Acta Scientiae Veterinariae, 2018. 46: 1525.

RESEARCH ARTICLE
Pub. 1525

ISSN 1679-9216

Virulence Factors and Antimicrobial Resistance Profile of *Escherichia coli* Isolated from Nursery Piglets and Drinking Water

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ABSTRACT

***Background*:** One of the most frequent health problems in the swine industry is the post-weaning diarrhea in nursery pigs, which leads to significant losses due to weight loss, dehydration, cost of medication and mortality. *Escherichia coli* (*E. coli*) is one of the main bacterial agents of the post-weaning diarrhea. To investigate the possibility of enterotoxigenic *E. coli* (ETEC) transmission through drinking water to nursery piglets, the objective of this study was to isolate, characterize by virulence factors, and compare the antimicrobial resistance profiles of *E. coli* from drinking water samples in nurseries and from rectal swabs of their piglets presenting post-weaning colibacillosis.

***Materials, Methods & Results*:** Fifteen rectal swabs from diarrheic piglets in their first three weeks after weaning and one water sample were collected from each of ten nurseries located in Rio Grande do Sul State, south of Brazil. After enrichment with a commercial broth medium, water samples were cultured in blood agar, as well as the rectal swab samples, and the characteristic colonies were identified by standard biochemical analysis. Following isolation and identification of *E. coli*, the colonies from water samples and their corresponding piglets' samples were characterized by multiplex PCR in order to determine specific ETEC fimbria and toxin genes. Finally, all *E. coli* isolates were submitted to antimicrobial susceptibility testing. Virulence factors and antimicrobial sensitivity could then be compared between water and piglets' samples. The difference in the antimicrobial resistance frequency for each of the sample groups were compared using the multi comparison test. *E. coli* was isolated in four out of the ten water samples, although none of the water samples presented ETEC virulence factors. From 60 rectal swab samples (15 from each of the four positive farms with *E. coli* isolated from water samples), 21 *E. coli* were isolated and seven demonstrated characteristic ETEC virulence factors. The fimbriae exhibited in higher frequency were F18 (62.5%) and F4 (25%) and the toxins were STb (100%) and STaP (75%). *E. coli* isolated from water samples presented higher resistance to the antimicrobials apramycin, florfenicol, lincomycin, lincomycin+spectinomycin, oxytetracycline, and sulfamethoxazole+trimethoprim; it did not present resistance to colistin and fosfomycin. The seven ETEC from rectal swab samples presented a higher resistance to lincomycin, and lower resistance frequency to fosfomycin. The other 14 *E. coli* non-ETEC from rectal swab samples presented a higher resistance to florfenicol and no resistance to colistin.

***Discussion*:** Enterotoxigenic *E. coli* is an important agent causing post-weaning colibacillosis, although, differently from other studies, this experiment did not find the agent in most of the sampled animals. In contrast to other authors, ETEC was not found in water, as the development of its virulence factors may depend on conditions presented exclusively in the animal. By the results we can conclude that, although *E. coli* was isolated from the drinking water, it was not a significant mechanism for nursery piglets' infection with ETEC in this experiment. The samples analyzed presented a wide range of resistance to different antimicrobials, including multi-resistance. In some cases, *E. coli* found in water presented different antimicrobial profile from the bacterium found in the rectal swab samples. Enterotoxigenic *E. coli* was susceptible to fosfomycin and its use may represent a prudent antimicrobial choice to the swine industry.

Keywords: antimicrobial susceptibility testing, colibacillosis, fimbria, PCR, toxin.

DOI: 10.22456/1679-9216.81810

Received: 22 September 2017 Accepted: 21 January 2018 Published: 6 February 2018

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INTRODUCTION

One of the most frequent problems in the swine industry is diarrhea in nursery pigs, which leads to significant losses due to weight loss, dehydration, and cost of medication [6,7]. Enterotoxigenic *Escherichia* (*E.*) *coli* (ETEC) is one of the main agents of the post-weaning diarrhea (between the first and third week post-weaning) and can cause a mortality rate as high as 25% [7].

E. coli is an intestinal commensal bacterium in pigs and its pathogenicity is directly related to fimbria and toxin genes expression. Fecal-oral is the main route of transmission, and water is an important contamination source [7]. In order to investigate the possibility of ETEC transmission through water, the aim of this study was to isolate, characterize the virulence factors and compare the antimicrobial resistance profile of *E. coli* isolated from drinking water and the related nursery pigs with post-weaning colibacillosis.

MATERIALS AND METHODS

Samples

Fifteen rectal swabs from piglets with clinical post-weaning diarrhea and one water sample (100 mL - pool of 50 mL from water drinker and 50 mL from water tank) were collected from each of 10 nurseries in Rio Grande do Sul, Brazil, totaling 150 rectal samples and 10 water samples. Water samples and swabs (Amies media)[1] were transported under refrigeration to the Swine Research Center at the Federal University of Rio Grande do Sul and bacteriologically tested in the same day.

Bacteriology

Rectal swab specimens were cultured on 5% ovine blood agar[2] plates and aerobically incubated for 24-72 h at 37°C. Typical *E. coli* colonies were cultured on MacConkey agar[3] and Trypticase Soy Agar (TSA)[2] and aerobically incubated for 24-72 h at 37°C. Lactose positive samples were submitted to specific biochemical tests for *E. coli* characterization [10]. The same techniques were applied to water samples after their enrichment by a commercial broth medium (Colilert)[4] for 24 h. *E. coli* isolates were aerobically incubated for 24-72 h at 37°C on the brain-heart infusion nutritional media (BHI)[2], and stored at -18ºC to preserve viable cells until processing. Only farms that had *E. coli* isolated from water samples had their corresponding *E. coli* isolates from rectal swabs analyzed. Specimens were treated as individuals in all tests.

Multiplex polymerase chain reaction (PCR)

E. coli isolates were submitted to multiplex PCR to amplify fimbriae (K88, K99, 987P, F18, F4) and toxins (LT, STb, STaP, and Stx2e) genes. Colonies from each *E. coli* isolate were suspended in sterile water and DNA was extracted using a commercial kit (PrepSeq Spin Column)[5]. Following supplier instructions, 750 µL from sample were added on single column, centrifuged at 1,077 *g* and the supernatant was discarded. Fifty µL of elution solution were added and heated at 98ºC for 10 min. Samples were centrifuged and the supernatant was used for amplification or stored at -20ºC. The reaction was performed as previously described [9]. Briefly, the reaction mix was composed by 300 nM of each primer[6], 1,5 µM MgCl27, 200 nM of dNTP (multiplex PCR kit)[7], DNAse free water and 1 µL of extracted DNA. Reaction conditions were as follow: one step of initial activation at 95ºC for 10 min followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 55ºC for 1 min, and extension at 70ºC for 2 min on a Verity™ thermocycler[5]. Amplicons were run in 2% agarose gel electrophoresis and the gel was stained with ethidium bromide and visualized under UV light. A 100bp ladder[8] was added on the gel electrophoresis in order to evaluate and interpret the results. Based on the results of this test, the samples were grouped as ETEC, non-ETEC and water non-ETEC for further analyses.

Antimicrobial sensitivity testing

E. coli isolates from rectal swabs and water were submitted to antimicrobial sensitivity testing, carried out by agar disk diffusion method [4]. The diameter of the disks' halos was measured, classifying samples in sensitive or resistant (intermediary inhibition was classified as resistant). The antimicrobial principles[9] tested were: apramycin (15 µg), colistin (25 µg), florfenicol (30 µg), fosfomycin (50 µg), gentamicin (10 µg), lincomycin (10 µg), lincomycin+espectinomycin (10 µg), neomycin (30 µg), oxytetracycline (30 µg), and sulfamethoxazole+trimethoprim (25 µg).

Statistical analysis

Data are presented as percentage of the frequency of resistance to the antimicrobial. Statistical

analysis was done by multiple comparison tests with Bonferroni adjustment using the R v3.2 software[10]. Significant differences among the three groups (ETEC, non-ETEC, water non-ETEC) were considered when *P*-values were lower than 0.05.

RESULTS

Among all water samples, four (40%) were *E. coli* positive, and from the 60 rectal swab samples (15 from each of the four farms), 21 (35%) were *E. coli* positive, resulting in 25 *E. coli* isolates. From the 21 *E. coli* isolates from the rectal swab samples, fimbriae and toxins were observed in seven (33.33%) isolates and only toxins were observed in one (4.76%) isolate. The most prevalent fimbriae and toxins from the rectal swab samples were F18 (62.5%, 5/8), F4 (K88) (25%, 2/8), STb (100%, 8/8), STaP (75%, 6/8), and LTb (37.5%, 3/8), resulting in a variety of virotypes. None virulence factor was detected on *E. coli* from the four water samples analyzed. Five ETECs (71.4%) produced the same fimbria and toxins combination - F18, STb and STaP (Table 1).

The percentages of resistance frequency among the three groups (ETEC, non-ETEC, water non-ETEC) to each antimicrobial are presented in Table 2. The seven *E. coli* isolates from rectal swab identified as ETEC (B1, B2, B3, B4, C1, C2, and C3) were resistant to lincomycin (100%), most resistant (>50%) to apramycin, gentamicin, lincomycin+espectinomycin, and sulfamethoxazole+trimethoprim, while showing lower resistance frequency (<50%) to colistin, neomycin, florfenicol, oxytetracycline, and one sample resistant to fosfomycin (14.2%). All 14 non-ETEC *E. coli* were resistant to florfenicol (100%), and none of the samples presented resistance to colistin, having varied resistance frequency to the other antimicrobials. Among the four non-ETEC *E. coli* isolates from water samples, 75% were resistant to apramycin, florfenicol, oxytetracycline, and sulfamethoxazole+trimethoprim; there was no resistance to colistin and fosfomycin. There was no significant statistical difference ($P > 0.05$) in the resistance frequency among the three groups (ETEC, non-ETEC and water non-ETEC) for lincomycin+spectinomycin and sulfamethoxazole+trimethoprim, being the three groups highly resistant to both principles. Both fosfomycin and lincomycin had a similar frequency of resistance for ETEC and non-ETEC groups, but significantly different ($P < 0.05$) from the water non-ETEC group. Non-ETEC and water non-ETEC groups generated a similar frequency of resistance to colistin, gentamicin and neomycin, but differed ($P < 0.05$) from the ETEC group. Apramycin, florfenicol and oxytetracycline, had all different resistance frequencies among the three groups.

Table 1. Virulence factors (fimbriae and toxins) identified in eight *E. coli* strains isolated from rectal swabs collected in three different nurseries in Rio Grande do Sul State.

Sample	Virulence Factors
A1	STb, LTb
B1	F18, STaP, STb
B2	F18, STaP, STb
B3	F18, STaP, STb
B4	K88, STb, LTb
C1	F18, STaP, STb
C2	K88, STb, STaP, LTb
C3	F18, STaP, STb

Samples are named with a letter (representing the nursery) and a number (representing the piglet) for rectal swab samples (e.g. A1 means barn A, piglet 1).

Table 2. *Escherichia coli* antimicrobial resistance frequency to ten antimicrobial drugs from 21 rectal swab samples (ETEC and non ETEC) and four water samples (W non ETEC) collected from piglets with post-weaning diarrhea and drinking water, respectively, in four nurseries in Rio Grande do Sul State.

Antimicrobial	ETEC	Non ETEC	W non ETEC
Apramycin	57.1% (4/7) [a*]	28.5% (4/14) [b]	75% (3/4) [c]
Colistin	28.5% (2/7) [a]	0% (0/14) [b]	0% (0/4) [b]
Florfenicol	42.8% (3/7) [a]	100% (14/14) [b]	75% (3/4) [c]
Fosfomycin	14.2% (1/7) [a]	7.1% (1/14) [a]	0% (0/4) [b]
Gentamicin	85.7% (6/7) [a]	35.7% (5/14) [b]	25% (1/4) [b]
Lincomycin	100% (7/7) [a]	92.8% (12/14) [a]	75% (3/4) [b]
Lincomycin+Espectinomycin	85.7% (6/7) [a]	85.7% (12/14) [a]	75% (3/4) [a]
Neomycin	28.5% (2/7) [a]	71.4% (10/14) [b]	75% (3/4) [b]
Oxytetracycline	42.8% (3/7) [a]	85.7% (12/14) [b]	25% (1/4) [c]
Sulfamethoxazole+Trimethoprim	85.7% (6/7) [a]	85.7% (12/14) [a]	75% (3/4) [a]

Multi comparison test. *Different letters in a row indicate a significant difference between values.

DISCUSSION

The most frequent fimbriae found in nursery pigs are F4ab, F4ac, F18ab and/or F18ac [7]. Likewise, the most important toxins for this age group are LT and STa and STb [7]. To be classified as enterotoxigenic, *E. coli* has to express at least one fimbria and one toxin simultaneously [8]. Thus, by this criterion, 11.6% (7/60) samples isolated from the analyzed rectal swab were classified as ETEC, different from other authors [10], who observed ETEC in 50.5% of rectal swab samples from diarrheic nursery piglets. Although *E. coli* is considered the main etiologic agent on post-weaning diarrhea, other agents may be found, as *Isospora suis*, Rotavirus type A, *Clostridium perfringens*, and *Salmonella Typhimurium* [7]. Besides, ETEC low detection might be related to the intensive use of antimicrobial therapy on nurseries.

The frequency of the virulence factors found in this work are consistent with previous works, where F18 and F4 (K88) were the most prevalent fimbriae detected on diarrheic post-weaning piglets [7,11]. Our study did not observe ETEC virulence factors on the water *E. coli* isolates, unlike other studies [3,15], which detected *E. coli* strains producing F6 (987P) and LT, and LT and ST, respectively. Swine farms assessed in this project are under a water quality control program, including addition of chlorine, acids, antimicrobial agents and vitamins to the water tanks to prevent diarrhea. Some of those products decrease the number of bacteria in the water and may reduce the agent's capacity to express virulence factors. Additional hypothetical factors are the individual differences between piglets, the animal's immunity status, the environmental conditions and the pressure of infection.

All *E. coli* isolates presented resistance from at least one, up to nine antimicrobials. From all 25 *E. coli* isolates, 24 (96%) presented resistance to at least three antimicrobials from different groups (characterizing multi-resistance), a similar result to other study [14], where 100% of the *E. coli* samples were multi-resistant, despite no multi-resistance being found in historical *E. coli* strains [13]. Five ETEC samples with the same virulence factors F18, STaP, and STb (B1, B2, B3, C1, and C3) were resistant to lincomycin and sulfamethoxazole+trimethoprim. From those five ETECs, at least two were resistant to apramycin, colistin, florfenicol, gentamicin, and lincomycin+spectinomycin, suggesting a relationship between virulence factors and antimicrobial resistance.

A higher ETEC resistance to oxytetracycline (93.6%) was found when comparing the resistance frequencies from previous studies [12], which found a resistance frequency of 50%. However, the same author found a similar resistance to florfenicol (95.2%). Our study showed a high resistance profile to lincomycin for ETEC and non-ETEC *E. coli* (100% and 92.8%, respectively), similarly to other study [1] that found a resistance of 96,4% for the same drug. The differences in the resistance frequency cited above might be attributed to the increase in

the antimicrobial resistance among enterobacteria [5], or to the regional variability, or to the type of antimicrobial treatment used in different barns, since antimicrobial resistance is closely related to the selection pressure of the agent and the drugs commonly used to treat the disease [2].

Non-ETEC swab and water samples exhibited high resistance frequencies to florfenicol and neomycin, unlike ETEC samples, which showed a significantly higher resistance to gentamicin and colistin than the two other groups. Although the groups had statistically different resistance frequencies for most of the antimicrobials, they presented high resistance frequency (>50%) to three antimicrobials: lincomycin, lincomycin+espectinomycin and sulfamethoxazole+trimethoprim, suggesting that similar antimicrobial resistance profiles can exist among specimens besides their different level of pathogenicity. The ETEC group had a lower frequency of resistance to fosfomycin, and its use may represent a prudent antimicrobial choice to the swine industry.

CONCLUSION

The results of this study showed that contamination of drinking water with *E. coli* was not a significant mechanism of ETEC transmission to nursery piglets, since no ETEC was detected in water samples. There were differences in virotypes among ETEC and non-ETEC from piglets and non-ETEC from the drinking water, as well as a wide range of resistance to different antimicrobials. However, the use of fosfomycin may represent a prudent antimicrobial choice to the swine industry. Although there were no ETEC specific virulence factors on *E. coli* isolated from water samples, this bacterium was present. Thereby, it is important to maintain good environmental and management conditions to avoid transmission of ETEC through other routes.

MANUFACTURERS

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[5]Applied Biosystems™. Foster City, CA, USA.

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[7]Qiagen. Hilden, North Rhine-Westphalia, Germany.

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***Declaration of interest*.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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http://dx.doi.org/10.22456/1679-9216.81810