

FREQUENCY OF VIRULENCE GENES IN *ESCHERICHIA COLI* STRAINS ISOLATED FROM PIGLETS WITH DIARRHEA IN THE NORTH PARANA STATE, BRAZIL

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Submitted: February 07, 2008; Returned to authors for corrections: May 09, 2008; Approved: February 25, 2009.

ABSTRACT

Identification of *Escherichia coli* causing porcine postweaning diarrhea requires knowledge regarding the prevalent pathotypes within a given region. A total of 100 *Escherichia coli* isolates from piglets with diarrhea in Londrina city, Parana State, South Brazil, were screened for the presence of genes for F4, F5, F6, F18, F41 fimbrial antigens by specific probes and for enterotoxins (STa, STb, LT and STx2e) by polymerase chain reaction (PCR). The results showed that 60% of the isolates were positive for one or more of the fimbrial antigens and 92% were positive at least for one of the virulence factors examined. Virulence factor genes detected were F4 (44%), F18 (38%), F5 (30%), F41 (32%), F6 (25%), LTP-I (71%), STa (40%), STb (47%) and STx2e (3%). Twenty four patterns of virulence factor according to the different virulence genes form were found and the most frequent virulence gene pattern was F4, F18, F41, STa, STb and LT. Most of the isolates that carried genes for adhesins also harboured genes for toxins.

Key words: porcine *Escherichia coli*, virulence genes, postweaning diarrhea, toxins.

INTRODUCTION

Postweaning diarrhea (PWD) in pigs is a disease of considerable economic importance and is characterized by watery diarrhea, dehydration, loss of body weight and sometimes death of infected pigs (17,20). Enterotoxigenic *E. coli* (ETEC) is an important cause of PWD, and its pathogenicity involves adherence of the pathogen to the small intestine by means of specific adhesion factors (fimbriae) and production of one or several exotoxins responsible for the disease. ETEC produce heat-stable (STa or STb) and/ or heat-labile (LT) enterotoxins that cause secretion of fluid and electrolytes, and STx2 that interfere with protein synthesis (17).

In ETEC strains, the fimbrial types F4 (K88) and F18 (F18ab, F18ac) are commonly found in pathogenic *E. coli* isolated from weaned pigs (4,18,25). There are also other fimbrial structures, such as F5 (K99), F6 (987P), F41, which are associated with

porcine ETEC strains, but are more frequently associated with *E. coli* causing neonatal diarrhea (18,24).

In Brazil, there is little information on the prevalence of virulence factors of porcine *E. coli*. Some studies with *E. coli* isolated from piglets with diarrhea have been showed different frequencies of the F4, F5, F6, F18 and F41 fimbriae and toxins in the São Paulo, Minas Gerais and Santa Catarina States (6,14,15).

The objective of this study was to evaluate the presence and distribution of virulence genes associated with *E. coli* strains isolated from piglets with diarrhea in Paraná State, Brazil

MATERIAL AND METHODS

Bacterial strains

Three hundred *Escherichia coli* strains were isolated from 100 piglets with diarrhea (3 strains/piglets) from four farms in the region of Londrina, Parana State, South Brazil. Samples were

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collected with swabs and transported in Stuart modified medium at 4°C to the laboratory. The samples were streaked on sheep blood agar and Mac Conkey agar and kept at 37°C for 48 hours. Putative *E. coli* colonies were identified by morphology and biochemical characteristics, including indole production, citrate utilization, glucose and lactose fermentation, hydrogen sulfate production, and β hemolysis. All isolates were stored at -20°C in Luria-Bertani (LB) broth to which 15% glycerol was added after growth, and they were tested for the presence of virulence factors.

The following reference of *E. coli* strains were included S02 (F4, LT) (21), B41 (F5, F41, STa) (1), S33 (F6), S25 (F18) (positive wild type isolate by specific antibody), *E. coli* ATCC 43889 (STx2e) and HB 101 (3).

Polymerase chain reaction assay (PCR)

PCR was used to detect the toxin genes and to prepare the chemiluminescent digoxigenin-labelled probes for fimbrial genes. The base sequences for specific oligonucleotide primers used in this study and the predicted sizes of amplified products are shown in Table 1.

Bacterial DNA to be amplified was released from whole organisms by boiling, and PCR was carried out in a total volume of 25 μ l containing 5 μ l of template DNA, 20 pmol of each primer, 200 μ M of dNTPs, 1x PCR buffer and 1.5 U of Taq DNA polymerase. PCR amplifications consisted of 30 cycles at 94°C

for 1 min, annealing temperature specific for each primer for 1 min (Table 1) and 72°C for 2 min in a Thermal Cycler (Gene Amp PCR System 9700/Perkin Elmer Corporation, Norwal CT, USA). The amplified DNA was visualized in 1.5% agarose gels stained with ethidium bromide. The 100-pb ladder (Promega, Madison, WI) was used as standard.

Detection of fimbrial and toxin genes by probe

Chemiluminescent digoxigenin (Boehringer Mannheim Biochemicals) labeled probes for fimbrial genes were prepared by PCR as described above using primers for each factor (Table 1). In order to promote colony hybridization, the colonies were transferred onto Whatman 541 filter paper, lysed, and the DNA was fixed as previously described (12). The filters were incubated for 1 h in the following prehybridization solution: 5 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS, 0.02% laurylsarcosine and blocking solution (Boehringer Mannheim Biochemicals). The probe was denatured by boiling for 10 min and was added to the prehybridization mixture, which was incubated overnight at 63°C. The filters were washed twice in 2 x SSC plus 0.1% SDS for 15 min, and in 1 x SSC plus 0.1% SDS for 30 min at 37°C. The digoxigenin-labeled probe was identified by means of alkaline phosphatase (AP)-conjugated anti-digoxigenin and color development reagent CDP-star (Amersham, Biosciences, England, UK).

Table 1. Oligonucleotide sequences, annealing temperature and predicted size of amplification products for different virulence-associated genes.

Target Gene	Oligonucleotide sequences (5' - 3')	Size fragment (bp)	Annealing Temperature (°C)	Reference
F4	GAA TCT GTC CGA GAA TAT CA GGT ACA GGT CTT AAT GG	505	53	(2)
F5	AAT ACT TGT TCA GGG AGAAA AAC TTT GTG GTT AAC TTC CT	230	50	(2)
F6	GTA ACTCCACCGTTT GTATC AAGTTACTGCCAGTCTATGC	409	53	(2)
F18	ATGCGTTTAAAATATATCTTG CTG TAT CTC GAAAAC AAT GG	900	50	This study DQ995282
F41	AGT ATC TGG TTC AGT GAT GG CCA CTA TAA GAG GTT GAA GC	612	53	(18)
Sta	CAA CTG AAT CAC TTG ACT CTT TTAATAACA TCC AGC ACA GG	158	50	(2)
Stb	TGC CTA TGC ATC TAC ACAAT CTC CAG CAG TAC CAT CTC TA	113	50	(2)
LTp-I	GGC GTT ACT ATC CTC TCT AT TGG TCT CGG TCA GAT ATG T	272	50	(26)
Stx2e	AAT AGT ATAGGG ACA GCG AT TCT GAC ATT CTG GTT GAC TC	733	50	(2)

RESULTS

Of 100 isolates examined, ninety two (92%) were positive at least for one of the virulence factors, and 60% presented one or more fimbrial genes, using probes for specific adhesions. The fimbrial genes for F4, F5, F6, F18 and F41 were detected in 44%, 30%, 25%, 38% and 32%, respectively (Table 2). Forty isolates (40%) did not harbor any of the colonization factor genes investigated (Table 3).

PCR analysis detected the gene for LTp-I in 71 strains (71%), which was the most prevalent gene for toxins, while the genes for STa, STb, STx2e were found in 44%, 47% and 3% of the isolates (Table 2). Forty eight (48%) isolates harboured more than one enterotoxin gene, and the most prevalent combination was STa/STb/LT, which was detected in 19 isolates. Only nine isolates did not harbour any of the toxin genes investigated (Table 3).

Table 2. Frequency of individual virulence genes from *E. coli* strains isolated from piglets with diarrhea.

Virulence factor	Frequency (%)
F4 (K88)	44
F5 (K99)	30
F6 (987P)	25
F41	32
F18	38
STa	40
STb	47
LTp-I	71
STx2e	3
none	8

Table 3. Distribution of genes for fimbrial and enterotoxins among *E. coli* strains isolated from diarrheic piglets.

Adhesins	N° of strains	STa	STb	LT	STa STb	STa LT	STb LT	STb STx2e	STa STb LT	None toxin
F4	4			2			2			
F18	2		1	1						
F5	1			1						
F6	2					2				
F41	2						1		1	
F4 + F5	3			3						
F4 + F18	1		1							
F4 + F41	1			1						
F5 + F6	1				1					
F5 + F18	2							2		
F6 + F18	2						2			
F18, F41	1	1								
F4+F5+F6	3			1		1			1	
F4+F6+F18	2			1	1					
F4+F6+F41	2	1	1							
F4, F18, F41	8	1			1	1			5	
F4+F6+F18	2					1			1	
F5+F6+F41	1			1						
F5+F18+F41	2					2				
F4+F5+F6+F18	2								2	
F4+F5+F6+F41	2						1			1
F4+F5+F18+F41	6	2	1						3	
F4+F6+F18+F41	3					1	1	1		
F4+F5+F6+F18+F41	5				1				4	
None	40	1	3	17		3	6		2	8
Total	100	6	9	28	5	11	13	3	19	9

We found 24 types of virulence factor patterns according to the different virulence gene form (Table 3). The combination of most frequent virulence genes was F4, F18, F41, STa, STb and LT. Most of the isolates that carried genes for adhesions also harboured genes for toxins.

DISCUSSION

The presence of ETEC virulence factors, fimbriae and enterotoxins production is a common feature of isolates associated with diarrhoea during the pre-weaning and weaning periods (17). This study shows high prevalence (92%) of genes for fimbriae and enterotoxins in the *E. coli* isolates from diarrheic piglets in the Parana State, and the most prevalent association of these genes was F4⁺ F18⁺ F41⁺ STa⁺ STb⁺ and LT⁺. The frequency of virulence genes in *E. coli* isolates from diarrheic piglets in the Minas Gerais State was of 42% (14).

Several studies have demonstrated variation in fimbriae frequencies, and this variation can be due to the different techniques utilized, number of isolates from each diarrhoeal pig and age of piglets.

A study with *E. coli* isolated from newborn piglets with diarrhea of São Paulo State showed that F18ac was expressed, but not fimbriae F4, F5, F6 or F41 (15). However, the F4 fimbriae are common in ETEC from swine diarrhea, and the prevalence of F4-positive strains in various countries has ranged from 3.7% to 65%. In this work, we found (44%) of F4-positive isolates, in agreement with the high frequency (64.6%) for F4 presented by PCR in the isolates from US (27). A significant proportion (23.9%) of *E. coli* isolated from 2 to 4-week-old piglets with diarrhoea in the Slovak Republic carried the gene for F4 fimbriae (22). Harel *et al.* (1991) (11) found 23.4% F4-positive isolates in Québec, Canada. Frydendahl (2002) (9) detected 44% F4 gene in isolates of *E. coli* from PWD in Denmark. However, a lower prevalence (14.3%) of F4-positive *E. coli* strains was found in the Minas Gerais State, Brazil (14); (9.8%) of F4-positive *E. coli* strains was found in China by agglutination tests (4); 3.75% by PCR (5); and 4.3% in Korea (13).

The fimbrial antigen F18 is one of the most important fimbriae associated with PWD (9,16,25). In this study we found the gene for F18 in 38% of *E. coli* strains examined, whereas 27.5% and 19% of F18-positive *E. coli* strains were found in the Santa Catarina and Minas Gerais States, respectively (6,14). Our findings are in accordance with those of other studies (5,9,23,27).

Several studies in many countries have reported that infection with ETEC carrying the F5, F6 or F41 fimbriae is age-related, occurring in pigs less than 2 weeks of age (10, 17, 18). In this study we detected the genes for F5 (30%), F41 (32%) and F6 (25%), similarly these genes were found in 32%, 9.5% and 14.3% in the Minas Gerais State (14). Martins *et al.* (2000) (15) did not detect F5, F6 and F41 in *E. coli* isolates from newborn piglets with diarrhea in São Paulo State, but used the

microhemagglutination assay and agglutination with specific antisera to detect fimbriae. The detection of F4, F5, F41 and especially F6 fimbriae can be difficult due to the fimbrial antigens expression *in vitro*. In other countries, the percentage of these fimbriae was low. Genes for the F5 (K99), F6 (987P) and F41 fimbriae were detected in 2%, 6% and 3.3% of the *E. coli* isolates, respectively, in Korea (13). The frequencies of genes for F5, F6 and F41 fimbriae were 0.9%, 5% and 0.9%, respectively, in the *E. coli* isolates from pigs with PWD in Slovakia (23). The prevalence of F6 fimbriae, in isolates of *E. coli* in China, was 15.6% (4).

In this study, the results of the PCR analysis showed that *E. coli* strains isolated from pigs with diarrhoea possessed the genes for LT or/and ST (STa or/and STb) enterotoxins. The gene for LTp-I was the most prevalent (71%) and the STa and STb genes were found in 40% and 47% of isolates. Macedo *et al.* (2007) (14) found 33.3% and 40% for STa and STb. Frydendahl (2002) (9) also found high frequency of the LT gene (61.6%) among swine ETEC, but the gene for STb was the most prevalent (77.6%). Several studies demonstrated that isolates positive for STb were also positive for other toxins, and the most prevalent gene combination was LT-STb (9,11). The presence of various toxins in ETEC can be explained by the presence of conjugative plasmids widely distributed among porcine ETEC strains. The ST genes, *sta* and *stb*, and a tetracycline resistance gene were located on a self conjugative 120-kb plasmid, called pTC (19).

The occurrence of enterotoxins is associated with specific fimbriae in *E. coli* from pigs and several strains produced more than one fimbrial antigen (17). We found 24 types of virulence gene patterns (Table 3), and the F4⁺ F18⁺ F41⁺ STa⁺ STb⁺ and LT⁺ was the most frequent. The presence of various virulence genes together is explained by linkages of resistance and virulence genes on plasmids. Preliminary results in our laboratory confirm this hypothesis, the association of F4 (K88) with conjugative plasmids codifying resistance to tetracycline, chloranfenicol and ampicilin were detected in ETEC strains isolated from piglets with diarrhea in Parana, Brazil (21). Similarly, the *E. coli* isolates associated with PWD in China could be assigned into 20 different virulence factor patterns; F18ab⁺, F4⁺ and/or intimin⁺ ETEC and high-pathogenicity island (HPI)⁺ and/or LEE⁺ *E. coli* are the dominant pathogens of PWD (5). The F4⁺STa⁺STb⁺LT⁺ pathotype was most commonly identified in Ontario and Vietnam (2,7). This pathotype was also the most prevalent in neonatal and 1-3 week old pigs, while the F18⁺STa⁺STb⁺LT⁺ were most prevalent in weaned pigs (8). The F4⁺ LT⁺STb⁺, F4⁺LT⁺STb⁺EAST1⁺ and F18⁺STa⁺STb⁺STx2e⁺ pathotypes were the most prevalent in the US (27).

In this study, only a small number of strains (3%) were stx2e-positive, in contrast of strains isolated in the US that presented 17,4% (27) and of strains isolated in Minas Gerais State, Brazil that presented 21,4% (14).

In conclusion, this study showed the frequency of virulence genes in *E. coli* strains isolated from piglets with diarrhea in the Londrina city, Parana State, South Brazil, and confirms the combination of various virulence genes in ETEC.

ACKNOWLEDGEMENTS

The authors are grateful to Jussevânia Santos Rubbo de Sá for technical assistance. This study was financially supported by CNPq and Fundação Araucária do Paraná.

RESUMO

Frequência de genes de virulência em *Escherichia coli* isolada de suínos com diarréia no norte do Estado do Paraná, Brasil

A identificação de amostras de *Escherichia coli* responsáveis por diarréia pós-desmame em suínos requer conhecimento dos patótipos prevalentes dentro de uma dada região. Cem amostras de *Escherichia coli* isoladas de leitões com diarréia no Estado do Paraná, Brasil, foram testadas para a presença dos genes que codificam antígenos fimbriais F4, F5, F6, F18, F41 e para a produção de enterotoxinas (STa, STb, LT and STx2e), através de sondas e da técnica da PCR (polymerase chain reaction). Os resultados mostraram que 60% dos isolados foram positivos para um ou mais antígenos fimbriais e 92% foram positivos para pelo menos um dos fatores de virulência examinados. Os genes de virulência detectados foram F4 (44%), F18 (38%), F5 (30%), F41 (32%), F6 (25%), LTP-I (71%), STa (40%), STb (47%) e STx2e (3%). Vinte e quatro padrões de virulência, de acordo com as diferentes combinações dos genes de virulência, foram encontrados e o mais prevalente foi F4, F18, F41, STa, STb e LT. A maioria das amostras que carregam genes para adesinas também transportam genes para produção de toxinas.

Palavras chaves: *Escherichia coli*, genes de virulência, diarréia pós-desmame, toxinas

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