



Prevalence of Fimbrial Antigen (K88 variants, K99 and 987P) of Enterotoxigenic *Escherichia Coli* from Neonatal and Post-weaning Piglets with Diarrhea in Central China

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ABSTRACT : Enterotoxigenic *Escherichia coli* is a major cause of diarrhea in neonatal and post-weaning piglets. To determine the most common fimbrial antigens of ETEC in piglets with diarrhea, two investigations were carried out on intensive pig farms in Hubei province, central China. In 2002-2003, 227 fecal samples from neonatal and post-weaning piglets with diarrhea were tested for the presence of the fimbrial antigen K88 and K99 of ETEC by the polymerase chain reaction (PCR). Twenty-three (10.1%) of 227 fecal samples were found to contain fimbrial antigen K88, which was identified as K88ac variant; and 13 (5.7%) samples containing K99. In 2004, another 179 fecal samples from diarrheic piglets, 1 day to 6 weeks of age, were tested for prevalence of fimbrial antigen K88, K99 and 987P. Forty-seven (26.3%) of the 179 samples carried at least one of the ETEC fimbrial antigens. K88 antigen was detected in 20.1%. In the 36 samples known to carry fimbrial antigen K88, 32 (88.9%) contained K88ad; and 4 (11.1%) contained K88ac; none of them carried K88ab. Fimbrial antigens K99 and 987P were detected in 1.1% and 6.1%, respectively. Our data indicate that K88 is the most common fimbrial antigen of ETEC associated with diarrhea in piglets in Central China. (**Key Words :** Enterotoxigenic *Escherichia Coli*, Fimbrial Antigen, PCR, Piglets, Diarrhea)

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is a major cause of diarrhea and death in neonatal and post-weaning piglets (Moon et al., 1986; Evelyn et al., 1988; Alexander, 1994; Hampson, 1994; Garabal et al., 1997). There are at least two established virulence properties: specific fimbriae and enterotoxins. The specific fimbriae enable ETEC to colonize the epithelial cells of the intestine and induce diarrhea by means of enterotoxins (Nataro and Kaper, 1998). Most ETEC strains isolated from diarrheic piglets possess K88 (F4), K99 (F5), 987P (F6), or F41 (Ojeniyi et al., 1994; Kwon et al., 1999). Moreover, K88 exists as three variants designated K88ab, K88ac and K88ad, (Guinee and Jansen, 1979; Broeck et al., 2000).

Antibiotics are used to protect neonatal and post-weaning piglets from diarrhea induced by *E. coli*. Because of increased incidence of antibiotic resistance in microorganisms and the pressure by regulatory agents to

ban or greatly restrict the use of antibiotics in the feed industry, alternative strategies are needed to control this intestinal disease. Using therapeutic antibodies, such as egg yolk antibodies to anti-fimbrial antigens, was reported as one effective alternative approach to control *E. coli*-induced diarrhea (Marquart et al., 1999; Owusu et al., 2003; Hong et al., 2004). Therefore, determination of the target antigen, as the first step for producing the specific therapeutic antibodies against ETEC, is very necessary.

The objective of this study was to investigate the prevalence of the fimbrial antigen of ETEC which causes diarrhea of neonatal and post-weaning piglets in intensive pig farms. This information may provide an important database for further production and application of specific therapeutic antibodies against ETEC.

MATERIALS AND METHODS

Samples

During 2002 and 2003, 227 samples of feces were collected from neonatal or weaned piglets with diarrhea, at several intensive pig farms located in Wuhan, the central

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Table 1. Primers used in PCR of ETEC fimbrial antigen

Gene	Region*	Primer sequences 5'-3'	Anneal temperature (°C)	Reference
K88	U	GGTGATTCAATGGTTTCGGTC	58	Choi and Chae, 1999
	D	AATGCTACGTTTCAGCGGAGCG		
K88ab	D	TGCAGCACCCGAAACAGTCGTCGT	58	
K88ac	D	CCCAGCCGACGATTCAGAACCCT	58	
K88ad	D	TGCAGAAATTCTGAACATTCGTCGG	58	
K99	U	GGTCATGGCACTGTAGTGGGA	60	
	D	TC A TAGATATGCCCGCAATG		
987P	U	CTGCCAGTCTATGCCAAGTG	56	Hua et al., 2002
	D	ACGGTGTACCTGCTGAACGAATAG		

* U: upstream region; D: downstream region.

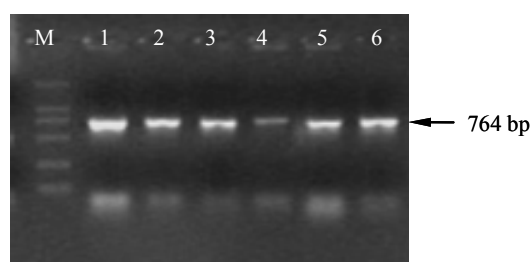


Figure 1. PCR product of K88, tested with reference strains. Lane 1 and 4: K88ab (C83901); Lane 2 and 5: K88ac (C83715); Lane 3 and 6: K88ad (C83923); Lane M: DNA ladder (2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp from the top).

area of Hubei province, central China. These fecal samples were tested for fimbrial antigen K88 variants and K99. In 2004, another 179 fecal samples were collected from diarrheic piglets, ranging in age from 1 day to 6 weeks, at various intensive pig farms located within a range of about 150 km radius around Wuhan, but not in Wuhan itself, in Hubei province. These samples were tested for three fimbrial antigens (K88, K99 and 987P). One sample was isolated from each piglet.

Reference strains

For control purpose the following *E. coli* strains were used: C83901 (O8: K87, K88ab), C83715 (O8: K87 K88ac), C83923 (O8: K87, K88ad), C83529 (K99), C836959 (O9: K103, 987P), which were purchased from the China Institute of Veterinary Drug Control.

Polymerase chain reaction (PCR)

Fecal samples were directly inoculated into tryptic soy broth (TSB) and incubated at 37°C for 18-20 h, centrifuged at 10,000 rpm for 5 min and suspended in distilled water, then heated at 100°C for 5 min and the supernatant of lysed bacteria was collected to perform the PCR. PCR for three K88 variants and 987P was carried out as previously described with slight modification (Changsun Choi and Chanhee Chae, 1999; Hua et al., 2002). Primers of K99 were designed to hybridize with a region of the K99 operon that code for the large structural subunit of the K99 fimbriae.

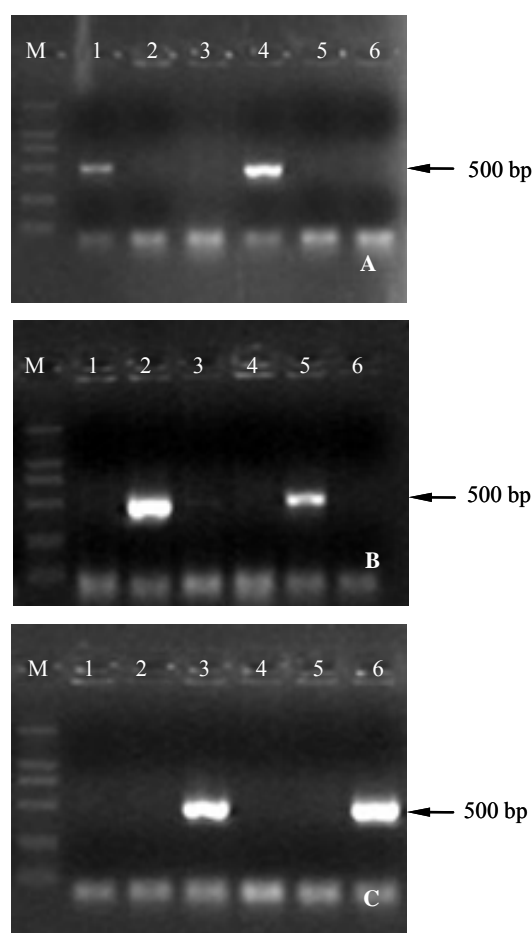


Figure 2. PCR products of K88 variants, K88ab (A), K88ac (B), and K88ad(C), tested with reference strains. Three K88 variants reference strains were tested by PCR with primers specific to K88ab, K88ac and K88ad, corresponding to 500 bp. Lane 1 and 4: K88ab (C83901); Lane 2 and 5: K88ac (C83715); Lane 3 and 6: K88ad (C83923); Lane M: DNA ladder (2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp from the top).

The characteristics of all primers are described in Table 1. Bacterial DNA amplification was performed by adding 1.5 µl of the samples to the PCR mixture consisting of 2.5 µl of dNTP (MBI, 2 mM of each of dATP, dCTP, dGTP, and dTTP, respectively), 2.5 µl of PCR buffer (10-times concentrated, without Mg⁺⁺), 1.5 µl of MgCl₂ (25 mM), 1.0

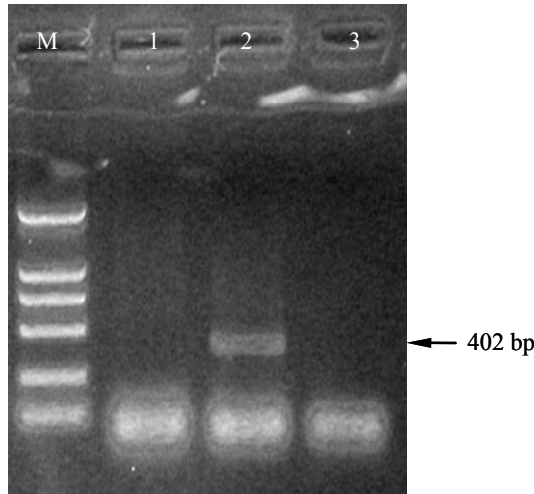


Figure 3. PCR product of K99, tested with reference strains. Lane 1: K88 (C83715); Lane 2: K99 (C83529); Lane 3: 987P (C836959); Lane M: DNA ladder (2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp from the top).

μl of each primer (10 μM), 1.0 U of *Taq* DNA polymerase (MBI), and distilled water to a total volume of 25 μl. The PCR profile used included a denaturing step at 94°C for 30 s, followed by annealing of the primers at corresponding temperature (Table 1) for 30 s, with an extension step at 72°C for 1 min. The 35 cycles were performed in a thermal cycler, followed by extension at 72°C for 10 min. The amplified product was visualized by standard gel electrophoresis of 10 μl of the final reaction mixture on 1.5% agarose gel. Amplified DNA fragments of specific sizes were located by ultraviolet fluorescence after staining with ethidium bromide. Their lengths were verified by a digested DNA marker (Takara) run simultaneously. The PCR reactions were repeated three times, and control DNA from reference strains were included in each reaction.

Statistical analysis

Data in this study were analyzed using the χ^2 test.

RESULTS

PCR products of ETEC reference strains

PCR products of various reference strains corresponded to expected lengths in the large structural subunit of the fimbrial gene operon. Primer for K88 produced a 764 bp

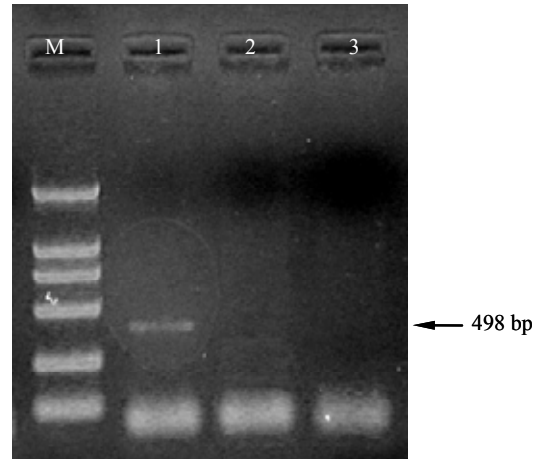


Figure 4. PCR product of 987P, tested with reference strains. Lane 1: 987P (C836959); Lane 2: K99 (C83529); Lane 3: K88 (C83715); Lane M: DNA ladder (2,000 bp, 1,000 bp, 750 bp, 500 bp, 250 bp, 100 bp from the top).

product that was common to all three K88 variants (Figure 1), but when primer specific for each K88 variant was used, a product of about 500 bp was obtained (Figure 2). PCR products of K99 and 987P reference strains corresponded to 402 bp and 498 bp respectively (Figures 3 and 4).

The prevalence of fimbrial antigens K88 and K99 during 2002 and 2003

Twenty-three (10.1%) samples contained ETEC K88 and 13 (5.1%) contained K99 in 227 samples collected during 2002 and 2003 (Table 2). All K88-positive samples carried antigen K88ac, none of *E. coli* strains carried antigen K88ab or K88ad.

The prevalence of fimbrial antigens K88, K99 and 987P in 2004

Fimbrial antigens K88, K99 and 987P were identified in samples isolated from 47 (26.3%) of 179 piglets with diarrhea in 2004 (Table 2). The most frequently detected fimbrial antigen was K88, found in 36 (20.1%) piglets, followed in order by 987P (6.1%) and K99 (1.1%). Among the 36 samples known to carry antigen K88, 32 (88.9%) samples contained antigens for K88ad and 4 (11.1%) samples contained antigens for K88ac, None carried antigens for K88ab. Moreover, in 2 of the 179 piglets, different fimbriated ETEC strains were detected in the same

Table 2. Fimbrial antigens in *E. coli* isolated from piglets with diarrhea

Year	Total No. of piglets	No. of piglets, positive to fimbrial antigens of ETEC						Total
		K88			K99	987P	K88+,987P+	
K88ab	K88ac	K88ad						
2002-2003	227	0	23	0	13	- ^a	-	36
2004	179	0	4	32	2	11	2 ^b	47

^a Not tested.

^b 2 samples positive to both fimbrial antigen K88 and 987P have also counted in No. of piglets positive to fimbrial antigen K88 and 987P respectively.

Table 3. Prevalence of fimbrial antigen K88, K99 and 987P in *E. coli* isolated from piglets with diarrhea at different growth stages^a

Age	No. of piglets	No. of piglets, positive to ETEC strains				No. of piglets with 3P ^{ab} strains/ total No. of piglets
		K88+987P-	K88-987P+	K99+	K88+987P+	
<1 week	81	8	9	1	1	19/81
2-4 weeks	75	15	0	0	1	16/75
4-6 weeks	23	11	0	1	0	12/23
Total	179	34	9	2	2	47/179

^aData for samples isolated in 2004; piglets were weaned at age of 28 days.

^bExpression one or more of K88, K99 or 987P fimbrial antigen.

piglets: both piglets harboured a mixture of K88⁺ and 987P⁺ ETEC strains. The occurrence of co-infections by mixtures of ETEC strains with diverse fimbrial antigens contributes to the complex nature of enteric infections caused by enterotoxigenic *E. coli* (Garabal, 1997).

Incidence rates of ETEC expressing one or more of K88, K99 or 987P fimbrial antigens, were statistically associated with growth stage of piglets ($p < 0.01$). Over half of post-weaning piglets (4-6 weeks) with diarrhea were found to carry fimbrial antigens for ETEC, which was a significantly greater ($p < 0.01$) proportion than in suckling piglets of <4 weeks of age, (Table 3). 987P fimbrial antigen was detected mostly in samples from newborn piglets (<1 week), whereas K88 and K99 were found in both newborn and weaned piglets. In samples from diarrheic piglets older than 4 weeks, K88 fimbrial antigen was detected most frequently.

DISCUSSION

ETEC fimbrial antigen identified by PCR

Classical methods for ETEC fimbrial antigen (K88, K99 and 987P) detection include hemagglutination inhibition (HI) and immunoserology reaction, such as the serum agglutination test (SAT), indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). HI and SAT require sufficient antigen production for success, and are not always reliable in characterizing the fimbrial antigen positive *E. coli* (Mullaney et al., 1991). Other immunoserology methods, such as IFAT and ELISA, allow better detection but still need large amounts of antigen production and the procedure is rather complicated. Investigating fimbrial antigen characteristics of ETEC strains by PCR using specific primers does not require high purity of sample (Franklin et al., 1996). The results indicated that the PCR test was likely to be one of the most effective methods for epidemiological studies of ETEC due to its sensitivity, simplicity and rapidity.

ETEC fimbrial antigen characteristics

To determine the most common fimbrial antigen of ETEC isolated from diarrheic piglets, the prevalence of fimbrial antigens K88, K99 and 987P in fecal samples was tested by PCR. The results indicated that K88 was the most common fimbrial antigen detected in suckling and weaned piglets in this study. This finding agrees with the results

from many other studies (Wilson et al., 1986; Nagy et al., 1990; Osek, 1999), but no detailed information about the prevalence of K88 variants was reported, including in China. In our work, the prevalence of K88 variants during 2002 and 2003 showed that K88ac was the most prevalent variant associated with diarrhea in piglets, while data in 2004 showed that K88ad was the most common being present in two-thirds of diarrheic samples from different areas. It is implied that diverse prevalence of K88 variants may relate to different areas.

987P fimbrial antigen was frequently encountered in ETEC from piglets less than 2 weeks old (Garabal et al., 1997); this observation is in agreement with our results, in which 987P fimbrial antigen was found mostly associated with piglets younger than one week, indicating that ETEC 987P is age-related. Cellular receptors for 987P fimbriae are present in the intestine of younger and older pigs, but swine develop an innate resistance to 987P ETEC by 3 weeks of age (Evelyn et al., 1989). This is because there are some functional glycolipid receptors in the mucus of older pigs that prevent 987P ETEC from colonizing the small intestines (Evelyn et al., 1994). In contrast, K88 receptors in the brush border are independent of age, so ETEC that produce K88 fimbriae are commonly associated with diarrhea in piglets of both ages (Broeck, et al., 2000; Jin and Zhao, 2000).

The three fimbrial antigens (K88, K99 and 987P) of ETEC were found more frequently in *E. coli* isolated from post-weaning diarrheic piglets (4-6 weeks) than from neonatal piglets (<1 week). This indicated that post-weaning piglets were more easily infected with ETEC and may be associated with the fact that, under commercial conditions, early weaning may involve complex changes which bring much more stress to piglets (Spreeuwenberg et al., 2001).

The present study suggested that K88 should be the major fimbrial antigen, and 987P also should be taken into consideration, when formulating a strategy to protect neonatal piglets. As regards post-weaning piglets, all-round management should be applied.

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