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A statistical approach to identify prevalent virulence factors responsible for post-weaning diarrhoeic piglets

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Abstract: A statistical approach was carried out to identify the prevalent virulence factors responsible for post-weaning diarrhoea (PWD). Healthy piglets' faecal samples and diarrhoeic piglets' rectal swab specimens were secured. Twenty-six (26) and 100 independent enterotoxigenic *Escherichia coli* (ETEC) strains were subsequently isolated. These strains were assessed utilising polymerase chain reaction to identify the encoding genes of six virulence factors: heat-labile enterotoxin (LT; encoded by *eltAB*), heat-stable enterotoxin A (STa; encoded by *estA*), heat-stable enterotoxin B (STb; encoded by *estB*), enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1; encoded by *astA*), Shiga toxin 2e (Stx2e; encoded by *stx2e*), and F18 fimbriae (encoded by *fedA*). The LT and ST secretions were investigated using enzyme-linked immunosorbent assays. From direct observation, no *stx2e* was evident in the 126 strains. Among the 26 strains retrieved from the healthy piglets, none harboured *fedA* or secreted LT; 23% (6/26) secreted ST, and 50% (13/26) carried *astA*. A statistical regression was applied on the 100 *E. coli* strains retrieved from the diarrhoeic piglets, where *fedA* was set as the dependent variable and the enterotoxin secretions were set as the independent variables. The results exhibit that the LT secretion was the only significant factor ($P < 0.000\ 1$) correlated to *fedA* in the diarrhoeic piglets; thus, it is concluded that the prevalent virulence factors for PWD were the ECET strain with F18 fimbriae adhesion and LT secretion, but not *astA* or *stx2e*.

Keywords: enteroaggregative heat-stable enterotoxin; F18 fimbriae; heat-labile enterotoxin; heat-stable enterotoxin; Shiga toxin 2e

Colibacillosis, a series of diseases of livestock caused by the pathogenic *Escherichia coli*, has increased in incidence and severity in recent years. The most significant colibacillosis occurrence in swine is the diarrhoeal disease of post-weaned piglets. If found severe, the diarrhoeal disease can cause death in piglets, and survivors usually have a decreased food intake and weight gain. This disease, known as post-weaning diarrhoea (PWD), frequently results in financial and economic damage in the swine industry. The addition of antibiotics to pig feed is the measure most effective

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at preventing PWD. Drug-resistant bacteria have emerged, however, and the use of antibiotics may lead to severe problems in the food chain and the environment (Cutler et al. 2007; Zhang 2014; Teng et al. 2021).

Studies have shown that enterotoxigenic *E. coli* (ETEC) is the leading cause of PWD (Fairbrother et al. 2005; Nagy and Fekete 2005; Zhang 2014). ETEC adheres to intestinal microvilli through fimbrial adhesion and secretes enterotoxins that alter the permeability of the intestinal epithelial cells, resulting in fluid and electrolyte leakage and cell death. Fimbrial adhesion and enterotoxin secretion are two significant virulence factors of ETEC (Zhang 2014). The fimbriae of ETEC strains of animal origin include F4 (K88), F5 (K99), F6 (987P), F7 (F41), F17, and F18 (Vu-Khac et al. 2007). F4 and F18 are the two most common types; they are further classified according to their antigen variants: F4ab, F4ac, and F4ad; F18ab and F18ac (Zhang 2014). Both F4 and F18 possess adhesive structures that play essential roles in binding to host cell receptors (Meijerink et al. 2000; Nagy and Fekete 2005); these different *E. coli* adhesion molecules are responsible for the sensitivity of a host toward infection by these particular strains of *E. coli* (Kim et al. 2010).

Porcine ETEC mainly secretes heat-labile enterotoxin (LT; encoded by *eltAB*) and heat-stable enterotoxin (ST; including STa encoded by *estA* and STb encoded by *estB*). LT can be inactivated completely upon incubation at 60 °C for 15 min, whereas ST can withstand heat at 100 °C for 5 min (Fairbrother et al. 2005). Both enterotoxins disrupt the intestinal homeostasis, ultimately leading to diarrhoea in the infected animal (Toledo et al. 2012; Zhang 2014). LT is closely related to the heat-labile toxin of *Vibrio cholera*, the cholera toxin. Upon binding to the monosialotetrahexosylganglioside 1 receptor of small-intestinal epithelial cells, LT enters the cell and stimulates the adenylate cyclase activity, resulting in excessive electrolyte secretion and, consequently, dehydration and fluid loss from the intestine (Nagy and Fekete 2005). Regarding ST, both STa and STb cause fluid loss from the enterocytes, albeit through different mechanisms. STa activates the guanylate cyclase pathway, which increases the level of cyclic guanosine monophosphate (cGMP) in the cell and inhibits the absorption of Na^+ and enhances the secretion of Cl^- (Nagy and Fekete 2005), resulting in fluid hypersecretion and the induction of diarrhoea. In contrast, STb is believed to decrease the net fluid and electrolyte absorption by stimulating the anion secretion and promoting the transport of water into the intestinal lumen, leading to watery diarrhoea (Dubreuil 2008).

In addition to LT and ST, some ETEC strains also secrete enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) and Shiga toxin type 2e (Stx2e) (Nagy and Fekete 2005; Zhang et al. 2007; Toledo et al. 2012; Zhang 2014). Stx2e is a member of the Shiga toxin family, associated with oedema disease and PWD in pigs (Marques et al. 1987; Fairbrother et al. 2005; Zhang 2014); Shiga toxins also inhibit protein synthesis in host cells (Gyles 2007). Although EAST1 toxin genes have been found in *E. coli* strains recovered from healthy and diarrhoeic piglets, little is been known regarding its role in porcine diarrhoea (Paiva de Sousa and Dubreuil 2001; Ngeleka et al. 2003; Zhang et al. 2007).

PWD has been observed at a commercial pig farm in Miaoli County in western Taiwan for years. A statistical approach was carried out in this study to identify and characterise its prevalent virulent factors. Faecal and rectal swab specimens were collected from diarrhoeic and healthy piglets over 10 months. The *E. coli* strains were independently isolated, identified, and characterised. Genes encoding six PWD virulence factors were detected via polymerase chain reaction (PCR). For the first time, two enterotoxins (heat-labile enterotoxin: LT and heat-stable enterotoxin: ST) were assayed via enzyme-linked immunosorbent assays. Observational and statistical analyses of the sampling data were performed and compared.

MATERIAL AND METHODS

Collection of faecal specimens

Two pig breeds, LY (Landrace × Yorkshire) and LYD (Landrace × Yorkshire × Duroc) (Choi et al. 2016), were tested on a commercial farm in Miaoli County in western Taiwan. Faecal samples of healthy and diarrhoeic piglets were collected. Initially, rectal swabs were taken from both the healthy and diarrhoeic piglets in the same pigsty, but no bacteria were recovered from the rectal swabs of the healthy piglets after the first four visits to the farm. Thus, rectal swabs were taken from the diarrhoeic

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piglets on the fifth to tenth visits. In contrast, the faeces were collected from the pigsties containing all the healthy piglets, and one specimen was collected from one healthy pigsty. In total, 15 faecal samples from the healthy piglets and 58 rectal swabs from the diarrhoeic piglets were collected that revealed the presence of *E. coli* (Table 1).

Isolation and identification of the *E. coli* strains

The *E. coli* strains in the faecal specimens and rectal swabs were isolated and identified using biochemical methods (Baron and Antonson 1997). Each faecal specimen or rectal swab was briefly spread onto a Levine eosin methylene blue (EMB) agar plate and incubated overnight at 37 °C. Colonies with a metallic sheen, but with different morphologies (i.e., rough vs. smooth and large vs. small), were transferred to blood agar plates (BAPs) and Levin EMB plates. Only one colony was selected for each specific morphology; thus, at most, four colonies (strains) were chosen for one faecal specimen or rectal swab. The plates were incubated overnight at 37 °C. A single colony from the Levin EMB agar plate was picked and examined for the following biochemical reactions: triple sugar iron agar test; citrate utilisation test; urease test; sulfide-indole-motility; semi-Voges Proskauer test; and ornithine decarboxylase, arginine dehydrolase, and lysine decarboxylase activities. At the same time, a single colony was picked from the BAP agar plate for the oxidase test. The results were used to identify the bacteria as *E. coli*. Many colonies were also confirmed using the VITEK 2 gram-negative microbial identification method (Crowley et al. 2012).

Table 1. Number of *E. coli* strains recovered from the collection of the faecal specimens of the healthy piglets and rectal swabs of the diarrhoeic piglets

Visit	Healthy piglets		Diarrhetic piglets	
	faecal specimen	*E. coli*	rectal swab	*E. coli*
1	–	–	6	9
2	–	–	4	4
3	–	–	8	10
4	–	–	4	6
5	2	3	1	1
6	3	3	6	12
7	3	4	6	12
8	3	6	9	14
9	2	7	7	20
10	2	3	7	12
Total	15	26	58	100

(–) not available

Identification of virulence factor genes

The *E. coli* strains were examined through the PCR analysis for the presence of genes encoding LT, STa, STb, Stx2e, EAST1, and F18 fimbrial adhesion molecules (Pass et al. 2000; Beutin et al. 2008). The PCR primers used are listed in Table 2. The PCR analysis was performed in a 50-µl volume containing 1.25 IU DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.4 mM deoxynucleotide triphosphates (dNTPs), 0.8 µM of each primer, and 5 µl of bacterial suspension as the template, obtained from a suspension of a fresh bacterial colony in 50 µl of ddH_2O. The PCR products were analysed using 2% agarose gel electrophoresis.

Enterotoxin analysis

The LT and ST enterotoxins secreted by the *E. coli* strains were detected using enzyme-linked immunosorbent assay (ELISA)-based VET-RPLA and COLIST-EIA kits (Denka Seiken, Tokyo, Japan). ATCC 43886 (serotype O25:K98:NM, LT enterotoxin secretion positive) (Wachsmuth et al. 1979) and ATCC 35401 (serotype O78:H11, LT and ST enterotoxin secretion positive) were used as the positive controls for LT and ST secretion, respectively (Skerman et al. 1972; Evans et al. 1973).

Genotype testing of pigs

The pigs were genotyped through the PCR for the presence of *ecf18R*, the receptor gene for *E. coli* F18 fimbriae (Frydendahl et al. 2003). The genomic DNA was extracted from the blood using a LabPrep Genomic DNA Mini Kit (Taigen Bioscience, Taipei, Taiwan). Amplification of a 162-base-pair (bp) DNA fragment of the *ecf18R* gene was conducted using the primers listed in Table 2, as described by Frydendahl et al. (2003).

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Table 2. Sequences of the PCR primers

Target gene (protein product)	Primers	Reference
eltAB (LT)	F: TGGATTCATCATGCACCACAAGG R: CCATTTCTCTTTTGCCTGCCATC	Pass et al. (2000)
estA (STa)	F: TTCTGTATTATCTTTCCCC R: TTATGATTTTCTCAGCACC	Beutin et al. (2008)
estB (STb)	F: CGCATTTCTTCTTGCATCTCTG R: TGCTGCAACCATTATTTGGG	Beutin et al. (2008)
astA (EAST1)	F: CCATCAACACAGTATATCCGA R: GGTCGCGAGTGACGGCTTTG	Beutin et al. (2008)
stx2e (Stx2e)	F: ATGAAGAAGATGTTTATAGCG R: GTTAAACTTCACCTGGGCAAAG	Beutin et al. (2008)
fedA (F18 fimbriae)	F: CTTTCACATTGCGTGTGGAG R: ACCACCTTTCAGTTGAGCAG	Beutin et al. (2008)
ecf18R (E. coli F18 receptor*)*	F: TTGGGAACCAGATGGGACAGTATG R: CCCGCCAAGGAGCGTGCCTGTCTA	Frydendahl et al. (2003)

F = forward; R = reverse

Statistical analysis

A fractional factorial model of up to two degrees was implemented under standard least-squares regression.

Instead of the response surface model commonly used for modelling quadratic surfaces (Zang et al. 2015), the second-degree fractional factorial model was chosen to analyse the correlation between the main effects and the two-degree interactions of the respective variables. The following linear model was constructed:

$$y_{p,k} = \alpha_0 + \sum_{i=1}^{I} \alpha_i x_{i,k} + \sum_{i \neq j}^{I,J} \beta_{i,j} x_{i,k} x_{j,k} \quad (1)$$

$$\varepsilon_k = y_{a,k} - y_{p,k} \quad (2)$$

where:

i, j – set of variables in the factorial model;
k – number of observations;
$y_{a,k}$ – actual response for the k^{th} observation;
$y_{p,k}$ – predicted response for the k^{th} observation;
$x_{i,k}$, $x_{j,k}$ – values of the x_i and x_j variables, respectively, for the k^{th} observation;
α_0, α_1, $\beta_{i,j}$ – parameters for the model;
ε_k – normally distributed residual term.

The sum of squared residuals was minimised as follows:

$$\min \sum_{k=1}^{K} \varepsilon_k \quad (3)$$

The standard least-squares approach was used to construct a linear model for the response data of *fedA* from the variables of LT, ST, and *astA*. In this case, all the main effects and interactions up to two degrees were created for the following reformulated linear model.

$$fedA_{p,k} = \alpha_0 + \alpha_1 ST_k + \alpha_2 LT_k + \alpha_3 astA_k + \beta_{STLT} ST_k LT_k + \beta_{STastA} ST_k astA_k + \beta_{LTastA} LT_k astA_k \quad (4)$$

$$\varepsilon_k = fedA_{a,k} - fedA_{p,k} \quad (5)$$

Bivariate fits were implemented to understand the independent correlation of *fedA* to each of LT, ST and *astA* in the form of a standard linear fit for each variable of interest, shown as follows:

$$fedA_i = \gamma_0 + \sigma_0 LT_i \quad (6)$$
$$fedA_i = \gamma_1 + \sigma_1 ST_i \quad (7)$$
$$fedA_i = \gamma_2 + \sigma_2 astA_i \quad (8)$$

where:

γ_i, σ_i – intercept and slope for the linear models.

All the statistical analyses are performed using JMP® v16.0 software (SAS Institute Inc, USA).

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RESULTS

Genotype testing

Our earlier studies indicated that some pigs on this particular farm carried the F18 fimbriae receptor gene, *ecf18R*. Two pig breeds, LY and LYD, were raised on the farm; two LY and three LYD pigs were chosen randomly. The genomic DNA was extracted from the blood and used as the template for the PCR to detect *ecf18R*. Figure 1 presents the results. Except for one LYD pig, the other four pigs were positive for *ecf18R*. Two PCR products, one from an LY pig and one from an LYD pig, were sequenced and confirmed as the 162-bp DNA fragment of *ecf18R*. Thus, some pigs on the farm carried the *ecf18R* gene, either in the homozygous or heterozygous state. Because piglets might carry the receptor gene (*ecf18R*) for the F18 fimbriae adhesion, and the adhesion–ligand interactions determine the sensitivity of piglets infected by pathogenic ETEC, we expected the piglets with the *ecf18R* gene to be the targets of the *fedA*-positive *E. coli* on the farm.

PCR and ELISA testing

We collected 15 faecal specimens from healthy piglets and 58 rectal swabs from diarrhoeic piglets during visits to the farm, where we recovered 26 and 100 independent *E. coli* strains, respectively (Table 1). These 126 strains were first assessed through a PCR analysis for genes encoding six virulence factors: LT (encoded by *eltAB*), STa (encoded by *estA*), STb (encoded by *estB*), EAST1 (encoded by *astA*), Stx2e (encoded by *stx2e*), and F18 fimbriae (encoded by *fedA*). The secretion of LT and ST by these *E. coli* strains was also evaluated using ELISA-based VET-RPLA and COLIST-EIA kits, respectively.

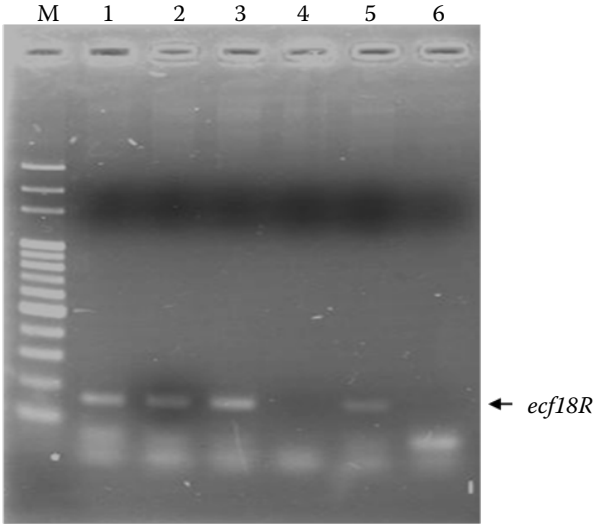

Figure 1. PCR for *ecf18R* using genomic DNA from two LY and three LYD pigs

Lane M, DNA size marker, from top to bottom, 3 000, 2 000, 1 500, 1 000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 base pairs; lanes 1 and 2, LY pigs; lanes 3–5, LYD pigs; lane 6, negative control using ddH_2O as the template

Table 3 provides the PCR data for *fedA*, *eltAB*, *estA*, *estB*, *astA*, and *stx2e*. All of the 126 *E. coli* strains were negative for *stx2e* through the PCR; therefore, *stx2e* and its toxin product, Stx2e, have been excluded from further discussion. Of the 26 strains recovered from the healthy piglets, none carried *fedA*, none carried *eltAB*, 23% (6/26) carried *estA*, 58% (15/26) carried *estB*, and 50% (13/26) carried *astA*. Of the 100 *E. coli* strains recovered from the diarrhoeic piglets, 41% (41/100) carried *fedA*, 41% (41/100) carried *eltAB*, 58% (58/100) carried *estA*, 73% (73/100) carried *estB*, and 35% (35/100) carried *astA*.

To identify the existence of enterotoxins, LT and ST were examined. Because of the lack of an EAST1 assay kit, the presence of *astA* was used to identify the possible presence of EAST1. Table 4 lists the results for the *E. coli* carrying *fedA*, LT, ST, and *astA*. None of the strains from the healthy piglets were positive for *fedA* and/or LT secretion; in contrast, 41% of the strains from the diarrhoeic piglets were positive for *fedA* and/or LT secretion, of which 39 were both $fedA^+$ and LT^+. Furthermore, just less than a quarter (23%) of the strains from the healthy piglets were ST^+ and half (50%) of the strains were $astA^+$; in contrast, among the strains from the diarrhoeic piglets, more than half (54%) were ST^+, but only 35% were $astA^+$.

Table 4 reveals that all of the LT secretions correlated with the presence of the *eltAB* gene. Thus, the *eltAB* expression was constitutive with the LT secretion in our cases. Regarding the ST secretion, it seemed that a combination of $estA^+$ and $estB^+$ was required for the ST expression, except in one case with $estA^-$ and $estB^+$. In addition, the presence of one or both of $estA^+$ and $estB^+$ did not ensure the expression and further secretion of ST. These results suggest that the production of ST was primarily due to the combination of *estA* and *estB*.

We expected that only the strains that were *fedA*-positive would possibly lead to infection. Table 5

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Table 3. PCR results for the presence of *fedA*, *estA*, *estB*, *eltAB*, *astA* and *stx2e* in the *E. coli* strains recovered from the healthy and diarrhoeic piglets

Isolations	Number of *E. coli* strains (%)						
	total	*fedA*$^+$	*eltAB*$^+$	*estA*$^+$	*estB*$^+$	*astA*$^+$	*stx2e*$^+$
Healthy piglets	26	0	0	6 (23%)	15 (58%)	13 (50%)	0
Diarrheic piglets	100	41 (41%)	41 (41%)	58 (58%)	73 (73%)	35 (35%)	0

Table 4. The presence of *fedA*, LT, ST, *astA* in the *E. coli* strains recovered from the healthy and diarrhoeic piglets

Isolations	Number of *E. coli* strains (%)				
	total	*fedA*$^+$	LT$^+$	ST$^+$	*astA*$^+$
Healthy piglets	26	0	0	6 (23%) (5 *estA*$^+$ *estB*$^+$, 1 *estA*$^-$ *estB*$^+$)*	13 (50%)
Diarrheic piglets	100	41 (41%)	41 (41%) (41 *eltAB*$^+$)*	54 (54%) (54 *estA*$^+$ *estB*$^+$)*	35 (35%)

*The corresponding positive gene number

Table 5. *E. coli* strains with/without *fedA* recovered from the diarrhoeic piglets

Isolations	Number of *E. coli* strains (%)			
	total	LT$^+$	ST$^+$	*astA*$^+$
fedA$^-$	59	2 (3%)	17 (29%)	33 (56%)
fedA$^+$	41	39 (95%)	37 (90%)	2 (5%)

Table 6. Enterotoxins phenotype in the *E. coli* strains (*fedA*$^+$) recovered from the diarrhoeic piglets

Number of *E. coli* strains			
No.	LT	ST	*astA*
36	+	+	–
2	+	–	–
1	+	–	+
1	–	+	–
1	–	–	+

lists the numbers of *fedA*-positive and -negative strains recovered from the diarrhoeic piglets. We found that 59% of the strains were *fedA*-negative, meaning that they belonged to the normal flora without infection. The number of strains without infection were quite similar to the enterotoxin secretion ratios of the healthy piglets in Table 4; that is, with low (0–3%) LT$^+$, medium (23–29%) ST$^+$, and high (50–56%) *astA*$^+$ ratios. Similar ratios for the enterotoxins support the notion that those strains were the normal flora without infection.

We expected the strains that were *fedA*-positive to be the main virulence factors leading to infection. The enterotoxin secretion patterns associated with these strains were entirely uncorrelated: 95% of the strains secreted LT and 90% secreted ST, but only 5% carried *astA*. The high LT and ST and low *astA*$^+$ appearance ratios suggested that they were associated with the PWD infection on this farm.

Table 6 separates the 41 strains carrying *fedA*$^+$ obtained from the diarrhoeic piglets concerning their enterotoxins. Interestingly, 36 strains were LT$^+$, ST$^+$, and *astA*$^-$; these were the dominant strains for the PWD infection. The other strains comprised two that were LT$^+$, one that was LT$^+$ and *astA*$^+$, one that was ST$^+$, and one that was *astA*$^+$; these strains were in the minority of the strains recovered from the diarrhoeic piglets. Thus, it appears reasonable to assume that both the LT and ST secretions were important for the PWD, although the ST secretion alone was not.

Statistical analysis on the diarrhoeic piglets

We performed a statistical analysis to identify the main causative agents for PWD. The data of 100 strains from the diarrhoeic piglets were chosen; the dependent variable was *fedA* and the independent variables were LT, ST, *astA* (EAST1), because we suspected these enterotoxin secretions to be the main virulence factors for PWD. For ease of analysis and interpretation, the positive and negative presence were identified by the numbers 1 and 0, respectively. All the analyses were conducted using the statistical software JMP® v16.0 (SAS Institute Inc., USA). Of all the diarrhoeic piglets (100 data),

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Table 7. Statistical analysis summary

Effects summary					
Source	LogWorth	*P*-value	*DF*	Sum of squares	*F* ratio
LT	14.764	0.000 00*	1	3.590 064 4	91.441 3
ST**astA*	1.297	0.050 44	1	0.154 223 8	3.928 2
ST*LT	0.820	0.151 24	1	0.082 212 3	2.094 0
astA	0.444	0.360 09	1	0.033 211 0	0.845 9
LT**astA*	0.064	0.862 38	1	0.001 186 3	0.030 2
ST	0.060	0.870 34	1	0.001 051 8	0.026 8
Analysis of variance (ANOVA)					
Source	*DF*	Sum of squares	Mean square	*F* ratio	
Model	6	20.538 741	3.423 12	87.189 2	
Error	93	3.651 259	0.039 26	prob > *F*	
Count total	99	24.190 000	–	< 0.000 1*	
R^2 = 0.849 059, R^2 Adj = 0.839 321, RMSE = 0.198 143					

*Statistically highly significant as $P < 0.001$

the significance of LT, ST, and *astA*, along with their interactions of up to two degrees in the factorial model of the positivity of *fedA*, was analysed using the standard least-squares model. Table 7 lists the results. The effect of LT had a value of LogWorth of 14.764, much higher than the significance threshold of 2 (*P*-value of 0.01). The interactions of ST**astA* come in second place with a value of LogWorth of 1.297 (*P*-value 0.050 44), slightly above the significance threshold of 0.05. Less important effects occurred for LT*ST (LogWorth 0.820, *P*-value 0.15), *astA* (LogWorth 0.444, *P*-value 0.36), LT**astA* (LogWorth 0.064, *P*-value 0.86), and ST (LogWorth 0.060, *P*-value 0.87). We performed bivariate fits of *fedA* with respect to LT, ST, and *astA*. The data for $fedA^+$ displayed positive correlations toward LT (slope = 0.917) and ST (slope = 0.606), but a negative correlation toward *astA* (slope = –0.526). These findings agree well with the parameters estimated for LT, ST, and *astA* from the standard least-squares model. Therefore, this statistical analysis confirmed that the hosts carrying *fedA* associated with LT were the prevalent virulent factors responsible for the PWD in the piglets on this farm in Taiwan.

DISCUSSION

Genotype testing

Our results indicate that only the *E. coli* strains recovered from the diarrhoeic piglets carried the F18 fimbriae adhesion gene *fedA*. Nevertheless, the piglets may/may not carry the receptor gene (*ecf18R*) for F18 fimbriae adhesion (Figure 1). Because adhesion–ligand interactions determine the sensitivity of the piglets toward pathogenic ETEC, our results indicate that the PWD on this particular farm was caused by *fedA*-positive *E. coli*.

We found that the *E. coli* strains from the healthy piglets, as part of the normal intestinal flora, were mainly *fedA*-negative and LT-negative, with either positivity or negativity observed for ST and *astA*. Among the 100 strains from the diarrhoeic piglets, excluding the 57 *E. coli* strains belonging to the normal flora (*fedA*-negative and LT-negative), we tested the 41 strains that were *fedA*-positive. We identified 36 strains that were LT- and ST-positive, but *astA*-negative. These 36 strains all carried *eltAB*, *estA*, and *estB* for the LT and ST expression and secretion. The remaining five *fedA*-positive strains, expressing only one or two other types of enterotoxins, might have been part of the normal flora or might have been fewer causative agents for PWD.

As revealed in Table 6, among the 41 *fedA*-positive strains recovered from the diarrhoeic piglets, 36 were LT- and ST-positive; the remaining five strains were LT- and *astA*-positive or positive for LT, ST, or *astA* alone. If we assume that all the strains carrying the *astA* gene also secreted the EAST1 enterotoxin, the number of strains recovered with the different enterotoxin types may have indicated the infection power of each type. Of the 41 *fedA*-positive strains retrieved from the diarrhoeic piglets, 36 were LT- and ST-positive but

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EAST1-negative; this type was the most infectious. Four less-infectious types were identified: one that was LT- and EAST1-positive, two that were LT-positive, one that was ST-positive, and one that was EAST1-positive. The strains expressing none of the three enterotoxins did not exhibit infectivity, as evidenced by the fact that no such types were recovered among the 41 *fedA*-positive strains from the diarrhoeic piglets. A previous study found that pathogenic porcine ETEC with deletion of the gene encoding LT could still express ST and EAST1 and cause diarrhoea in piglets (Erume et al. 2008).

The observation might lead to a conclusion that ETEC harbouring *fedA*, and secreting both enterotoxins LT and ST are the causative agents for the PWD. However, the statistical analysis reveals that ETEC, harbouring *fedA* and secreting only enterotoxin LT, was most responsible for the PWD of piglets. The reason is that the appearance of LT was the highest with the $fedA^+$ strains in the diarrhoeic piglets, whereas LT^+ was lowest among the $fedA^-$ strains. In contrast, ST always coexisted with $fedA^+$ at a high level, whereas its appearance with $fedA^-$ was always higher than 0.25. Therefore, the attribution of ST to PWD was not significantly as critical as that of LT.

Earlier studies indicated that some clinical *E. coli* strains carry $estA^-$ or *eltAB*-containing plasmids and that these plasmids are transferable from one strain to another by conjugation (McConnell et al. 1980; Yamamoto and Yokota 1983). Porcine strains carrying an $estA^-$ or *eltAB*-containing plasmid have also been reported (Wasteson and Olsvik 1991). Furthermore, a porcine strain has been found to harbour a plasmid containing both *eltAB* and *estB*, with *estB* located on a transposon that could move from one plasmid to another in the same bacterium (Lee et al. 1985). In this present study, we found that the two *fedA*-negative strains recovered from the diarrhoeic piglets that secreted LT and ST (Table 5) were not the causative agents of PWD; in other words, the three genes *eltAB*, *estA*, and *estB* were likely acquired from the horizontal transfer of a plasmid and/or transposon from another *fedA*-positive and LT- and ST-positive *E. coli* strain in the same diarrhoeic piglet. In fact, these two *fedA*-negative strains were recovered from the same rectal swab from a diarrhoeic piglet from which the main causative agents were also recovered.

Fimbrial adhesion is one of the most important virulence factors of PWD-causing agents and is considered a good target for vaccines (Zhang 2014). Nevertheless, it appears that the prevalence of PWD caused by F4-positive *E. coli* and F18-positive *E. coli* in Asia is different from that in the USA and Europe. Indeed, studies in China, South Korea, and Malaysia have revealed that F18-positive *E. coli* is more frequently associated with porcine PWD than are F4-positive bacteria (Cheng et al. 2006; Lee et al. 2008; Ho et al. 2013). In contrast, research in Hungary, the United States, Slovakia, and Denmark has indicated a high prevalence of F4-positive *E. coli* isolated from cases of PWD (Vu-Khac et al. 2007; Zhang et al. 2007). In this study, we obtained 100 *E. coli* strains from the diarrhoeic specimens, and found that 41 of them (41%) carried the F18 gene (*fedA*). This prevalence is higher than that found in China (3.25%) (Yang et al. 2019) and South Korea (15%) (Kim et al. 2010), but lower than that in Malaysia (71.4%; 5/7) (Ho et al. 2013). However, it might be expected that F18-targeting treatments against PWD would be more effective in Asia.

In conclusion, it could be stated that the PWD of weaned piglets caused by ETEC occurs worldwide. The *E. coli* strains recovered from healthy and diarrhoeic piglets were analysed and identified. We searched for the presence of gene encoding F18 fimbrial adhesion molecules, and five virulent factor genes namely *eltAB*, *estA*, *estB*, *astA*, and *stx2e*. We also assessed the secretion of the enterotoxins LT and ST. From a comparison of the strains obtained from healthy and diarrhoeic piglets, a statistical analysis of the diarrhoeic piglets indicated that the prevalent virulent factors for PWD was a *fedA*-positive ETEC strain secreting LT. This statistical assessment enables us to search for an effective vaccine or drug treatment for controlling PWD on pig farms.

Conflict of interest

The authors declare no conflict of interest.

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