

## Phylogenetic and pathotype analysis of *Escherichia coli* swine isolates from Southern Brazil<sup>1</sup>

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**ABSTRACT.**- Girardini L.K., Siqueira F.M., Krewer C.C., Krewer C.C., Costa M.M. & Vargas A.C. 2012. **Phylogenetic and pathotype analysis of *Escherichia coli* swine isolates from Southern Brazil.** *Pesquisa Veterinária Brasileira* 32(5):374-378. Departamento de Medicina Veterinária Preventiva, Universidade Federal de Santa Maria, Camobi, Santa Maria, RS 97105-900, Brazil. E-mail: [agueda.vargas@gmail.com](mailto:agueda.vargas@gmail.com)

The current study evaluated the presence of virulence factors by a multiplex PCR technique and then phylogenetically classified the studied strains into groups A, B1, B2 and D, according to Clermont et al. (2000), in 152 intestinal and extraintestinal swine isolates of *Escherichia coli*. Seventy seven isolates tested were positive for virulence factors. Phylogenetic characterization placed 21 samples into group A, 65 into B1, 19 into B2 and 47 into D. Fourteen urine samples were classified as uropathogenic *E. coli* (UPEC), nine were both UPEC and enterotoxigenic *E. coli* (EPEC) and four were EPEC only. The most common phylogenetic classifications were B1 and D groups. Of the analyzed fecal samples, 25 were classified as EPEC. Phylogenetically, the group of higher occurrence was B1, followed by B2, A and D. For the small intestine samples, 20 were classified as EPEC. Phylogenetic analysis found groups B1 and A to be the most commons in these samples. Six isolated tissue samples were classified as EPEC and most of them were designated as group D by phylogenetic classification. The phylogenetic analysis could be employed in veterinary laboratories in the *E. coli* isolates screening, including the possibility of vaccine strain selection and epidemiological searches.

INDEX TERMS: *Escherichia coli*, *E. coli* typification, phylogeny, uropathogenic *E. coli*, enterotoxigenic *E. coli*.

**RESUMO.**- [Análise filogenética e de patotipos de *Escherichia coli* isoladas de suínos no Sul do Brasil.] O presente estudo teve por objetivo avaliar a presença de diferentes fatores de virulência em 152 isolados de *Escherichia coli* intestinais e extra-intestinais provenientes de suínos pela técnica de PCR multiplex e classificá-los nos grupos filogenéticos A, B1, B2 e D, de acordo com Clermont et al. (2000).

Setenta e sete isolados foram positivos para pelo menos um fator de virulência. Através da caracterização filogenética, 21 isolados foram caracterizados como pertencentes ao grupo A, 65 ao grupo B1, 19 ao grupo B2 e 47 isolados ao grupo D. Quatorze isolados de urina foram caracterizados como *E. coli* uropatogênica (UPEC); nove apresentaram fatores de UPEC e *E. coli* enterotoxigênica (EPEC) simultaneamente e quatro foram classificados como EPEC. Na classificação filogenética, os isolados provenientes de amostras de urina classificaram-se principalmente nos grupos D e B1. Das amostras de fezes analisadas, 25 demonstraram fatores de virulência característicos do patotipo EPEC. Filogeneticamente, o grupo de maior ocorrência foi o B1 seguido de B2, A e D. Em relação às cepas isoladas de intestino delgado, 20 foram caracterizadas como EPEC. Pela filogenia, 23 isolados classificaram-se nos grupos A ou B1. Seis isolados de tecidos foram qualificados como EPEC e a maioria deles foram designados como pertencentes ao gru-

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po D, pela classificação filogenética. A análise filogenética pode ser empregada em laboratórios de diagnóstico veterinário como um *screening* para isolados de *E. coli*, incluindo a possibilidade de seleção de cepas vacinais e levantamentos epidemiológicos.

TERMOS DE INDEXAÇÃO: *Escherichia coli*, tipificação de *E. coli*, filogenia, *E. coli* uropatogênica, *E. coli* enterotoxigênica.

## INTRODUCTION

The bacterium *Escherichia coli* is a normal inhabitant of human and animal intestine. Some *E. coli* strains can cause a wide variety of intestinal and extraintestinal diseases, such as diarrhea, urinary tract infections and septicemia (Orskov & Orskov 1992). Diseases incidence is associated with many factors, which includes animal characteristics, poor nutrition, inappropriated handling (Brito et al. 1999), and the pathogenicity characteristics of the involved strain (Gyles & Fairbrother 2010).

*E. coli* isolates are characterized into different pathotypes according to the presence of specific virulence factors. The main pathotypes in swine diseases are enterotoxigenic *E. coli* (ETEC), characterized by the presence of the toxins STa, STb and LT and F4, F5, F6, F18 or F41 fimbria; enteropathogenic *E. coli* (EPEC), which carries the *eae* gene; Shiga toxigenic or Verotoxigenic *E. coli* (STEC or VTEC), characterized by the presence of factors such as F18a/b fimbria and Stx2 toxin, and finally uropathogenic *E. coli* (UPEC), which carries at least one of the following genes: *cnf*, *hly*, *bfp*, *eae*, *sfa*, *pap*, *iha* and *usp* (Brito et al. 1999, Campos et al. 2008, Afset et al. 2008).

Diagnosis of illness is carried out by phenotypic tests for microorganism identification; however, these tests are limited due to the evaluation subjectivity and to the individual variations of each strain. Serotyping can also be used to classify the species, but specific anti-sera are not readily available for serotype identification. Therefore, genotyping of *E. coli* strains based on the presence or absence of the virulence factors by polymerase chain reaction (PCR) has been widely used to characterize pathotypes (Beier et al. 2005). Some studies have attempted to identify the evolutionary origin of extraintestinal *E. coli* by using pathogenic marker analysis to classify the isolates into different phylogenetic groups (Johnson et al. 2001; Luna et al. 2010; Moulin-Schouleur et al. 2007). Phylogenetic analyses have shown that strains of this agent can be placed into four main groups: A, B1, B2 and D. The virulent strains usually belong to group B2 and, less commonly, to group D. Conversely, the commensal isolates belong to groups A and B1 (Clermont et al. 2000).

In Brazil, there are a limited number of studies involving the molecular characterization of *E. coli* strains of swine origin (Brito et al. 1999, Costa et al. 2006, Costa et al. 2008, Costa et al. 2010) as well as its classification into phylogenetic groups. The aims of the current study were the characterization of swine *E. coli* isolates by multiplex PCR (mPCR) and to classify these isolates into phylogenetic groups.

## MATERIALS AND METHODS

A total of 152 isolates of *Escherichia coli* were used in the present study: Seventy gilt urine samples of animals from piglets with reproductive and urinary problems (however the status of each animal was unknown), 35 fecal samples of piglets presenting problems of diarrhea, 35 samples of intestinal content from piglets with no clinical signs of disease and 12 tissue samples (6 livers, 3 lungs, 2 brains and 1 gall bladder) of piglets with clinical signs of diarrhea and dehydration. The samples were collected from three different swine breeding farms from Rio Grande do Sul State and 15 different swine breeding farms from Santa Catarina State, Brazil. The samples were previously identified by biochemical tests (Quinn et al. 1994) and kept lyophilized until bacterial DNA extraction by thermal extraction method. Isolated colonies on ovine blood agar 5% were diluted in sterile deionized water and boiled for 7 minutes, and then, centrifuged at 13,000 rpm for 5 minutes. The DNA was obtained from the supernatant and was used for different PCR methods.

The *E. coli* samples were genotyped using two mPCR assays to detect fimbria and toxins by amplifying the following gene encoding for regions: STa (158 bp), STb (113 bp), LT (272 bp), STx (758 bp), F4 (499 bp), F5 (230 bp), F6 (520 bp), F18 (313 bp) and F41 (613 bp), for the intestinal, fecal and tissue samples; and *cnf* (543 bp), *hly* (1117 bp), *bfp* (326 bp), *eae* (368 bp), *pap* (328 bp), *iha* (827 bp), *sfa* (410 bp), *cnf* (543 bp) and *usp* (440 bp) for the urinary isolates, as previously described (Brito et al. 2004, Costa et al. 2008, Blanco et al. 1996, Blanco et al. 1997). The mPCR was carried out with reactions of 25 µl containing 100ng of DNA, 30 pmol of each primer, 1X Taq buffer (10 mM Tris, 50 mM KCl, 2.5 mM MgCl<sub>2</sub>), 200 µM each deoxyribonucleotide triphosphate and 1 U of Taq DNA polymerase. *Thermocycling* conditions were: 35 cycles of 45 seconds at 94°C, 1 minute at 55°C, 45 seconds at 72°C and a final extension of 7 minutes at 72°C.

*E. coli* isolates were classified by phylogenetic PCR as previously described (Clermont et al. 2000). The PCR reaction mix was the same as described above. The amplification of genes *chuA* (279 bp) and *yjaA* (211 bp) and TspE4.C2 (152 bp) DNA fragment was obtained with a denaturation step for 5 minutes at 94°C, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C and 30 seconds at 72°C and a final extension of 7 minutes at 72°C.

Aliquots of 10 µl amplification products were subjected to electrophoresis for 45 minutes at 100V in 1.5% agarose gel stained with ethidium bromide. DNA fragments were compared with a 50 bp DNA ladder. Gel was visualized and photographed under ultraviolet light. Group B2 includes isolates *chuA*+/*yjaA*+; group D, *chuA*+/*yjaA*-; group B1, *chuA*-/TSPE4.C2+ and group A, *chuA*-/TSPE4.C2-. Analysis was performed to compare the results than genotyping mPCR and phylogenetic PCR of *E. coli* strains using the chi-square test. The level of significance was established at a *p* value of 0.05. The statistical tests were performed using the SAS package (SAS Institute, 2000).

## RESULTS AND DISCUSSION

The phylogenetic classification performed in the current study demonstrated that the most representative groups observed among swine *Escherichia coli* isolates were B1 and D, while other studies with human isolates showed groups A and B2 as the most prevalents (Picard et al. 1991). Strains of human origin are expected to be in the B2 *E. coli* group. Animal's strains are more resistant to antibiotics, less virulent and classified in non B2 groups (Sabaté et al. 2008). These differences may also be correlated to geographical variations, individual characteristics of the hosts and

due to the insufficiency of studies involving phylogenetic analyses of swine samples and also the lack of standard reference strains of natural populations (*Escherichia coli* Reference Collection).

The urine's phylogenetic classification, small intestine, fecal and tissue isolates compared to the presence or absence of virulence factors by mPCR is described in Table 1. From 152 *E. coli* isolates tested by mPCR, 51% (77) were positive for at least one of the virulence factors, while phylogenetic characterization found 42% (65) in of isolates in group B1, 31% (47) in D 14% (21) in A and 13% (19) in group B2.

**Table 1. Phylogenetic classification of urine, feces, small intestine and tissue *Escherichia coli* isolates from swine in comparison with the presence or absence of virulence factors by multiplex polymerase chain reaction**

Phylogenetic group	Urine (N=70)		Feces (N=35)		Small intestine (N=35)		Tissue (N=12)		Percentage of agreement
	+ <sup>a</sup>	-	+	-	+	-	+	-	
A*	0	8	3	2	2	3	0	3	76.00%
B1*	9	14	13	8	10	8	1	2	49.23%
B2**	5	2	6	0	5	1	0	0	84.20%
D**	12	20	3	0	3	3	5	1	48.93%
Total	26	44	25	10	20	15	6	6	

<sup>a</sup> + Presence of virulence factors; - absence of virulence factors; \*Classified as Commensal or \*\*virulent group by phylogenetic PCR (Clermont et al. 2000).

The majority of urine isolates (62.8%) were negative for virulence factors when tested by mPCR, which is consistent with previous findings (Costa et al. 2008). The remaining 37.1% (26/70) were positive for one or more virulence factors, 20% of these (14/70) were classified as UPEC, 12.8% (9/70) as both UPEC and ETEC and 5.8% (4/70) as ETEC (Fig.1A). In the phylogenetic grouping, 55.8% (39/70) were classified into groups B2 and D, of which 45.8% (32/70) were classified as group D. The 44.2% (31/70) remaining ones were classified as either group A or B1 (23/70). From the 31 samples phylogenetically classified as commensals, 29% (9/31) contained one or more virulence factors using mPCR, while out of the 39 samples considered pathogenic, 53.8% (21/39) did not show any virulence gene ( $P < 0.05$ ).

Seventy one per cent (25/35) of fecal samples analyzed, contained one or more ETEC virulence factors (Fig.1B). In the phylogenetic analysis, the most abundant group was B1, followed by B2, A and D, respectively. Studies carried out with *E. coli* from fecal samples of several domestic animal species found phylogroups A, B1 and D were prevalent, but in swine no virulence genes were associated (Unno et al. 2009). The same author report that major phylogroup found in feces were A group. The same results were described for swine feces in Brazil by Carlos et al. (2010) that suggest B1 group as the most environmental resistant group of *E. coli*.

Seventy four per cent (26/35) of samples were classified as commensals, of which 38.4% (10/26) did not show

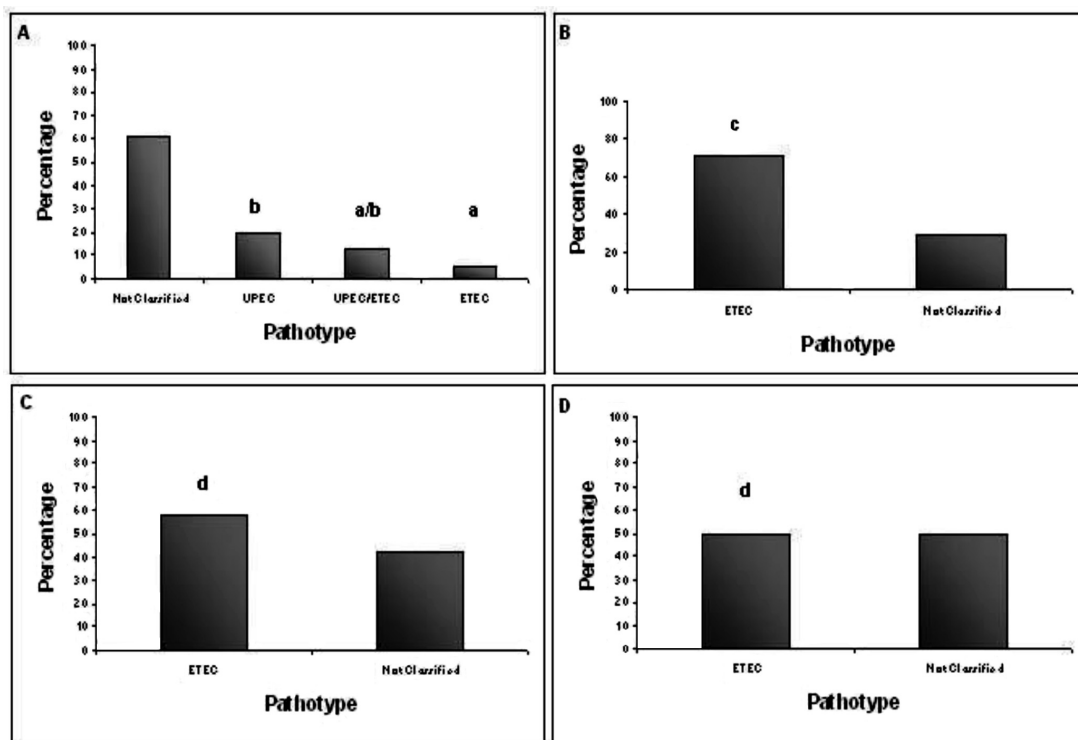


Fig.1. Pathotype characterization and virulence factors of urine (A), feces (B), small intestine (C), and tissue (D) *E. coli* isolates from swine. a = presence of at least one virulence factors (STa, STb, STx, LT); b = presence of at least one virulence factors (iha, pap, hly, sfa, usp); a/b = presence of at least one of both virulence factors characteristic of enterotoxigenic *E. coli* (ETEC) and uropathogenic *E. coli* (UPEC) pathotypes; c = presence of at least one virulence factors (F4, F5, F6, F41, STa, STb, STx, LT); d = presence of at least one virulence factors (F4, F5, F6, STa, STb, LT); Not Classified = negative for virulence factors tested by multiplex polymerase chain reaction.

any virulence factor by mPCR (Table 1). Accordingly, Chapman et al. (2006) in swine, the most commensal strains belonged to groups A and B1, with occasional predominance of phylogroup A (Schierack et al. 2007). In another study from commensal samples the phylogenetic groups A and B1 were prevalent, but ampicillin treatment led to a shift in the composition of the fecal *E. coli* population, with selection for ampicillin-resistant strains belonging to phylogroup A (Bibbal et al. 2009). In contrast with the most of the studies, group B2 clones were most frequently isolated, followed by group A, D, and B1 clones isolates from *E. coli* microflora of wild boars (Schierack et al. 2009).

The analysis of small intestine samples, characterized 57.2% (20/35) as ETEC (Fig. 1C). In the phylogenetic analysis, 65.6% (23/35) of the isolates were classified in groups A or B1. The same results were described by Picard et al. (1991) and Chapman et al. (2006), which can reflect a preference of specific virulence factor coded by plasmids from certain genetic groups (Turner et al. 2006). A total of 34.4% were placed into groups B2 (6/35) and D (6/35). The phylogenetic group B2 is not common in intestinal samples from domestic swine (Chapman et al. 2006) and boars (Schierack et al. 2009). From the samples classified in pathogenic phylogenetic groups, 66.6% (8/12) were found to be positive for virulence factors by mPCR, which was also found in 52.2% (12/23) of the samples that were considered commensals. Using mPCR, none of the fecal or small intestine isolates was classified as EPEC or STEC pathotypes in the current study. These results support previous findings of *E. coli* pathogenesis in isolates from Brazil (Costa et al. 2006).

Fifty per cent of tissue isolates (6/12) were classified as ETEC (Fig.1D) and fifty per cent (6/12) of the samples were classified into group D, followed by group A and B1, each one containing 25% of the samples (3/12). These data differ from previous ones which observed that human pathogenic extraintestinal strains were typically classified in group B2 (Gyles & Fairbrother 2010). One (16.6%) commensal isolate showed virulence factors ( $P<0.05$ ). Accordingly to Picard et al. (1999), B1 and D phylogenetic group can be highly virulent for animal models, but B2 phylogenetic group strains are clearly the most virulent strains, which are genetic divergence of strains among the *E. coli* species.

Some urinary samples were classified as UPEC, while others were positive for UPEC and ETEC virulence factors, which is also consistent with previous findings that demonstrated the presence of these two pathotypes in swine samples (Brito et al. 2004). Urinary strains demonstrating only ETEC factors were also observed. This data reinforces the fact that urinary tract infections in swine can be ascendant of gastrointestinal tract infections (Brito et al. 2004). Phylogenetic analysis demonstrated that the urinary samples classified according to the pathogenic groups were grouped primarily into group D, different from previous studies which observed that human pathogenic extraintestinal strains were typically classified in group B2 (Gyles & Fairbrother 2010). According to Sabaté et al. (2008), animal *E. coli* isolates are classified in non B2 groups. In the present study, a higher number of urine samples were

classified in commensal groups, most of them in group B1, while previous findings showed nonpathogenic strains typically assembled into group A (Clermont et al. 2000). Commensal *E. coli* of animals and humans demonstrate diversity in the distribution of phylogenetic groups of different populations (Picard et al. 1999).

Fifty two percent of the commensal isolates presented virulence genes by mPCR. This was also determined in 29% of the urine samples. Clinical and commensal *E. coli* strains are diverse in their genetic traits. Commensal *E. coli* are considered silent autochthones bacteria that can sometimes harbor virulence genes. In the current case, lack of the correct gene association may be related to no disease development. The presence of virulence genes is not equivalent to its expression. Genes that are turned to facilitate infections had their expression dependent on environmental and immunologic conditions (Chapman et al. 2006).

The remaining 21 fecal and small intestine samples were classified into groups B2 and D. Thirty three per cent (4/12) of the pathogenic isolates were negative for all of the tested virulence genes. However, the absence of these genes does not mean that the strains are not virulent, since the pathogenesis of *E. coli* can be evaluated by some other less studied genes (Geyid et al. 1996) that were not tested in the present study. The combination of virulence genes cannot fully define each pathotype, but may contribute to typify disease syndrome (Chapman et al. 2006). Under different selection pressures clones that possess nonadvantageous virulence genes could be eliminated and replaced with less frequent ones. These changes may be associated with the use of *E. coli* in immunization programs of sows to produce protective antibodies in the colostrums (Chapman et al. 2006).

The direct search of virulent determinants by mPCR is essential for the correct evaluation of *E. coli* pathogenesis. This search is limited, however, because only the main known factors were investigated, which most likely does not reflect reality (Picard et al. 1991). Results of the current study diverged from studies using human strains (Picard et al. 1991), but confirms previous studies on the subject of *E. coli* swine and others animal species isolates (Chapman et al. 2006, Sabaté et al. 2008). No absolute definition of virulence in *E. coli* is possible considering differences in species pathogenesis. Studies have suggested that *E. coli* isolates may have a multi-ancestral origin, one from pathogenic lineage and another from non-pathogenic lineage that possibly evolved by the horizontal acquisition of virulent genes (Silveira et al. 2002).

As mPCR is a technique that analyzes specific virulence factors, the phylogenic technique can be used for grouping swine pathogenic isolates while no essential virulence genes would be determined. With this, we propose the use of the phylogenetic analysis technique in veterinary laboratory diagnostic in screening of potential virulent *E. coli* isolates.

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