Virulence factors, antimicrobial resistance, and plasmid content of *Escherichia coli* isolated in swine commercial farms

[*Fatores de virulência, resistência aos antimicrobianos, presença de plasmídeos em* Escherichia coli *isoladas de amostras clínicas e ambientais de suínos*]

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ABSTRACT

Virulence factors and antimicrobial resistance patterns of *Escherichia coli* isolates were evaluated. A total of 80 *E. coli* isolates were evaluated, being 64 from clinical samples (intestinal content and fragments of organs from diarrheic piglets), seven from feces of clinically healthy piglets and sows, and nine environmental samples (five from facilities, two from feed, one from insect, and one from waste). Molecular characterization was performed by PCR detection of fimbriae and toxin genes and plasmid content determination. The isolates were also characterized according to their resistance or sensitivity to the following drugs: ampicillin, trimethoprim:sulfamethoxazole, tetracycline, amikacine, colistin, norfloxacin, florfenicol, enrofloxacin, cefalexin, trimethoprim, neomycin, chloramphenicol, and gentamicin. From 80 *E. coli* isolates, 53.8% were classified as enterotoxigenic *E. coli* (ETEC), 2.5% were shiga toxin-producing *E. coli* (STEC), and 43.8% showed a non specific pattern and were unclassified. One fecal isolate from non-diarrheic piglet was classified as ETEC by PCR. Clinical isolates showed resistance mainly for tetracycline and trimethoprim:sulfamethoxazole. Plasmidial DNA was observed in 70 isolates, being 78.5% of clinical isolates, 8.57% of non-diarrheic feces, and 12.8% of environment.

Keywords: swine, colibacillosis, fimbriae, toxin, antimicrobial resistance

RESUMO

Os fatores de virulência e a resistência aos antimicrobianos foram avaliados em Escherichia coli. *Um total de 80 isolados de* E. coli, *sendo 64 de amostras clínicas (conteúdo intestinal e fragmentos de órgãos de leitões diarreicos), sete das fezes de porcas e leitões saudáveis e nove de amostras ambientais (cinco de instalações, dois de alimentos, um de inseto e um de esterqueira). A caracterização molecular feita pela PCR objetivou detectar fimbrias e toxinas, bem como a determinação do conteúdo de plasmídeos. Os isolados foram caracterizados quanto à resistência ou sensibilidade às seguintes drogas: ampicilina, sulfazotrim, tetraciclina, amikacina, colistina, norfloxacina, florfenicol, enrofloxacina, cefalexina, trimetoprim, neomicina, cloranfenicol e gentamicina. Dos 80 isolados, 53,8% foram classificados como* E. coli *enterotoxigênica (ETEC), 2,5% como* E. coli *produtora de shiga toxina (STEC) e 43,8%, por não apresentarem padrão específico, não foram classificadas. Pela PCR, um isolado de fezes de suíno sem diarreia foi classificado como ETEC. Os isolados das amostras clínicas foram principalmente resistentes à tetraciclina e à sulfazotrim. Em 70 isolados, observaram-se DNA plasmidial, destes 78,5% foram obtidos de amostras clínicas, 8,57% de leitões sadios e 12,8% de amostras ambientais.*

Palavras-chave: suíno, colibacilose, fímbrias, toxinas, resistência antimicrobiana

INTRODUCTION

Neonatal colibacillosis is one of the important disease of swine herds around the world (Blickwede and Schwarz, 2004; Hart et al., 2004). Different pathotypes are associated with neonatal and post-weaning diarrhea as enterotoxigenic *Escherichia coli* (ETEC) and attaching-effacing or enteropathogenic *E. coli* (AEEC). The edema disease is caused by a Shiga toxin-producing *E. coli* (STEC). These diseases are associated with high mortality rates in young piglets (Choi et al., 2002; Hariharan et al., 2004). ETEC adheres to small intestine by fimbriae (F4, F5, F6, F41, and F18) and produce enterotoxins (STa, STb, and LT) that interact with enterocytes causing water and electrolytes hyper secretion and impairing nutrient absorption (Bertschinger and Fairbrother, 1999; Amezcua et al., 2002). The hemolysins are considered an important marker of ETEC virulence from swine (Docic and Bilkei, 2003). The piglet contamination occurs from sow or environmental contact and the disease produces very high mortality rates in the first 12 hours of life. Host susceptibility is associated to several factors such as the presence of intestinal *E. coli* fimbriae receptors, poor hygiene, stress, and colostrum ingestion quantity (Bertschinger and Fairbrother, 1999).

Antimicrobials have been commonly used to control and prevent *E. coli* infections in swine herds (Docic and Bilkei, 2003; Hariharan et al., 2004; Kummerer, 2004). However, the abusive administration of the antimicrobial drugs causes many problems as residual contamination of pork meat and potential risks for consumers (Dunlop et al., 1999; Beier et al., 2005). Considering that human, bovine, and swine ecosystems are highly interconnected by food production chain, the dissemination of resistant bacteria should be avoided (Petersen et al., 2002; Parveen et al., 2006; Schierack et al., 2006).

The occurrence of multi-resistant *E. coli* isolates has rapidly increased in recent years (Schroeder et al., 2002). In swine, a survey performed in Sweden for the detection of STEC, showed the presence of strains with high resistance to tetracyclines, sulfamethoxazole, and streptomycin, drugs widely used as grow promoters (Sherley et al., 2004). Similar results were also reported in *E. coli* isolates from beef cattle and poultry (Hart et al., 2004). Significant

differences in antimicrobial susceptibility patterns were found between swine ETEC and commensal bacteria, showing that sick animals are important sources of resistant bacteria to healthy animals (Boerlin et al., 2005).

Plasmids carrying multiple drug resistance genes are frequently detected in *E. coli* (Schroeder et al., 2002; Schierack et al., 2006). Plasmids may carry genes with different functions as: antibiotic resistance, adhesins, toxins, resistance to heavy metals, and assimilation of unusual nutrients (Schroeder et al., 2002). Therefore, the antimicrobial therapy may not be the unique selection pressure to raise resistant bacteria and the use of disinfectants could be considered important to co-selection of pathogenic resistant bacteria to antimicrobial compounds (Yates et al., 2004; Wallmann, 2006). The co-selection of genes coding for resistance and enterotoxin production was reported as well as the multiple antimicrobial drugs resistance among clinical isolates (Dunlop et al., 1999). In many cases, the resistance to some antibiotic may be extended to the others (Blickwede and Schwarz, 2004; Harada et al., 2006).

The drug resistance of *E. coli* isolates is a very important issue and this problem can only be solved by interdisciplinary efforts (Stephan and Schumacher, 2001). The correct use of antimicrobial therapy by clinicians is very important to reduce possible failures (Choi et al., 2002). According to Dunlop et al. (1998), a reduction in the use of growth promoters can help to minimize the dissemination of resistant *E. coli* for both the environment and the man. However, it has been demonstrated that once acquired by *E. coli* the resistance genes are not rapidly lost (Enne et al., 2005). The aim of the present study was to characterize the virulence and the drug resistance profile of swine *E. coli* isolates from Santa Catarina, Brazil.

MATERIAL AND METHODS

E. coli strains were isolated from clinical cases of neonatal colibacillosis (intestine contents and fragments of liver and lungs), from non-diarrheic piglets and sows feces, and from the environment (feed, insects, and facilities waste). Samples were collected from swine breeding farms in Santa Catarina state, Brazil, in sterile bags and swabs, and were transported in Stuart modified

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medium at 4°C to the laboratory. The samples were streaked on ovine blood agar and Mac Conkey agar and were kept at 37°C for 48 hours. Putative *E. coli* colonies were identified by morphology and biochemical tests according to Quinn et al. (1994). One *E. coli* colony was used to DNA extraction and sensitivity test.

The Kirby-Bauer disc diffusion test (Bauer et al., 1966; Performance…, 1999) was used to determine the resistance to antimicrobial drugs of *E. coli* isolates. The follow drugs were tested: ampicillin (10μg), trimethoprim: sulfamethoxazole (25μg), tetracycline (30μg), amikacine (30μg), colistin (25μg), norfloxacin (10μg), florfenicol (30μg), enrofloxacin (5μg), cefalexin (30μg), trimethoprim (25μg), neomycin (30μg), chloramphenicol (30μg), and gentamicin (30μg).

Total DNA was extracted by boiling methodology. Briefly, one-colony cultures were suspended in sterile Milli-Q water, boiled for 5min and immediately immersed on ice. After centrifugation at 13.000RPM for 2min, the DNA was recovered from the supernatant. The *E. coli* isolates were characterized by multiplex PCR for fimbrial and toxin genotypification by amplification of the follows regions: STa, STb, LT, STx F4, F5, F6, F41, and F18, as previously described (Costa et al., 2006). The presence of attaching-effacing *E. coli* (AEEC) was evaluated, as described by Nakazato et al. (2004). The PCR reactions were performed in 25μL reactions containing 100ng DNA template, primers (30pmol each), *Taq* buffer (10mM Tris, 50mM KCl, 2.5mM MgCl₂), 200mM dNTPs, and 1U *Taq* DNA polymerase (Cenbiot-Enzymes, UFRGS). Amplification was performed in 35 cycles of 45s at 94°C, 1min at 55°C, and 45s at 72°C. After amplification, a 7μL aliquot was submitted to electrophoresis for 30 min at 100V in a 1.5% agarose gel stained with ethidium bromide. Amplification products were visualized and photographed under UV illumination. Amplification identities were confirmed by sequencing in an automated DNA sequencer MEGABACE 1000 using the Dynamic ET Dye Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech).

Plasmidial extraction from *E. coli* isolates was performed by alkaline lysis, as previously described (Birnboim and Doly, 1979). The presence of plasmidial DNA was confirmed by electrophoresis in 0.7% agarose gels. The plasmids were visualized and photographed under UV illumination.

The difference among the *E. coli* isolates resistance patterns was evaluated by the Z test of two proportions with normal approximation. The level of significance at 0.05 and the cut off point for Zp5% at 1.96 were set. The relationship among the frequencies of haemolysis and virulence factors was done by chi-square test.

RESULTS AND DISCUSSION

The results of haemolysis in blood agar and PCR for fimbrial and enterotoxin determination in the *E. coli* isolates are summarized in Table 1. Haemolysis is normally a marker of virulence in *E. coli* (Bertschinger and Fairbrother, 1999). In this study, 57 hemolytic isolates were found, being 79.7% (51/64) of diarrheic piglets, 57.1% (4/7) from feces of healthy swines, and 22.2% (2/9) from environment. The haemolysis showed statistically relationship (P<0.05) with detection of virulence factors by PCR.

Table 1. Distribution of virulence factors in neonatal colibacilosis, feces of healthy swines, and environment *Escherichia coli* isolates

Isolate	Virulence factor										
	n[a]	H[b]	STa[c]	STb[c]	LT[c]	STx[c]	F4[c]	F5[c]	F6[c]	F41[c]	F18[c]
Clinical	64	51	13	27	23	2	12	8	0	7	17
Non-diarrheic feces	7	4	0	1	0	0	0	0	0	0	0
Environment	9	2	0	0	0	0	0	0	0	0	0
Total	80	57	13	28	23	2	12	8	0	7	17

[a]number of *E. coli* isolates;
[b]haemolytic in blood agar;
[c]PCR results for fimbrial and toxins amplifications.

From the 64 clinical isolates, 43 were classified as ETEC and two as STEC. Nineteen *E. coli* clinical isolates gave negative results in the multiplex PCR for virulence markers and could not be typified. One isolate from feces of healthy swine was classified as ETEC. None isolate was confirmed as AEEC in this study.

In the clinical isolates, the most prevalent virulence factors were STb (27), LT (23), F18 (17), F4 (12), and STa (13). F18, 987P, and F4 were described to diarrheic piglets in Brazil (Macêdo et al., 2007). Thirteen clinical isolates presented LT and STb genes, six isolates only the STb gene, and six isolates the STa and STb genes. The detection of genes coding for all enterotoxins was observed in two isolates. According to Celemin et al. (1995), the enterotoxin coding genes LT and STb, or LT, STa, and STb are expected to be found in ETEC. The same findings were found in the study made by Macêdo et al. (2007) in diarrheic piglets, in which where LT and STb were the prevalent virotype. Only in one isolate from non-diarrheic piglet feces the STb gene was detected by PCR. Therefore, *E. coli* isolates from healthy swine and environment samples show a lower pathogenic potential. The STb alone causes only lost of fluids on the piglets intestinal mucosa, without drastic effects proper of colibacillosis diarrhea (Casey et al., 1998). However, the pathogenicity of ETEC may be increased by associated infections with other intestinal pathogens (Nakamine et al., 1998). New vaccines strategies are being developed, using fimbrial and enterotoxins purified from *E. coli* (Bertschinger and Fairbrother, 1999). Therefore, the knowledge of the molecular patterns of *E. coli* isolates is very important.

The determination of the pathogenic bacteria susceptibility patterns to antimicrobial drugs is important to guide the therapy, that will reduce the economic loses due to *E. coli* infections (Choi et al., 2002; Hariharan et al., 2004). The resistance patterns of *E. coli* isolates from Santa Catarina State are presented in Table 2. The majority of the clinical *E. coli* isolates revealed resistance to tetracycline and trimethoprim:sulfamethoxazole (P<0.05) and very few isolates were resistant to florfenicol. These results are in accordance to others studies that evaluated the resistance patterns of *E. coli* isolates from slaughtered swine, specially to susceptibility to florfenicol and resistance to tetracycline and trimethoprim:sulfamethoxazole (Hariharan et al., 2004; Hart et al., 2004; Sherley et al., 2004; Beier et al., 2005; Macêdo et al., 2007). From eighty *E. coli* evaluated isolates, 52 (65%) were simultaneously resistant to four or more antimicrobial groups. This criterion is considered to multi-drug resistant isolates (Rjavek et al., 2006).

Table 2. Percent of resistant *Escherichia coli* isolates from clinical, non-diarrheic swines, and environment to antimicrobial drugs

Drug	Isolate*		
	Clinical[1] (n = 64)	Non-diarrheic pigs feces[2] (n = 7)	Environment[3] (n = 9)
Ampicillin	42.19	42.86**	30.00
Trimethoprim:sulfamethoxazole	62.50**	28.57	40.00
Tetracycline	68.75**	42.86	60.00
Amikacine	37.50	42.86	20.00
Colistin	28.13	42.86	10.00
Norfloxacin	35.94	28.57	20.00
Florfenicol	12.50**	14.92**	00.00**
Enrofloxacin	29.69	28.57	20.00
Cefalexin	43.75	28.57	40.00
Trimethoprim	42.19	14.29	40.00
Neomycin	32.81	28.57	50.00
Chloramphenicol	20.31**	28.57	20.00
Gentamicin	39.06	71.43**	60.00

n: number of isolates.
*Non-statistically difference between *E. coli* groups (P>0.05);
[1]Statistically difference in clinical isolates group (P<0.05);
[2]Statistically difference in non-diarrheic swines feces *E. coli* isolates (P<0.05);
[3]Statistically difference in environmental *E. coli* isolates (P<0.05);
*Statistical difference by the Z test with significance level of 5%.

Boerlim et al. (2005) found a significant difference in antimicrobial susceptibility patterns among pathogenic and commensal *E. coli* isolates. In this study, the resistance was higher for all *E. coli* isolates tested and non-significative (P>0.05) differences were detected and were related to the source of those samples (diarrheic, non-diarrheic piglets, and environment). The drugs tested in the present study are widely employed to animal production in Brazil, including confined cattle (Bastos et al., 2006; Pigatto et al., 2008). The improper veterinary use of these drugs increases the resistance to animal pathogenic bacteria and could generate impact to human health by food and feces residues from swine herds (Petersen et al., 2002; Parveen et al., 2006; Schierack et al., 2006). The potential risk of the dissemination of resistant bacteria from animal farming to the environment and the man was established by detection and determination of conjugative plasmids in bacteria isolated from water (Petersen et al., 2002), meat producing animals, and man (Schierack et al., 2006). According to Wallmann (2006), strategies to avoid the spread of resistance to antimicrobial drugs involve the control of antimicrobial use in the food-producing chain.

Plasmid was observed in 55 (85.9%) clinical and six (85.7%) non-diarrheic feces and in nine (100%) environmental *E. coli* isolates (data not shown). The plasmid occurrence was higher among clinical isolates showing simultaneous resistance from five to seven antimicrobial drugs. Plasmids in *E. coli* carry multiple resistance genes. The higher resistance of *E. coli* is related to wide distribution of bacteria and ability to maintain mobile genetic elements (Blickwede and Schwarz, 2004; Yates et al., 2004; Harada et al., 2006; Schierack et al., 2006). The co-selection of resistance and enterotoxin production genes were reported, as well as the multiple antimicrobial drugs resistance among clinical isolates (Dunlop et al., 1999). Many clinical and environmental *E. coli* isolates revealed multiple resistances even in the absence of plasmidial DNA. The resistance to antimicrobial drugs may be associated to others different genetic mechanisms. Chromossomal mediated resistance genes are described for several antimicrobial compounds, as enrofloxacin beta lactams and biocides, as chlorexidine (Piddock et al., 1998; Beier et al., 2005).

CONCLUSIONS

Multi resistance was detected among *E. coli* isolates. The majority of *E. coli* isolates were molecularly characterized as ETEC, by using a multiplex PCR technique. The most frequent virulence factors were STb and LT.

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