in the spleen ($P < 0.01$). In conclusion, our study unraveled the involvement of *HEG1* and *MUC20* genes in piglets' susceptibility to diarrhea caused by ETEC and could be used as molecular markers in the selection of piglets that are resistant to ETEC-F4ac diarrhea in the swine's breeding program.

Keywords: ETEC-F4ac diarrhea, Adhesion, Resistance, *HEG1, MUC20*

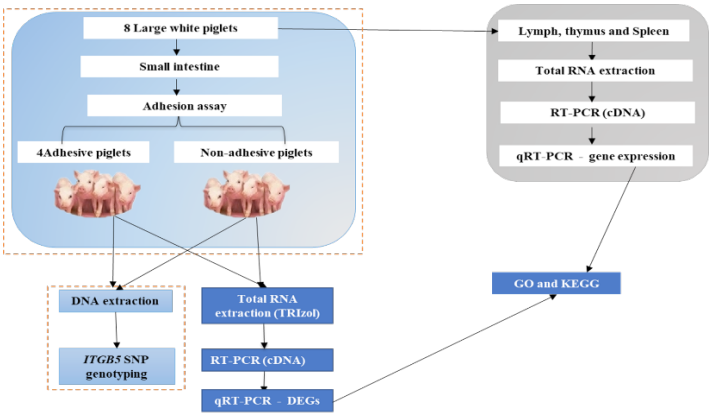
Introduction

Economic benefit or progress is the fundamental goal of swine industries, while one aspect that hinders economic progress is porcine disease risks. In pig industries, diarrhea is considered one of the top major diseases that hinder economic progress.

Diarrhea that happened in neonatal or recently-weaned piglets leads to huge economical losses in many small and middle-sized swine farms [1,2], due to high morbidity and mortality rate [3]. Resistance or susceptibility to enterotoxigenic *Escherichia*

coli (ETEC) that possess F4 fimbriae in their cell walls which causes piglets diarrhea, is dominantly inherited following Mendelian inheritance [4,5] Three major F4 fimbriae antigenic variants have so far been described, namely F4ab, F4ac and F4ad [6,7]. Colonizing ETEC F4 release harmful enterotoxins that lead to more secretion of electrolytes into the gut lumen and stop fluids' homeostasis, consequently permitting water flows into the gut lumen causing diarrhea [8,9]. However, some pigs are naturally resistant to ETEC F4, because they don't have receptors (F4R) on their epithelial cell brush borders to which the fimbriae bind [8,10]. In swine, our recent study identified *HEG1* (Heart of glass homology 1) as a novel candidate and most promising gene that underlie F4ab/F4ac susceptibility to ETEC diarrhea [11]. These findings provided new evidence for understanding the mechanism of genetic resistance behind the risk of diarrhea in piglets [11]. On the other hand, *MUC20* (*Mucin 20*), a cell surface-associated protein coding gene, was first identified as a novel MUC protein, up-regulated in immunoglobulin A nephropathy (IgAN) in human patients [12,13] The expression profiles of *HEG1* and *MUC20* in different types of tissues in swine with adhesion and non-adhesion to ETEC F4 are yet to be defined. Therefore, the current study aimed mainly at: First, to investigate the expression patterns of *HEG1* and *MUC20* as well as inflammation-related genes in the small intestine and establish evidence of their involvement in susceptibility/ resistance to ETEC diarrhea in Large White piglets in different adhesion reactions to ETEC F4 fimbriae. Second to investigate the expression of *HEG1* and *MUC20* in lymph, spleen, thymus as immune-related tissues and evaluate their immunity roles in baby pigs.

Material and Methods



Experimental animals and sampling

The experiment was conducted according to the regulations and guidelines established by the Animal Care and Use Committee of China Agriculture University (Permit Number: DK996). A total of 8 Large White piglets aged under two months were selected for this study on the basis of their adhesion to F4ac fimbriae of Enterotoxigenic *Escherichia coli* (ETEC) after the adhesion test was performed (n = 189) [14]. Then they were divided into two groups (adhesive and non-adhesive groups) of four animals each. Samples of the small intestine, lymph, spleen and thymus were collected from each piglet, placed in liquid nitrogen, immediately transferred to our lab and stored in the refrigerator at -80º C until used for total RNA extraction.

Adhesion Assay

An in vitro adhesion test was done as previously described by Rapacz & Hasler-Rapacz (1986) and Baker et al. (1997) cited in [15]. Briefly, first, a jejunal segment of about 10 cm was collected within 2 hours after slaughter, cut along its longitudinal axis, and washed with a hypotonic EDTA solution (5 mmol / L EDTA, pH 7.4). Small intestinal epithelial cells were prepared by scraping the mucosal surface of jejunal tissue with a microscope slide and subjecting it to subsequent treatment. The intestinal epithelial cell brush border cell suspension and ETEC F4 suspension (0.1 mL each) were mixed with 0.4 mg/mL mannose and incubated for 30 minutes at room temperature. Microscopic observation of bacterial adhesion, according to the number of bacteria adhering to the intestinal epithelial cells, the piglets not adhering to ETEC F4 were classified as ETEC F4 resistant individuals, and the small intestinal epithelial cells and ETEC F4 adherent piglets were classified as ETEC F4 susceptible individuals. Twenty well-separated and intact brush borders were scored for each specimen. In cases where fewer than four brush borders bound more than two bacteria, an additional 20 brush borders were examined. According to the criteria proposed by [16], specimens were classed as adhesive (susceptible) to ETEC F4 strains if at least 10% of brush borders bound more than two bacteria. Specimens with all brush borders bound by fewer than two bacteria were judged non-adhesive (resistant) animals. Otherwise, specimens were considered weakly adhesive.

RNA extraction and cDNA synthesis

Total RNA extraction from the small intestine, lymph, spleen and thymus of large white piglets in both adhesive and non-adhesive groups, was performed using TRIZOL Reagent following the manufacturer's protocol. The concentrations of RNA were determined using a NanoDrop spectrophotometer and yielded OD 260/280 values > 1.8. The quality of RNA was assessed by gel electrophoresis on 1% agarose gel, which revealed three distinct bands of 28S, 18S and 5S and stored at –80°C until used for cDNA synthesis. Complementary DNA (cDNA) was synthesized from total RNA using PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (TAKARA BIO INC.) following the manufacturer's instructions. Reverse transcription reactions were performed in a final volume of 20 µl using the following PCR

reaction conditions 37° C for 15 minutes and 85° C for 5 seconds then stored at – 20°C until used for RT-qPCR.

Real-time quantitative PCR (RT-qPCR)

The real-time quantitative PCR (RT-qPCR) reactions were performed in a final volume of 20 µL with the Roche SYBR Green PCR Kit (Roche, Hercules, CA, USA) using a Light- CyclerH 480 Real-Time PCR System (Roche, Hercules, CA, USA) to quantify *HEG1* and *MUC20* mRNAs. Porcine *GAPDH* was used as a reference gene. The primers used to amplify all the genes' mRNAs were designed using online Primer 3 software [17] and were further checked for primer-dimer and primer self-complementarity using oligo6 software. The primer sequences used for qPCR are listed in Table 1.

Gene	Primer's sequence (5' – 3')		Size (bp)
HEG1	F	GCTGAGTACCCCAAGAACCC	162
	R	GGCCGGGTAGAGTCCATTTC	
MUC20	F	AGAAGACCTCACTGACCCCA	73
	R	CGTAAGCACCTGCAGTTCAC	
ER1	F	CTGGGTCTGCACGTACATCTC	115
	R	GCCTGTTCAGAAGAGCCAAAT	
KRIT1	F	GTTGGGAGATGCTGATACTTGT	176
	R	TGTGGTGGCTTAGGTATCAGTT	
LMLN	F	AGACAGGACCAGAGAGCAGTT	101
	R	GAGGGTACGCCACTGAGTT	
DNMT1	F	TGTATGTTGGCCAAAGCACGG	127
	R	ATATCAGTGCACGTTGGGGA	
DNMT3A	F	CCAGCATTTCCCCGTCTTCA	101
	R	TGGAGACGTCGGTATAGTGGA	
DNMT3B	F	GCCCCTTTGACCTGGTGAT	133
	R	GGGCGTGTGTAATTCAGCAG	
TET1	F	GCTTCATCGCTGCCACTGT	122
	R	GGGGTCGGTGAGTAGGTTTTA	
TET2	F	GCTTCATCGCTGCCACTGTT	86
	R	GGGGTCGGTGAGTAGGTTTTA	
TET3	F	GGAGCCGCAGAACCACTT	105
	R	TTCACGCTGTAGGGGTCGG	
SLC12A8	F	ACCAACTTCTTTCTACACCCG	136
	R	GAAATACACAATGGCAGCAAC	
UMPS	F	AGAGGCGGGTCTTATCAGGT	205
	R	CAGATCGATGTACACGGGGG	
IL-6	F	TGTCGAGGCTGTGCAGATTA	104
	R	CGGCATTTGTGGTGGGGTTA	
IL8	F	ACCAGAGCCAGGAAGAGACTA	177
	R	GGAAAGGTGTGGAATGCGTA	
TNF-α	F	TATCGGCCCCCAGAAGGAAG	99
	R	ACGGGCTTATCTGAGGTTTGAG	
NF-kB	F	CATGAGCTCGTGGGGAAAGAC	163
	R	GGAAGGGGTTGTTGTTGGTCT	
GAPDH	F	CCACGGTCCATGCCATCACT	268
	R	GCCTGCTTCACCACCTTCTTG	

Table 1: Primers of genes used in differential expression analysis in the comparison of non-adhesive vs. adhesive groups using qPCR.

The cycling conditions were as follows: one cycle of 95°C for 5 minutes; 45 cycles of 95°C for 20 seconds, 58°C for 30 seconds, and 68°C for 45 seconds; and one cycle of 72°C for 10 minutes. A single peak in the melting curves was used to validate the specificity of the RT- qPCR amplification. Duplicate qRT-PCRs were conducted on each cDNA sample and the average Ct was used for the analysis.

Statistical analysis

The difference in cycle times, ΔCt, was determined as the difference between the tested gene and the reference housekeeping genes. The ΔΔCt was obtained by finding the difference between samples. The relative mRNA expression levels of all the genes investigated in this study were analyzed against housekeeping gene using the $2^{-\triangle\triangle Ct}$ method and Graph Pads PRISM version 7.0 was used for generating graphs and *t*-test statistical analyses were performed.

Gene ontology (GO) annotation and KEGG

Gene ontology (GO) enrichment and KEGG pathway analyses were performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) [18,19].

String analysis

Search Tool for the Retrieval of Interacting Genes/ proteins (STRING) was used to identify the *HEG1* and *MUC20* genes interaction network [20,21].

Results

Differential expressions of candidate genes in the porcine small intestine with resistance and susceptiple phenotypes to ETEC F4ac

For measuring the relative quantification of *HEG1* and *MUC20* mRNAs in the small intestine of Large white piglets in both adhesive and non-adhesive groups and to reveal their involvement in diarrhea resistance and susceptibility. We performed real-time qPCR (RT-qPCR), first we extracted total RNA from all small intestines epithelial cells using TRIZOL reagent followed by cDNA synthesis. Real-Time qPCR (RT-qPCR) revealed that the expression levels of *HEG1* and *MUC20* genes were both significantly differentially expressed in the small intestine ($p < 0.05$) (Figure 1. A).

To investigate the role of inflammation-related genes and their association with diarrhea pathological features in the small intestine of large white piglets in adhesive and non-adhesive groups, the mRNA expression levels of inflammation-related genes (*IL-6, IL-8, TNF-α and NF-kB*) in the small intestine were performed. The same cDNAs were used as templates in RT-qPCR. Real-Time qPCR (RT-qPCR) revealed that none of the inflammatory genes was found significantly differentially expressed in small intestine tissue ($p > 0.05$) (Figure 1. B).

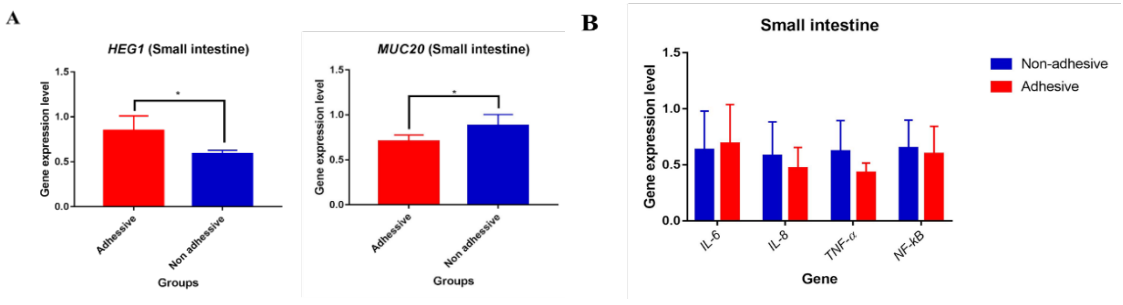


Figure 1: Differential gene expressions. (A) Differential gene expression of HEG1 and MUC20 small intestine of piglets in the adhesive and non-adhesive group. (B) Differential gene expression of four inflammatory genes in the small intestine of piglets in the adhesive and non-adhesive groups. * P-value < 0.05.

Expression of *HEG1* and *MUC20* genes in immune-related tissues

Furthermore, *HEG1* and *MUC20* mRNAs were measured and compared in several immune-related tissues (lymph, spleen and thymus) between the adhesive and non-adhesive Large White piglets (Figure 2). And we found that there were no significant differences for *HEG1* in the three immune-related tissues between the adhesive and the non-adhesive piglets (Figure 2A), while *MUC20* was significantly differentially expressed ($p < 0.05$) in spleen tissue (Figure 2B). In addition, the trends of the expression levels of *MUC20* in lymph between the adhesive and the non-adhesive piglets were similar to that in the small intestine and spleen tissues, which all showed higher expression in the non-adhesive piglets than the adhesive ones.

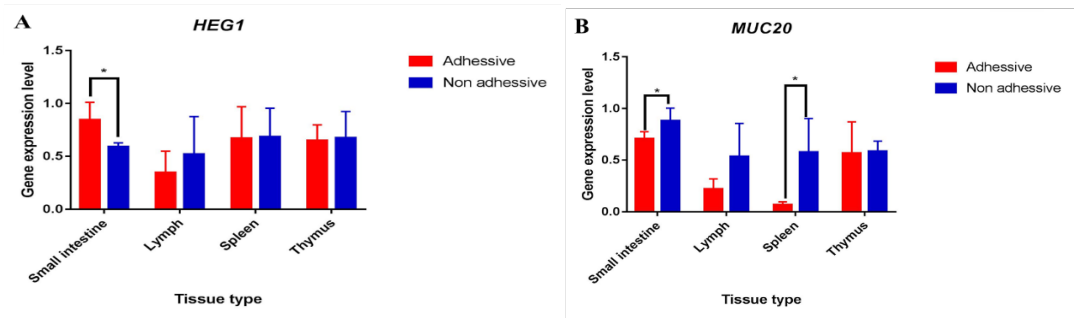


Figure 2: (A) Differential gene expression of *HEG1* in immune-related tissues of piglets in the adhesive and non-adhesive group. (B) Differential gene expression of *MUC20* in immune-related tissues of piglets in the adhesive and non-adhesive group.

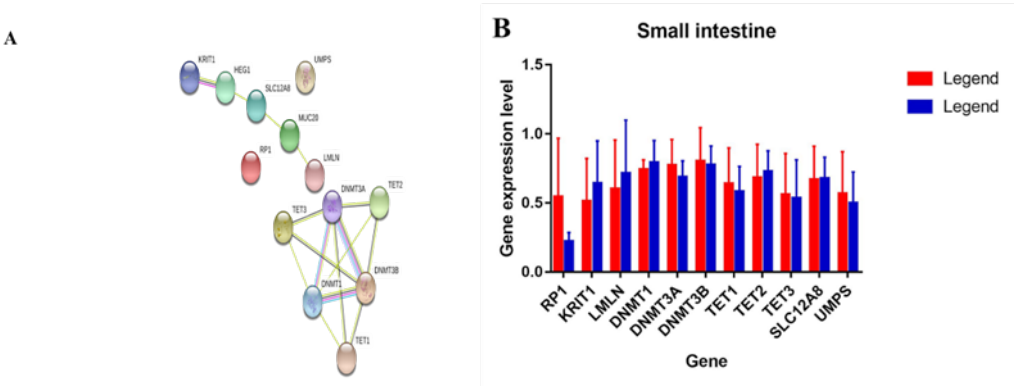


Figure 3: String and differential gene expressions. (A) Protein-protein interaction of 13 genes. (B) Differential gene expression of 13 genes in the small intestine of piglets in the adhesive and non-adhesive groups. * *P* value < 0.05.

Gene ontology (GO)

To show the possible biological processes, cellular components and molecular function, Gene ontology (GO) annotation was performed. Gene ontology term for biological process (GO term BP) revealed that *HEG1* is mostly involved in biological processes such as morphogenesis, development and growth factors whereas *MUC20* is involved in activation of *MAPK* activity, hepatocyte growth factor receptor signaling pathway, and protein homooligomerization (Table 2). GO term for cellular components (GO term CC) revealed that *HEG1* is enriched in cell-cell junction and external side of plasma membrane and *MUC20* is enriched in the plasma membrane and basal plasma membrane. GO term for molecular function (GO term MF) revealed that *HEG1* is involved in calcium ion binding and *MUC20* was not involved in any molecular function (Table 2).

Gene	GO term_BP	GO term_CC	GO term_MF
HEG1	vasculogenesis, in utero embryonic development, endothelial cell morphogenesis, lymph vessel development, lymph circulation, cardiac atrium morphogenesis, ventricular trabecula myocardium morphogenesis, ventricular septum development, cell-cell junction assembly, heart development, post-embryonic development, lung development, multicellular organism growth, venous blood vessel morphogenesis, regulation of body fluid levels, cardiac muscle tissue growth, pericardium development, positive regulation of fibroblast growth factor production,	cell-cell junction, external side of plasma membrane	calcium ion binding,
MUC20	activation of MAPK activity, hepatocyte growth factor receptor signalling pathway, protein homooligomerization,	plasma membrane, basal plasma membrane	

Table 2: Gene Ontology (GO) of *HEG1* and *MUC20.*

Protein-protein interaction networks

In order to gain insights into a deep understanding of the relationships between *HEG1* and *MUC20* genes biologically, STRING was performed. We found that *HEG1* had interactions with six genes including *MUC20*, of which five had protein of unknown 3D with text-mining evidence of interactions and one of which is *KRIT1* had protein of known 3D with two interactions evidence of text-mining and gene fusion (Figure 3A). In addition, *MUC20* interacted with five genes including *HEG1.* When gene expression of all the six interacted genes plus three porcine DNA methyltransferases (*DNMT1, DNMT3A, DNMT3B*) and three porcine DNA demethylases (*TET1, TET2, TET3*) genes was performed, we observed that, all genes except *HEG1* and *MUC20* ($p < 0.05$) were non-significantly differentially expressed between the non-adhesive piglets and the adhesive ones ($p > 0.05$) (Figure 3B).

Discussion

Searching for the genes underlying resistance/ susceptibility to piglets' diarrhea to be used as molecular markers in the selection of resilient piglets in the breeding programme could help in reducing the economic burdens inflicted as a result of diarrhea on farmers and producers in swine industries and eventually add more economic benefits.

The main goal of the current study was first, to investigate the mRNA expression of *HEG1* and *MUC20* as well as inflammation-related genes in the small intestine in order to uncover evidence of their involvement in resistance/ susceptibility to diarrhea caused by ETEC strain in Large White piglets in different adhesion reactions to F4 fimbriae; second to investigate the expression of *HEG1* and *MUC20* in lymph, spleen, thymus as immune-related tissues and evaluate their roles in piglets' immunity.

Heart of glass homolog 1 (*HEG1*), a transmembrane receptor found at Endothelial Cell (EC)–cell junctions [22] has been extensively studied in humans, mice and zebrafish. Protein *HEG1* was first described as the heart of glass gene that regulates the concentric growth of the heart in zebrafish [23]. In mice, the *HEG1* gene has been linked to the development of cardiovascular organs [24]. Furthermore, molecular genetic studies in mice and fish have shown that *HEG1*, *PDCD10, KRIT1* and *CCM2* function combinatorially in endothelial cells during the genesis of the heart and vasculature [25]. In humans, the proliferation of Malignant Mesothelioma (MM) cells partly depends on the expression of *HEG1*, the greater the expression of *HEG1* in mesothelioma cells, the more they proliferate. Shoutaro et al. identified sialylated *HEG1* as a novel mucin-like membrane protein, and that its expression supports the survival and proliferation of mesothelioma cells in humans [23]. In the current study, *HEG1* was significantly differentially expressed in the small intestine of piglets ($p < 0.05$) i.e. higher in adhesive group than in non-adhesive group. This followed similar expression trend of our RNA-Seq data analysis of the same piglets [26]. Considering all of the above findings, it is unequivocally that, *HEG1* has a different and specific function in different organs in different species [27,28]. No previous publications on *HEG1* expression studies in pigs against which to compare our results.

Our study strongly confirmed a previous GWAS study in our laboratory, which identified *HEG1* as the most promising new candidate gene that may underlie F4ab/F4ac susceptibility to ETEC diarrhea in piglets in which the authors suggested that *HEG1* could be a possible candidate gene and deserve follow-up validation in the future [11] and this actually what our study did. In addition, [4] also indicated that the *HEG1* is an important adhesion molecule, which could serve as a genetic marker for selecting ETEC F4ac-resistant pigs in breeding programs.

On the other hand, *MUC20* was significantly differentially expressed in the small intestine ($p < 0.05$). Xiuying Xiao et al. showed that *MUC20* expression was significantly higher in colorectal cancer (CRC) in humans ($p < 0.05$) [12]. Toshio Higuch et al. also reported that *MUC20* mRNA was moderately expressed in many tissues including the digestive system such as the colon, esophagus, and rectum [13]. Similarly, Mette et al. in their study entitled characterization of five candidate genes within the ETEC F4ab/ac candidate region in pigs reported that *MUC20* had the highest expression in the duodenum ($p < 0.05$) but was also expressed in the three other intestinal tissues (colon, ileum and jejunum) at a relatively high level [29]. They concluded that no differences in expression levels of the five tested candidate genes (including *MUC20*) were detected between resistant and susceptible animals (four piglets of crosses between the Landrace, the Yorkshire and the Duroc breeds). They reached this conclusion because their findings showed that none of the polymorphisms they had identified was obvious causative candidate mutations, and further concluded that they cannot exclude that these genes are bona fide candidates for susceptibility to ETEC F4ab/ac infection in piglets.

Contrary to our findings, *MUC20* mRNA was reported moderately expressed in the small intestine, and weakly in the lung, thymus, stomach and liver of the White Duroc x Erhualian F2 intercross [5]. The differences in expression could be due to breed variations and alternatively, could be owing to within breed variations. Furthermore, could also be due to cell and tissue type-specific gene expression. This is not surprising because our study was carried out on Large White pig breeds, which were further classified on the basis of their adhesion reactions to ETEC fimbriae F4ab/ac.

Because the differential pattern in inflammatory gene expression levels can reveal their genetic association with the diseases, we thus, investigated their mRNA expression levels by RT-qPCR. The investigated inflammatory genes exhibited almost similar expression levels in the piglets' small intestines in both adhesive and non-adhesive groups. This suggests that they are not directly associated with the diarrhea pathological features or inflammation due to diarrhea in the small intestine of Large White piglets in the adhesive group. Albeit, intriguingly, three of the inflammatory genes (*IL-8, TNF-α* and *NF-Kb*) showed slightly greater expression in the non-adhesive group as compared to the adhesive group whereas *IL6* showed vice versa expression.

When the expression of *HEG1* and *MUC20* were investigated in immune-related tissues, the expression of both genes was similar in the thymus and followed the same trend in the lymph. Lin et al. when comparing human-mouse tissues, indicated that tissues within the same species are more similar than tissues between species, and concluded that gene expression profiles of different tissues within an organism do exhibit similarities in gene expression, this is consistent with what we obtained [30]. Nevertheless, surprisingly *MUC20* was more expressed in the spleen of the non-adhesive group than the adhesive group ($p < 0.01$) compared to *HEG1, which* exhibited a similar expression level in the spleen of both adhesive and non-adhesive piglets. This

could indicate that the spleen provides the resistance phenotype conferred upon piglets in the non-adhesive group since thymus and lymph both showed non-significant expression levels. This is very intriguing, the thymus being a specialized primary lymphoid organ of the immune system, which is critical to the adaptive immune system, where the body adapts specifically to foreign invaders.

HEG1 is methylated in the promoter region (Figure 3) with 653 bases overlapping with the first exon, for this reason, DNA methyltransferase and DNA demethylase genes expression were performed to reveal their role in the regulation of *HEG1* gene. To our surprise, none of them was found significantly differentially expressed in the small intestine.

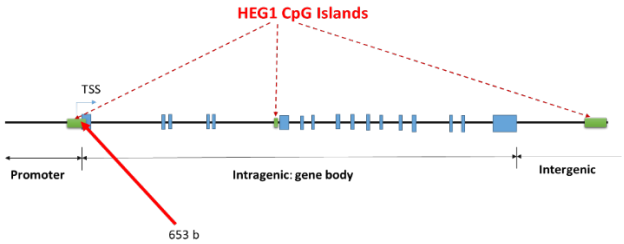


Figure 4: Showing TSS, CpG Islands, Promoter region is methylated, and methylation has 653 b overlapped with first exon, gene body and intergenic regions, the green and blue colours indicate CpG islands and exons respectively.

This suggests that neither DNA methyltransferases nor DNA demethylases influenced the regulation of *HEG1*. The regulation could be by either histone modification at the transcription level or miRNA at the translation level, this is worth further follow-up by epigenetic studies.

Conclusion

Considering the findings of this study, it could be concluded that, firstly, RT-qPCR confirms both *HEG1* and *MUC20* genes were significantly differentially expressed in the small intestines of Large White piglets. Secondly, none of the inflammatory genes had a direct link to the diarrhea pathological features or inflammation due to diarrhea in the small intestine of Large White piglets in the adhesive group. Thirdly, *MUC20* was significantly expressed in the spleen of non-adhesive piglets, which could indicate that spleen as immune-related tissue is behind the resistance phenotype conferred upon piglets in the non-adhesive group. Fourthly, DNA methyltransferases and DNA methylases did not affect *HEG1* regulation. Finally, our study was the first to draw the curtain and unravel the involvement of *HEG1* and *MUC20* genes in piglets' susceptibility to diarrhea caused by ETEC and could be used as molecular markers in the selection of piglets resistant to ETEC diarrhea in a breeding program in swine industries.

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Author Contribution

SA, conducted all the experiments, designed the primers and wrote the manuscript, XQ, assisted in samples collection and conducted RT-qPCR, MS, and EO, assisted in the discussions of the results, ZQ, assisted in cross-checking of the results and proofreading of the final version of the manuscript, YY, designed the experiments, cross-checked the results and proved read the final version of the manuscript. All authors have read and approved the final version of the manuscript.

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