Virulence profiles of enterotoxigenic *Escherichia coli* isolated from piglets with post-weaning diarrhea and classification according to fecal consistency[1]

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The aim of this study was to assess the frequency and association of virulence factors of *Escherichia* (*E.*) *coli* isolated from weaned piglets with diarrhea and to correlate it with fecal consistency. A total of 152 rectal swabs were collected from 25-40 day-old piglets with diarrhea, in farms of Southern Brazil. Phenotypical and molecular techniques were used for bacterial isolation, characterization and classification of enterotoxigenic *E. coli* (ETEC) pathotypes. Statistical analysis was carried out to determine the frequency of virulence factors and virotypes, of fimbriae F4, F5, F6, F18, F41 and toxins LT, STa, STb and STx2e. Out of *456 E. coli* isolates, 287 (62.9%) samples showed significant growth of *E. coli.* Among them, 194 (67.6%) samples showed at least one virulence factor, indicating that ETEC is an important etiological agent of diarrhea in weaned piglets. Higher frequencies were found of fimbria F4 and F18 and enterotoxins LT, STa and STb. Significant association was found to F4, LT, STa and STb; between F18 and STa and STx2e; between F5 and LT, STa and STb. The most frequent virotypes were F18-STa, F4-LT-STa-STb, F4-STa, F4-LT-STb and F18-STa-STx2e. Beta-hemolysis was observed in 47.4% of samples and there was significant association between hemolytic samples and virulence factors F4, F18, STa and STx2e. Regarding fecal consistency, there was significant association of liquid feces and F4 fimbria, STa toxin and virotypes F4-STa and F4-F5-LT-STa-STb. Since there was significant association of ETEC and liquid feces in nursery piglets, it is important to prioritize the sampling of liquid feces for the diagnosis etiologic cause of diarrhea.

INDEX TERMS: ETEC, swine, diarrhea, enterotoxigenic, *Escherichia coli,* piglets, virulence factors, fecal consistency.

RESUMO.- [**Perfis de virulência de *Escherichia coli* enterotoxigênica isoladas de leitões desmamados com diarreia do Sul do Brasil e classificação das amostras de acordo com a consistência fecal.**] O objetivo deste estudo foi avaliar a frequência e associação de fatores de virulência de *Escherichia* (*E.*) *coli* isoladas de leitões desmamados com diarreia e correlacioná-la com consistência fecal. Suabes retais foram coletados em leitões com 25-40 dias de idade com sinal clínico de diarreia, em granjas do Sul do Brasil, totalizando 456 amostras. Foram utilizadas técnicas fenotípicas e moleculares para isolamento bacteriano, caracterização e classificação de patotipos de *E. coli* enterotoxigênica (ETEC). A análise estatística foi realizada para determinar a frequência de fatores de virulência e vi-

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rotipos, de fímbrias F4, F5, F6, F18, F41 e toxinas LT, STa, STB e STx2e. Duzentas e oitenta e sete (62,9%) amostras apresentaram crescimento significativo de *E. coli*. Entre os quais, 194 (67,6%) amostras apresentaram pelo menos um fator de virulência, indicando que ETEC é um importante agente etiológico de diarreia em leitões desmamados. As frequências mais elevadas foram encontradas para as fímbrias F4 e F18 e enterotoxinas LT, STa e STb. Associação significativa foi encontrada para F4, LT, STa e STb; entre F18 e STa e STx2e; entre F5 e LT, STa e STb. Os virotipos mais frequentes foram F18-STa, F4-LT-STa-STb, F4-STa, F4-LT-STb e F18-STa-STx2e. Beta-hemólise foi observada em 47,4% das amostras e houve associação significativa entre amostras hemolíticas e fatores de virulência F4, F18, STa e STx2e. Quanto consistência fecal, houve associação significativa de fezes líquidas e fímbria F4, toxina STa e virotipos F4-STa e F4-F5-LT-STa-STb. A associação significativa da ETEC e fezes líquidas em leitões de creche, é importante para priorizar a amostragem de fezes com essa consistência para no diagnóstico etiológico da diarreia.

TERMOS DE INDEXAÇÃO: ETEC, suíno, diarreia, *Escherichia coli* enterotoxigênica, fatores de virulência, consistência fecal.

INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is one of the main causes of diarrhea in pigs and is associated with neonatal colibacillosis and post-weaning colibacillosis (Fairbrother & Gyles 2006), resulting in significant economic losses to pig production (Ewing & Cole 1994, Barcellos et al. 2011). In order to cause diarrhea, *E. coli* must be able to adhere to enterocytes, avoiding elimination through peristalsis (Menin et al. 2008) and to produce enterotoxins (Fairbrother & Gyles 2006). Adhesion is mediated by fimbriae, being F4 (K88), F5 (K99), F6 (P987), F7 (F41) and F18 the most commonly found in pathogenic *E. coli* (Francis, 2004). Among the enterotoxins associated with ETEC strains, the thermolabile (LT) and thermostable (STa, STb) are the most relevant ones (Dubreuil 2008).

In Brazil, the routine diagnostic of colibacillosis in piglets is often accomplished only by *E. coli* isolation, without determining the virulence factors in the isolates. However, to confirm that the isolate is pathogenic, it is essential to identify the set of virulence factors (fimbriae and toxins), and, consequently, classification in virotypes (Nataro & Kaper 1998). The association between hemolysis in blood agar cultures and certain *E. coli* virotypes has been previously described (Frydendahl 2002, Chapman et al. 2006, Do et al. 2005). Therefore, hemolytic *E.coli* strains isolated from diarrheic fecal samples have often been identified as pathogenic. However, this criterion is not enough to characterize enteropathogenicity (Frydendahl 2002), being the virotype determination the best approach.

The detection of genes codifying fimbriae and enterotoxins in *E. coli* isolates by polymerase chain reaction (PCR) has contributed to make a more accurate and straightforward diagnosis of virotypes (Fairbrother 2006). Multiplex PCR improved the diagnosis, allowing the investigation of different virulence factors in the same reaction (Francis 2004), speeding results and reducing genotyping costs (Ruiz-Rueda et al. 2011). Real-time polymerase chain reaction (qPCR) generate results in higher speed, sensitivity and specificity when compared to traditional PCR methods (Elizaquivel et al. 2010). The objective of this study was to investigate the ETEC virotypes associated with post-weaning diarrhea in pig farms from Southern Brazil, using a multiplex qPCR protocol.

MATERIALS AND METHODS

Sampling was performed in thirteen pig farms located from seven municipalities of the following states of southern Brazil: Rio Grande do Sul (Lajeado and Casca), Santa Catarina (Concordia and Videira) and Parana (Cascavel, Marechal Candido Rondon and Toledo). These farms adopted a similar housing, feed formulation and medication managements. Pigs were weaned around the 21 days of age, and housed in cages, with fully slatted metal floor. Pigs between 25 and 40 days of age that presented diarrhea and had not been previously medicated were included in the study. Animals were selected for sampling based on diarrhea staining on the hindquarters; reddened and/or humid perineal area; apathy and dehydration. The presence of liquid feces on the floor, walls or below crates was also assessed. Immediately before sampling, feces consistency was evaluated with the following visual criteria: normal; pasty; creamy and liquid.

Rectal swabs were collected from 12 to 15 piglets in each farm. Immediately after collection, swabs were individually stored in transport medium (Stuart - Larbor Import©), shipped under refrigeration to the laboratory, and processed within 48 hours. Swabs were streaked on Blood agar base (Difco®) enriched with 5% defibrinated sheep blood and MacConkey agar (Difco®), and incubated for 24h at 37°C. Plates showing a significant growth (at least 90% phenotypically similar colonies) were considered positive. From each plate, three typical colonies of *Escherichia coli* (smooth and shiny, with 2-3mm size on Blood agar plates; pink, lactose fermenters on MacConkey agar) were transferred to Blood agar plates for further biochemical confirmation (Oliveira 2000). Based on the hemolytic activity on blood agar plates, colonies were classified as beta-hemolytic (well delimitated hemolysis ring) or non-hemolytic.

Isolates phenotypically confirmed as *E. coli* were submitted to DNA extraction and detection of virulence genes using NewGene Prep, NewGene Preamp and NewGene Amp kits, following manufacturer´s instructions (Simbios Biotecnologia, Canoas, RS).

The NewGene Amp kit consisted of a multiplex qPCR, in which, genes of the nine virulence factors (toxins LT, STa, STb and STx2e; fimbriae F4, F5, F6, F18 and F41) were amplified in three distinct *multiplex* reactions (Multiplex 1, Multiplex 2 and Multiplex 3), according to the manufacturer instructions. Briefly, 2µL of purified DNA was added to 28µL of the amplification mix (Master mix and *Taq* Polimerase), and submitted to cycling conditions of 3 minutes at 95ºC, followed by 45 cycles of denaturation for 15 seconds at 95ºC, annealing for 1 minute at 60ºC. Reactions were performed in the thermocycler StepOnePlus (Applied Biosystems™). The results were analyzed by Software StepOnePlus™ v2.2 (Applied Biosystems™).

The frequency of virulence factors was carried out using the FREQ procedure from SAS (SAS, 2005). The association between fimbriae, toxins, hemolysis and fecal consistency was carried out with the chi square test or Fisher´s test.

RESULTS

Four hundred fifty six *E.coli* isolates were obtained from 152 rectal swabs, from pigs with diarrhea. Two hundred

Table 1. Virotypes of fimbriae and toxin genes detected by Real-time multiplex PCR in *Escherichia coli* isolates from weaned pigs with diarrhea

	F4 % (n)	F5	F18	F4+F5	F4+F18	F4+F5+F18	No fimbriae	TOTAL
LT	0.70 (2)	-	0.35 (1)	-	-	-	-	1.05 (3)
STa	7.32 (21)	-	11.15 (32)	-	0.70 (2)	-	3.14 (9)	22.30 (64)
STb	0.70 (2)	-	-	-	-	-	1.39 (4)	2.09 (6)
LT+STa	0.70 (2)	-	-	-	-	-	0.35 (1)	1.05 (3)
LT+STb	6.27 (18)	-	-	-	-	-	4.18 (12)	10.45 (30)
LT+STa+STb	9.76 (28)	-	-	4.18 (12)	1.05 (3)	0.35 (1)	0.70 (2)	16.03 (46)
STa+STb	-	-	0.35 (1)	-	-	-	2.09 (6)	2.44 (7)
STa+STx2e	1.05 (3)	1,05 (3)	4.53 (13)	-	-	-	-	6.62 (19)
STa+STb +STx2e	-	-	0.35 (1)	-	-	-	-	0.35 (1)
No toxins	5.23 (15)	-	-	-	-	-	32.40 (93)	37.63 (108)
TOTAL	31.71 (91)	1,05 (3)	16.72 (48)	4.18 (12)	1.74 (5)	0.35 (1)	44.25 (127)	100.0 (287)

and eighty seven isolated showed a significant bacterial growth (62.9%). One hundred and nighty isolates (41.7%) were obtained from liquid feces, 188 (41.2%) from creamy feces, and 78 (17.1 %) from pasty feces. *Escherichia coli* has been not isolated (169) or nor simultaneously fimbriae and toxin genes (93) were detected in 262 (57.4%) strains of the pigs feces.

Using qPCR kit, we detected no target genes for any fimbriae or toxins in 93 (32.4%) *E. coli* isolates, while only genes for toxin production were detected in 34 (11.8%) isolates and only genes for fimbriae were present in 15 (5.2%) *isolates*. Among the genes codifying fimbriae, F4 (n=109; 38.0%) was the most prevalent, followed by F18 (n=54; 18.8%) and F5 (n=16; 5.6%). Genes for fimbriae F6 and F41 were not detected. Among the investigated toxin genes, STa (n=140; 48.8%) was the most frequent, followed by STb (n=90; 31.4%), LT (n=82; 28.6%) and STx2e (n=20; 7.0%) (Table 1). The presence of more than one fimbriae was detected in 18 (6.3%) isolates, while multiple genes codifying toxins were present in 106 (37.0%) isolates. There was a significant association between F4 and F5 as well as LT and STa, LT and STb, STa and STb, STB and STx2 (Table 2, 3).

At least one fimbria and one toxin gene was detected in 145 (50.5%) isolates, indicating that the isolate was pathogenic. Considering the simultaneous presence of genes coding for fimbriae and toxins, there was a significant association (P≤0.0005) between F4 or F5 with LT, STa or STb. Regarding F18 gene, this fimbriae gene was statistically associated (P<0.0001) with STa or STx2e genes (Table 2). A total of 17 virotypes were identified, being F18-STa (n=32; 11.1%), F4-LT-STa-STb (n=28; 9.8%), F4-STa (n=21; 7.3%), F4-LT-STb (n=18; 6.3%) and F18-STa-STx2e (n=13; 4.5%) the most frequent ones. In all farms at least one *E. coli* virotype was found, and most farms (n=8) presented up to two virotypes.

One hundred thirty six strains (47.4%) were beta-hemolytic. Non-hemolytic strains were significantly associated with absence of fimbriae and toxin genes (Table 3). Hemolytic strains had significant association (P<0.0005) with the F4 and F18 genes, as well as STa and STx2e toxin genes. The most common virotypes of hemolytic strains were F18-STa and F4-STa.

Among the 287 fecal samples positive for *E. coli*, the majority had liquid (48.4%) or creamy (40.8%) consistency (Table 3). The absence of ETEC virulence factors in the *E. coli* isolates was associated with pasty feces (P<0.012).

Liquid feces had a significant association (P≤0.05) with F4 and STa genes; and *E.coli* virotypes F4-STa and F4-F5-LT-STa-STb.

Table 2. Association between fimbriae from *Escherichia coli* strains isolated from weaned pigs with diarrhea

Virulence factors		F18 % (n)	F4 % (n)	*Fisher`s test
F4	Negative	44.21 (103/233)	-	-
	Positive	11.11 (6/54)	-	
	P value	<0.0001	-	
F5	Negative	6.44 (15/233)	1.69 (3/178)	*0.3214
	Positive	1.85 (1/54)	11.93 (13/109)	
	P value	0.1857	0.0002	

Chi square test and Fisher´s test.

Table 3. Association between toxins from *Escherichia coli* strains isolated from weaned pigs with diarrhea

Virulence factors		STa	STb	LT
STa	Negative	-	43.65(86/197)	44.39(91/205)
	Positive	-	60.00(54/90)	59.76(49/82)
	P value	-	0.0102	0.0102
STb	Negative	-	-	6.83(14/205)
	Positive	-	-	92.68(76/82)
	P value	-	-	<0.0001
STx2e	Negative	0.00(0/147)	9.64(19/197)	9.76(20/205)
	Positive	14.29(20/140)	1.11(1/90)	000(0/82)
	P value	<0.0001	0.0084	0.0034

Chi square test.

Table 4. Association between fimbriae and toxins from *Escherichia coli* strains isolated from weaned pigs with diarrhea

Virulence factors		Positive strains			
		LT% (n)	STa% (n)	STb% (n)	STx2e% (n)
F4	Negative (n= 178)	9.0 (16)	38.2 (68)	14.6 (26)	9.5 (17)
	Positive (n= 109)	60.5 (66)	66.1 (72)	58.7 (64)	2.7 (3)
	P value	<0.0001	<0.0001	<0.0001	0.0281
F5	Negative (n= 271)	25.5 (69)	45.8 (124)	28.4 (77)	6.3 (17)
	Positive (n= 16)	81.2 (13)	100.0 (16)	81.2 (13)	18.7 (3)
	P value	<0.0001	<0.0001	<0.0001	0.0903
F18	Negative (n= 233)	33.0 (77)	37.3 (87)	36.0 (84)	2.6 (6)
	Positive (n= 54)	9.3 (5)	98.1 (53)	11.1 (6)	25.9 (14)
	P value	0.0005	<0.0001	0.0004	<0.0001

Chi square test.

Table 5. Association of the most frequent virotypes detected among ETEC isolates from pigs with post-weaning diarrhea with hemolysis detected on blood agar and fecal consistency observed by clinical examination of the affected animals

Virotype	Hemolysis			Fecal consistency			
	Absence (%) (n=151)	Beta-hemolysis (%) (n=136)	P value	Liquid (%) (n=139)	Creamy (%) (n=117)	Pasty (%) (n=31)	P value
F4+STa	0	15.4	<0.0001	12.9a*	2.6b	0b	<0.050
F4+LT+STb	2.6	10.3	0.0076	8.6	5.1	0	0.1610
F4+LT+STa+STb	10.6	8.8	0.6133	10.1	10.3	6.4	0.8051
F4+F5+LT+STa+STb	7.3	0.7	0.0056	7.9a	0b	3.2ab	<0.002
F18+STa	2.0	21.3	<0.0001	13.7	8.5	9.7	0.4151
F18+STa+STx2e	1.3	8.1	0.0059	5.0	5.1	0	0.4382
No ETEC	50,3	12,5	<0,0001	33.3a	25.9a	58.1b	<0.012

Chi square test. * Different letters in a row indicate a significant difference between values.

DISCUSSION

Fimbriae F4 and F18 were the most predominant in the ETEC cases, as observed in other studies (Post et al. 2000, Frydendahl 2002, Francis 2004, Do et al. 2005 Nagy & Fekete 2005, Fairbrother & Gyles 2006). The lack of amplification of genes codifying F6 and F41 fimbriae was expected, since these fimbriae are most frequently detected in *E.coli* isolates from neonatal colibacillosis, because there is lack of receptors in enterocyte surface for these fimbriae in weaned pigs (Fairbrother & Gyles 2006). On the other hand, fimbria F5, which is usually absent in in *E. coli* isolates from pigs with post-weaning colibacillosis, was found in a low frequency (5.6%) in this study. It has been suggested that piglets, which carried *E.coli* strains with F5 during the first weeks of life, may remain colonized close after the weaning (Macêdo et al. 2007).

Regarding the toxins, ETEC strains associated with post--weaning diarrhea produce LT, STa and STb toxins or their combinations (Fairbrother & Gyles 2006, Doo et al. 2006). In this study, at least one toxin gene was detected and STa was the most prevalent (62.4%). Toxin STx2e was found in a low proportion of isolates (7.0%) and is related to edema disease rather than to neonatal or post-weaning diarrhea (Francis, 2004). However, some strains can cause simultaneously edema disease and post-weaning diarrhea (Fairbrother & Gyles 2006). The detection of STx2e in this study was significantly associated with fimbriae F18, a commonly toxin associated with edema disease in nursery pigs (Francis 2002), demonstrating gut colonization with isolates capable to cause this illness.

In order to be able to cause colibacillosis, an ETEC strain needs to simultaneously carry at least one fimbria and one toxin gene (Francis 2002). Considering this criterion, 50.5% of the isolates in the present study could be classified as ETEC. The other isolated strains might have been non-pathogenic or belonged to other pathotypes such as EPEC and the fimbrial type AIDA (Almeida et al. 2007), which were not investigated in this study. The most frequent virotypes were F18-STa, F4-LT-STa-STb, F4-STa, F4-LT-STb and F18--STa-STx2e. Variation in the frequency of ETEC virotypes is common and may be related to the plasmid-location of virulence genes, and the widespread transfer of these conjugative plasmids among ETEC strains in the gut (Vidotto et al. 2009). The most common virotypes in weaned piglets with diarrhea are F4-LT-STb (41.1%) and F18-STa-STb--STx2e (32.0%) (Post et al. 2000). The virotype F4-LT-STa--STb was the most prevalent in the study of Boerlin et al. (2005) and Do et al. (2006), and F4-LT-STb (42.5%) was one of the most prevalent in a study conducted in the USA (Zhang et al. 2007). The significant association between different types of fimbriae and toxins, as well as between certain types of fimbriae and toxins, also indicate that these genes may be located in a common mobile genetic element and were transferred together among *E.coli* isolates.

Although in most of the farms the predominance of a unique ETEC virotype was observed, in three farms up to nine different virotypes were present. This fact may be related to the comingling of piglets, originated from various farms, which may be carrying different ETEC virotypes in the gut. The diversity of ETEC virotypes circulating in the nursery may be a challenge for the immune system and for any control program.

Hemolysin is not considered itself a significant virulence factor in ETEC; however it is well-known the association between hemolysis and pathogenicity in *E. coli* (Frydendahl 2002, Do et al. 2005, Chapman et al. 2006). Previous studies demonstrated the association of hemolysin production with the presence of fimbriae F4 and F18 (Francis 2002). The hemolytic serogroup O149:K91 expressing the F4-LT-STa-STb virotype was also found at higher frequency by Do et al. (2006) in post-weaning diarrhea cases. In our study, we found a significant association between hemolysis and the most frequent virotypes, while the non-pathogenic strains showed a significantly lower frequency of hemolysis on blood agar. Hemolysis has been used as an auxiliary criterion for ETEC identification in the clinical laboratory. Our results demonstrate that it can be further used for a preliminary diagnostic, although the virotype identification by qPCR constitutes a more accurate diagnostic tool that gives quick information for control programs. Since there was significant association of ETEC and liquid feces in nursery piglets, sampling of liquid feces is crucial for the diagnosis of diarrhea.

Because 262 strains (57.4%) of the pigs with diarrhea were not associated with ETEC strain nor fimbriae of toxins, we assume they were not post-weaning colibacillosis cases. Infectious and non-infectious agents have been described as the cause of post-weaning diarrhea, such as rotavirus

and feed-induced intestinal flow disturbances (nutritional diarrhea), respectively (Morés & Moreno 2012).Therefore, they were assumed as. Even though *E. coli* infection is the main cause of diarrhea in this age group (Fairbrother & Gyles 2006), several.

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