

# Adhesion phenotypes of pigs of Chinese and Western breeds and a White Duroc-Erhualian crossbreed with regard to susceptibility to enterotoxigenic *Escherichia coli* with fimbrial adhesins K99, 987P, and F41

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**Objective**—To investigate adhesion phenotypes of pigs of Chinese and Western breeds and a specific crossbreed with regard to enterotoxigenic *Escherichia coli* (ETEC) with fimbrial adhesins K99 (F5), 987P (F6), and F41 (F7).

**Animals**—Purebred 6- to 8-week-old pigs of 3 Western breeds introduced into China (n = 144) and 12 Chinese breeds (148) and 1,330 adult White Duroc-Erhualian crossbred pigs.

**Procedures**—Brush border preparations were prepared from jejunal specimens collected from each pig following euthanasia. Each preparation was incubated with ETEC strains that had fimbrial adhesins K99, 987P, or F41; an ETEC K88<sup>-</sup> strain was used as a negative control sample. The mean number of brush border-bound bacteria in aliquots of the bacteria-brush border suspensions (determined via phase-contrast microscopy) was used to determine each pig's adhesion phenotype for ETEC K99, 987P, and F41 strains; the phenotype was classified as adhesive (susceptible) if  $\geq 10\%$  of examined brush borders bound  $> 2$  bacteria.

**Results**—Most purebred and crossbred pigs had nonadhesive phenotypes with regard to ETEC K99 and 987P strains. For the F41 strain, 34.9% and 65.1% of all purebred pigs had adhesive and nonadhesive phenotypes, respectively; among crossbred pigs, these values were 39.2% and 60.8%, respectively. The percentage of pigs with the F41 adhesive phenotype was higher among Western breeds than it was among Chinese breeds (38.9% vs 31.1%).

**Conclusions and Clinical Relevance**—Results suggested that the ETEC F41 strain, but not the K99 or 987P strain, might be a cause of diarrhea in 6- to 8-week-old pigs in China. (*Am J Vet Res* 2011;72:80–84)

Enterotoxigenic *Escherichia coli* are an important and global cause of diarrhea in neonatal and weaned pigs.<sup>1</sup> Strains of ETEC depend on 2 main types of virulence factors (adhesins and enterotoxins) to cause enteric colibacillosis in pigs.<sup>2</sup> The essential step in the pathological mechanism of ETEC is the interaction of the bacterial fimbriae with receptors on the small intestinal epithelium, which allows ETEC to colonize

ABBREVIATION	
ETEC	Enterotoxigenic <i>Escherichia coli</i>

the mucosal surface of the small intestine. After colonization, ETEC strains produce enterotoxin (typically heat-stable [STa or STb] or heat-labile proteins or peptides).<sup>3</sup> These toxins cause diarrhea in affected animals by changing the water-electrolyte balance of the small intestine.<sup>3,4</sup> The most common fimbrial adhesins produced by porcine ETEC strains include F4 (K88), F5 (K99), F6 (987P), F7 (F41), and F18.<sup>3</sup>

Susceptibility or resistance of pigs to ETEC strains is correlated with whether the brush borders of the small intestinal epithelium are capable of bacterial binding. The adhesion of the bacteria to brush border vesicles can be observed via phase-contrast microscopy. By use of an in vitro microscopic brush border adhesion assay involving ETEC K88 bacteria, Sellwood et al<sup>5</sup> initially described 2 pig phenotypes (termed adhesive and nonadhesive) and showed that the 2 phenotypes were genetically determined. Cox and Houvenaghel<sup>6</sup> com-

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pared the adhesion of ETEC strains that had fimbrial adhesins K88, K99, 987P, or F41 and determined that the number of bacteria adhering to villi was highest for K88ab and K88ac strains, followed by (in decreasing order) K88ad, 987P, K99/F41, K99, and F41 strains. The intestinal protein receptors for 987P are histone H1 proteins<sup>7</sup>; the receptors for K88, K99, or F41 fimbriae remain undetermined.

In China, there are at least 72 pig populations that have been classified into 6 ecotypes—North China type, South China type, Central China type, Southwest China type, Lower Yangtze River Basin type, and Plateau type—according to their appearance, geological location, and performance.<sup>8</sup> As in other countries, colibacillosis is the most common enteric disease of neonatal pigs in China. It has been reported that pigs of Chinese Minzhu and Fengjing breeds are mainly resistant to ETEC F4ac<sup>9,10</sup> and that Chinese Meishan pigs have predominant resistance to K88 strains.<sup>11</sup> We have previously reported the distribution of ETEC F4 (K88) adhesion phenotypes in pigs of 12 Chinese and 3 Western breeds.<sup>12</sup> However, the prevalence of susceptibility to other ETEC strains including K99, 987P, and F41 in indigenous Chinese pig breeds remains unknown. The purpose of the study reported here was to investigate the adhesion phenotypes of 12 indigenous Chinese pig breeds (representing 6 ecotypes), 3 Western commercial pig breeds, and a White Duroc–Erhualian crossbreed (F<sub>2</sub> and F<sub>3</sub> individuals) with regard to susceptibility to *E. coli* with fimbrial adhesins K99, 987P, and F41.

## Materials and Methods

**Animals**—Experimental animals purchased for this study included 292 purebred pigs (age range, 6 to 8 weeks old) and 1,330 adult crossbred pigs (age range, 237 to 243 days old). The 292 purebred pigs represented 12 Chinese indigenous breeds and 3 Western commercial breeds. The Chinese indigenous breeds included Bama Xiang, Erhualian, Hang, Laiwu, Lantang, Jiangquhai, Jinhua, Rongchang, Shaziling, Tongcheng, Tibet, and Yushan Black breeds. The Western commercial breeds included Duroc, Landrace, and Large White breeds. These pigs had been used in another investigation of F4 adhesion phenotypes performed by our group.<sup>12</sup> The purebred pigs were selected from at least 3 unrelated sire families (ie, there was no common ancestry for 3 generations) in each breed except for the Lantang breed, from which only 1 sire family was used in the study. The 1,330 hybrid pigs included 883 F<sub>2</sub> individuals and 447 F<sub>3</sub> individuals, which were from a 4-generation White Duroc × Erhualian intercross resource population. Briefly, the intercross was developed from 2 White Duroc boars and 17 Chinese Erhualian sows. Nine F<sub>1</sub> sires and 59 F<sub>1</sub> dams were interbred to generate F<sub>2</sub> animals through 6 batches, from which 62 F<sub>2</sub> boars and 149 F<sub>2</sub> sows were selected to produce F<sub>3</sub> animals. The crossbred pigs had been used in another investigation of F4 adhesion phenotypes<sup>12</sup> and a linkage mapping study<sup>13</sup> performed by our group. All adult F<sub>2</sub> and F<sub>3</sub> pigs and young purebred pigs were euthanized for isolation of brush borders of the small intestines following the Guide of the Care and Use of Experimental Animals established by the Ministry of Agriculture of China.

**Bacteria**—The ETEC strains C83524 (O139:K+:K88<sup>-</sup>), C83608 (O141:K99), C83694 (O20:987P), and C83707 (O101:K30:F41) were obtained from the National Center of Veterinary Culture Collection, China Institute of Veterinary Drug Control.<sup>a</sup> Strain C83524 was used as a negative control. Before performing each assay, expression of specific fimbriae by the ETEC strains was confirmed by use of an agglutination assay, as described previously.<sup>14</sup> Bacteria were then cultured in broth culture medium,<sup>b</sup> after which the bacteria were allowed to grow in trypticase soy broth medium<sup>c</sup> for 18 hours at 37°C. Bacterial cells were suspended in PBS solution (15mM KH<sub>2</sub>PO<sub>4</sub>, 8mM Na<sub>2</sub>HPO<sub>4</sub>, 137mM NaCl, and 2.6mM KCl; adjusted to pH 7.4 with NaOH); the solution was diluted to achieve an optical density of approximately 1.0 at 560 nm for the microscopic brush border adhesion assay.

**Brush border adhesion assay**—The *in vitro* microscopic brush border adhesion test was performed, as described previously.<sup>15,16</sup> Briefly, a 2-cm-long segment of the jejunum was harvested from the small intestine of each pig within 30 minutes after death. The specimen of jejunum was washed free of contents and then was immersed in EDTA solution for 20 minutes. Epithelial cells were removed by scraping the mucosal surface of the jejunum and subsequently immersed in EDTA solution for 30 minutes. After that, enterocytes were homogenized with a T18 basic tissue grinder<sup>d</sup> at 13,000 × g for 20 seconds and then filtered. The filtrate was centrifuged at 3,600 × g for 10 minutes to pellet brush borders, which were then suspended in 50 mL of PBS solution and centrifuged again. Brush border pellets were suspended again in PBS solution with 100 μL of gentamicin sulfate and sodium azide; preparations were adjusted to a final concentration of 1 × 10<sup>6</sup> cells/mL and stored at 4°C. Each brush border preparation was tested for adhesion of ETEC K99, 987P, F41, and K88<sup>-</sup> strains. For each assay, equal volumes (100 μL) of bacterial suspension and brush border preparation were incubated with 50 μL of mannose (0.4 mg/mL) at 37°C for 30 minutes with gentle shaking. A drop of the bacteria–brush border suspension was then placed on a glass slide and examined via phase-contrast microscopy.<sup>e</sup> For each bacteria–brush border combination, 20 well-separated brush borders or brush border vesicles were examined and the number of bacteria bound to each brush border was recorded. In instances in which < 4 brush borders bound > 2 bacteria, 20 additional brush borders were examined. According to criteria proposed by Baker et al,<sup>16</sup> the phenotype of the pig from which the specimen was derived was classified as adhesive (susceptible) to ETEC strains if ≥ 10% of brush borders bound > 2 bacteria; otherwise, the phenotype of the pig from which the specimen was derived was classified as nonadhesive.

## Results

The ETEC adhesion phenotypes in the 15 purebred (outbred) populations and the F<sub>2</sub> and F<sub>3</sub> individuals from the White Duroc × Erhualian intercross were identified (Table 1). In the *in vitro* agglutination test, the negative control strain (K88<sup>-</sup>) did not adhere

Table 1—Adhesion phenotypes (adhesive [+] or nonadhesive [-]) with respect to ETEC K99, 987P, and F41 strains in 292 purebred pigs (6 to 8 weeks old) of 12 indigenous Chinese breeds and 3 commercial Western breeds and 1,330 adult White Duroc-Erhualian crossbred pigs.

Breed (breed ecotype)	Location	Total No. of pigs	No. of sire families	K99		987P		F41	
				+	-	+	-	+	-
Western									
Large White	Jiangxi, Guangdong, Hunan, and Zhejiang provinces	66	21	0	66	1 (3.5 ± 0.69)	65	20 (4.6 ± 2.90)	46
Duroc	Jiangxi, Jiangsu, Guangdong, Hunan, and Zhejiang provinces	46	18	0	46	0	46	26 (6.1 ± 3.27)	20
Landrace	Guangdong, Hunan, and Zhejiang provinces	32	8	0	32	0	32	10 (4.2 ± 2.74)	22
Chinese									
Erhualian (Lower Yangtze River Basin type)	Wuxi city, Jiangsu province	14	5	0	14	0	14	3 (4.0 ± 1.38)	11
Jiangquhai (Lower Yangtze River Basin type)	Taizhou city, Jiangsu province	12	3	0	12	0	12	5 (4.9 ± 2.11)	7
Jinhua (Central China type)	Jinhua city, Zhejiang province	11	4	0	11	1 (2.0 ± 1.23)	10	8 (7.1 ± 2.95)	3
Shaziling (Central China type)	Xiangtan city, Hunan province	8	4	0	8	0	8	4 (3.9 ± 2.25)	4
Yushan Black (Central China type)	Yushan county, Jiangxi province	24	6	0	24	0	24	7 (6.6 ± 3.38)	17
Hang (Central China type)	Xiushui county, Jiangxi province	10	3	0	10	1 (1.9 ± 1.33)	9	4 (5.6 ± 1.81)	6
Tongcheng (Central China type)	Tongcheng county, Hubei province	10	3	0	10	0	10	5 (5.1 ± 2.51)	5
Lantang (South China type)	Xinfeng county, Guangdong province	5	1	0	5	0	5	0	5
Rongchang (Southwest China type)	Rongchang county, Chongqing City	13	4	0	13	0	13	2 (3.1 ± 1.79)	11
Bama Xiang (South China type)	Bama county, Guangxi autonomous region	16	6	0	16	1 (1.9 ± 1.17)	15	6 (5.3 ± 2.52)	10
Laiwu (North China type)	Laiwu city, Shandong province	13	4	0	13	0	13	2 (4.75 ± 1.46)	11
Tibet (Plateau type)	Tibet autonomous region	12	10	0	12	0	12	0	12
Crossbreed F <sub>2</sub> and F <sub>3</sub> pigs	White Duroc × Erhualian intercross	1,330	NA	17 (2.3 ± 0.72)	1,294	125 (2.6 ± 1.35)	1,157	500 (5.8 ± 2.28)	775

For each ETEC strain, the number of pigs that had each phenotype is reported. For pigs with the adhesive phenotype, data in parentheses represent the mean ± SD number of bacterial cells per isolated brush border segment.  
 NA = Not applicable.  
 Adhesive phenotypes were not successfully recorded for some samples in F<sub>2</sub> and F<sub>3</sub> pigs.

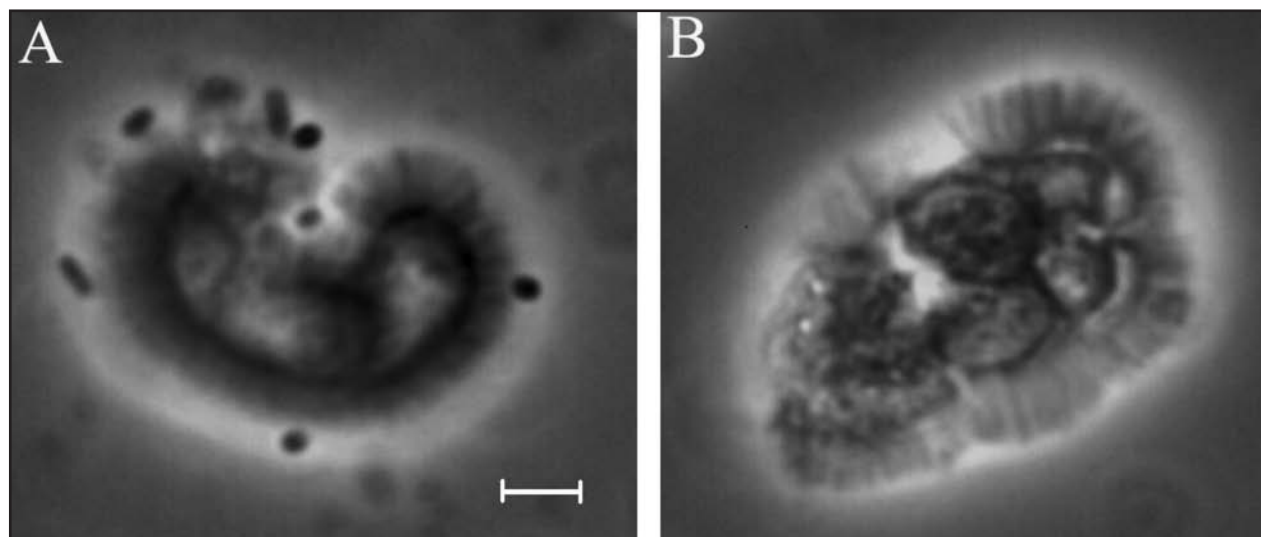


Figure 1—Photomicrographs to illustrate the ETEC adhesion patterns observed via phase-contrast microscopy in brush border preparations derived from pigs that had the adhesive (A) or nonadhesive (B) phenotype for a given ETEC strain. A preparation of the brush borders of jejunal epithelium was obtained from each pig, and aliquots were incubated separately with 4 bacterial suspensions (ETEC K99, 987P, F41, or K88<sup>-</sup> [negative control sample]). Samples were examined microscopically, and the pig was classified as having the adhesive phenotype (susceptible) when at least 10% of the brush border segments examined had bound > 2 bacteria; otherwise, the pig was classified as having the nonadhesive phenotype. In these images, a brush border segment from each of 2 White Duroc-Erhualian crossbred pigs following incubation with ETEC F41 is displayed; 7 bacteria are bound to the brush border segment in panel A. Scale bar = 5 μm.

to small intestinal epithelium brush borders obtained from any of the study pigs (data not shown). For each ETEC strain except the K99 strain, 2 adhesion phenotypes (adhesive and nonadhesive) were detected in the various purebred pigs and the crossbred pigs, especially in the adult F<sub>2</sub> and F<sub>3</sub> animals (Figure 1). For most of the pigs with the adhesive phenotype, the brush borders bound > 2 bacteria.

The brush borders from all purebred pigs and most (1,294/1,311 [98.7%]) of the crossbred adult pigs were nonadhesive for K99 bacteria. Most pigs were also re-

sistant to adhesion of the ETEC 987P strain (Table 1); however, 38.9% (56/144) of the purebred Western pigs and 31.1% (46/148; range, 0/12 to 8/11 [0% to 73%] depending on the breed) of indigenous Chinese pigs had adhesive phenotypes with regard to F41 fimbriae.

## Discussion

It is known that the fimbriae of ETEC strains interact with receptors on the small intestinal epithelium to cause disease,<sup>1,3</sup> but results of a microscopic adhe-

sion assay indicated that there was no difference in adhesion intensity for the 987P and F41 strains among different small intestinal segments in pigs.<sup>6</sup> However, Duchet-Suchaux et al<sup>11</sup> reported that challenge-exposure strains that have K99, 987P, or F41 fimbriae more intensively colonize the jejunum in pigs. Thus, in the present study, we selected the jejunum as the source of brush border preparations for the *in vitro* adhesion assay to investigate susceptibility of pigs to ETEC strains of interest.

The K99 strain mainly causes diarrhea in neonatal pigs within 15 days after birth.<sup>17</sup> The resistance to adhesion by K99 fimbriae might be age related because the expression of K99 receptors gradually decreases with increasing host age.<sup>18</sup> All of the pigs in the present study were > 40 days old, which may be the main reason why most of the animals had resistance to K99-mediated adhesion. The age of the pigs may also be the reason that our findings were inconsistent with earlier observations, which indicated that adhesions of ETEC F41 strains were found mostly in association with adhesions of ETEC K99 strains<sup>4</sup>; in that previous study, the pigs were 14 to 28 days old.

It has been reported<sup>19–23</sup> that ETEC 987P isolates mainly cause diarrhea in neonatal (< 6-day-old) pigs and are not the predominant fimbrial strains associated with diarrhea in weaned pigs. Thus, it was not unexpected that most of the pigs in the present study had the nonadhesive 987P phenotype because of their age (> 40 days old). Interestingly, Dean-Nystrom et al<sup>24</sup> and Dean-Nystrom and Samuel<sup>25</sup> reported that pigs that are ≥ 6 days old become resistant to ETEC 987P, although that strain will adhere to purified brush border preparations from those pigs. The age-related resistance to ETEC 987P resulted from overexpression of the glycolipid receptors for 987P; free receptors were shed into the intestinal lumen and covered the 987P fimbriae, thereby blocking adhesion.<sup>25</sup>

In the present study, the adhesive phenotype for the ETEC F41 strain was evident in 38.9% (56/144) of the purebred Western pigs, 31.1% (46/148) of the purebred indigenous Chinese pigs, and 39.2% (500/1,275) of the F<sub>2</sub> and F<sub>3</sub> adult crossbred animals. Among the indigenous Chinese pig breeds, Tibet pigs were highly resistant to ETEC F41 strains. In the remaining purebreds (Bama Xiang, Erhualian, Jinhua, Jiangquhai, Shaziling, Yushan Black, Rongchang, Laiwu, Hang, and Tongcheng breeds and the 3 Western commercial breeds [Duroc, Large White, and Landrace]), pigs that were resistant to the F41 strain and pigs that were susceptible to the F41 strain were identified. Although K88 fimbriae are the main colonization virulence factors of ETEC that cause neonatal diarrhea and postweaning diarrhea, F41 fimbriae have also been detected in some diarrhea-causing strains.<sup>19,26</sup> High prevalence of the F41 strain has been detected in pigs with diarrhea, especially in Sweden,<sup>27</sup> Canada,<sup>28</sup> and Spain.<sup>17</sup> To date, it is not known whether the presence of the receptor for F41 is genetically determined, as is the receptor for F4ab/ac. In the population of crossbred pigs used in the present study, we have previously mapped a significant quantitative trait locus for susceptibility to ETEC F41 on porcine chromosome 4 (SSC4) by use of a genome scan,<sup>29</sup> shedding light on the genetic basis of the F41 receptor.

To the authors' knowledge, there are few data regarding the relative susceptibility of different pig breeds to ETEC strains K99, 987P, and F41, except for results of a study by Duchet-Suchaux et al<sup>11</sup> of the susceptibility of Chinese Meishan and European Large White pigs to ETEC strains. In the present study, Large White pigs were more susceptible (adhesive phenotype) to ETEC F41, compared with susceptibility of Erhualian pigs. This observation was inconsistent with the finding of a study<sup>11</sup> in which Large White pigs and Meishan pigs (a consanguineous population of Erhualian pigs<sup>8</sup>) had similar susceptibility to an ETEC strain expressing F41. Furthermore, although indigenous Chinese pigs and Western commercial pigs had similar resistance (nonadhesive phenotype) to 987P fimbriae in the present study, Western pigs were more susceptible to the ETEC F41 strain than were some of the indigenous Chinese pig breeds, such as Rongchang, Laiwu, and Tibet pigs.

In the present study, most pigs had the nonadhesive phenotype with regard to the ETEC 987P strain, but 38.9% of purebred Western pigs and 0% to 72% of Chinese indigenous pigs had the adhesive phenotype with regard to F41 fimbriae. Our earlier observations in these pig populations indicated that 52.0% (77/148) of pigs of the indigenous Chinese breeds were susceptible to the K88 bacteria.<sup>12</sup> In addition, Cheng et al<sup>30</sup> reported that the detection rate of F18 fimbriae was 26.25% among 240 clinical *E. coli* isolates associated with postweaning diarrhea in pigs from different swine farms in the Jiangsu Province of China. These results suggested that in China, ETEC F41, K88, and F18, but not K99 or 987P, might be able to cause diarrhea in pigs that are 6 to 8 weeks old.

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  - b. Broth culture medium, Sigma-Aldrich Corp, Shanghai, China.
  - c. Trypticase soy broth medium, Sigma-Aldrich Corp, Shanghai, China.
  - d. T18 basic tissue grinder, IKA, Staufen, Germany.
  - e. Phase-contrast microscope, Leica, Wetzlar, Germany.
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