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Antimicrobials resistance patterns and the presence of *stx1*, *stx2* and *eae* in *Escherichia coli*

Padrões de resistência antimicrobianos e da presença de stx1, stx2 e "eae" em "Escherichia coli"

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SUMMARY

The objectives of this study were to investigate whether antimicrobial resistance (AMR) or the presence of resistance genes was associated with the occurrence of the virulence genes, *stx1*, *stx2* and *eae*. Three virulence genes and 11 AMR phenotypes were examined using polymerase chain reaction (PCR) and antimicrobial susceptibility tests. From 800 samples collected in this study, 561 samples were isolates *E. coli* strains , being: 90 (16.0%) carriers of *stx1*, 97 (17.3%) of *stx2* and 45 (8.0%) of *eae* genes singly. Thirty seven (6.6%) isolates were carriers of *stx1* and *stx2*, 110 (19.6%) were carriers of *stx1* and *eae* and 67 (11.9%) were carriers of *stx2* and *eae*. The most common virulence gene detected was *stx1* followed by *stx2*. The findings showed no relationship between presence of virulence factors and antimicrobial resistance. Also was not found relationship between serogroup and virulence factors.

Keywords: antimicrobial resistance, dairy cow, *Escherichia coli*, serogroups, STEC

RESUMO

Objetivou-se com este estudo investigar se a resistência antimicrobiana (RAM) ou a presença de genes de resistência foi associada com a ocorrência dos genes de virulência, stx1, stx2 e *eae* e os sorogrupos de *Escherichia coli* isoladas a partir de vacas-vacas leiteiras. Três genes de virulência e 11 fenótipos RAM foram examinados através de PCR e testes de susceptibilidade aos antimicrobianos. Das 800 amostras colhidas neste estudo, 561 amostras foram isoladas cepas de *E. coli*, sendo: 90 (16,0%) portadoras de stx1, 97 (17,3%) de stx2 e 45 (8,0%) dos genes eae, separadamente. Trinta e sete (6,6%) isolados foram portadores de stx1 e stx2, 110 (19,6%), foram portadores de *stx1* e *eae* e 67 (11,9%) foram portadores de *stx2* e *eae*. O gene de virulência mais comum detectado foi *stx1* seguido por *stx2*. O sorogrupo predominante entre os isolados portadores de *stx1* foi O119. Entre as cepas portadoras de *stx2, eae* e também estirpes com nenhum fator de virulência, o sorogrupo predominante foi O9. O RAM dos isolados, medido fenotipicamente, não foi associado com a presença ou ausência de genes de virulência na população saudável de vaca leiteira-nem a sua

ocorrência a qualquer um dos sorogrupos foi associada com genes *stx1* e *stx2* e *eae.*

Palavras-chave: *Escherichia coli*, resistência antimicrobiana, sorogrupos, STEC, vaca leiteira

INTRODUCTION

Currently, Shiga toxigenic *Escherichia coli* (STEC) strains are the most important emerging groups of foodborne pathogens (BEUTIN et al., 2002). These strains are producer of one or two cytotoxins called Shiga toxins (Stx1 and Stx2) (PATON & PATON, 1998). Intimin is another virulence factor responsible for intimate attachment of STEC and it is encoded by the chromosomal gene *eae*, which is part of a large cluster of virulence genes on a pathogenicity island termed the locus for enterocyte effacement (LEE) (KAPER et al., 1998).

There have been recent reports suggesting that antimicrobial resistance (AMR) levels in STEC is increasing (GALLAND et al., 2001; MENG et al., 1998). The natural evolution of bacteria has been changed by antibiotics use. In specific situations, there is a great probability of association between the presence of virulence factors and antimicrobial resistance (NAGASHINTA et al., 2008). It occurs because both AMR and virulence genes are carried in a similar fashion, may be linked and then co-selected (MARTINEZ & BAQUEIRO, 2002). When the association between the presence of virulence factors and antimicrobial resistance happens, the antimicrobial use may potentially enhance the selection of bacteria carrying virulence genes, accelerating the spread of virulence genes within bacterial populations (BOERLIN et al., 2005). Contrary, some studies have reported that the acquisition of antimicrobial resistance by some bacterial strains may have a fitness cost which leads to decreased virulence (CALHAU et al., 2013; CLARK et al., 2012).

Gow & Waldner (2009) demonstrated that antimicrobial resistance is not substantially more likely to be identified in *stx* positive than in *stx*-negative *E. coli* isolates from healthy beef calves. However, there are dearth information in the literature if this association occurs at dairy cow herds and also if there is an association between serogroups of *E. coli* with both *stx* and *eae* genes. Therefore, the aims of this study were to investigate if the antimicrobial resistance phenotype was associated with the occurrence of virulence genes, and also investigate if the presence or absence of virulence genes is associated with the serogroups of *E. coli* isolated from dairy cows.

MATERIAL AND METHODS

Fecal samples were collected in 10 different farms located in São Paulo State, Brazil, from January 2012 to February 2013. From each farm, faeces from the rectum of 80 animals were sampled using sterile swabs. In each farm, the samples were collected in a single day and represented more than 50% of each population at the time of sampling. Isolates were obtained by directly spreading the samples on MacConkey agar and sorbitol MacConkey agar. After incubation, a minimum of three colonies from each plate were analyzed biochemically (KONEMAN et al., 2001).

Up to two *E. coli* isolates from each fecal sample were characterized using PCR. To perform PCR, the DNA template was obtained using a thermal cell lysis procedure (KESKIMAKI et al., 2001) and *stx1*, *stx2* and *eae* were collected using primers and PCR conditions describe by China et al. (1996).

Antimicrobial disk susceptibility tests were performed using the disk diffusion method recommended by Clinical Laboratory Standard Institute (WATTS et al., 2002). Drug-impregnated disks (CEFAR, São Paulo, Brazil, accessed in 2014) were placed on the surface of Muller-Hinton agar using a disk dispenser.

The serogroup was determined by standard agglutination methods (EDWARDS & EWING, 1972). The serogroups were either commercially prepared (Probac, Brazil). The following "O" antigens, which include most of the serogroups reported in cow or associated with severe enteric-disease in humans, were used: O5, O6, O8, O9, O20, O26, O55, O75, O86, O91, O101, O146, O149, O153, O157, O158 and O172.

Chi-square and Duncan exact tests were performed for the analysis of associations using software R version 2.12.0 (http://www.r-project.org/) accessed in 2014. A P value of $p<0.01$ was considered to be statistically significant.

RESULTS AND DISCUSSION

From 800 samples collected of dairy cow herds, it was isolated 561 *E. coli* strains. From these strains, 90 (16.0%) harbored only *stx1*, 97 (17.3%) harbored only *stx2* and 45 (8.0%) harbored only *eae*. Thirty seven *E. coli* strains (6.6%) harbored both *stx1* and *stx2*; 110 (19.6%) harbored *stx1* and *eae*; and 67 (11.9%) harbored *stx2* and *eae*. Interestingly no strain was harbored to three genes, *stx1*, *stx2* and *eae* together. The number of *E. coli* strains harboring at least one virulence gene was 446 (79.5%) and the number of strains presenting no gene was 115 (20.5%) (Table1).

Table 1. Frequency of *Escherichia coli* isolates harbored at least one virulence factor for Shiga like toxin (STEC) followed by total of strains harbored of genes for STEC, total of strains without genes for STEC, total of *E. coli* isolates, total of samples without *E. coli* and total of samples collected

Virulence gene *Eschericia coli*	Number of isolates	Frequency isolates (%)
stx1	90	16.0
stx2	97	17.3
eae	45	8.0
stx1+stx2	37	6.6
stx1+eae	110	19.6
stx2+eae	67	11.9
Number of isolates with genes	446	79.5
Number of isolates without genes	115	20.5
Total of isolates	561	100.0
Total of samples without gene	239	29.9
Total of samples isolates of the total collected	800	70.13

The frequencies of resistance to antimicrobial agents were verified with all *E. coli* strains carrying a single virulence factor and also with all strains carrying no virulence factor. The 90 *E. coli* isolates that carried *stx1* only showed highest antimicrobial resistance to streptomycin (85.6%), followed by kanamycin (72.2%) and nalidixic acid (70.0%). For this same group, the lowest antimicrobial resistances levels were for

cephalotin (20.0%), ampicillin (30.0%) and cefoxitin (35.6%) (Table2).

For *E. coli* strains carrying *stx2* only, highest antimicrobial resistance levels were related to streptomycin (73.2%), followed by kanamycin and nalidixic acid (70.1%) and the lowest antimicrobial resistance levels were due to cephalotin (14.4%), ampicillin (29.9%) and amoxicillin – clavulanic acid (33.0%) (Table 3).

Table 2. Frequency of resistance to antimicrobial agents among *E. coli* Shiga like toxin (STEC) isolates harboring *stx1* from dairy-cow herds (n=90)

Antimicrobial agent	Percentage of resistance - % (-number of resistant/total number of isolates)
Ampicillin	30.0 (27/90)
Amoxicillin – clavulanic acid	40.0 (36/90)
Cefoxitin	35.6 (32/90)
Ceftriaxone	45.6 (41/90)
Cephalothin	20.0 (18/90)
Streptomycin	85.6 (77/90)
Kanamycin	72.2 (65/90)
Gentamicin	55.6 (50/90)
Amikacin	67.8 (61/90)
Tetracycline	38.9 (35/90)
Nalidixic acid	70.0 (63/90)

Table 3. Frequency of resistance to antimicrobial agents among *E. coli* Shiga like toxin (STEC) isolates harboring *stx2* from dairy-cow herds (n=97)

Antimicrobial agent	Percentage of resistance (nº of resistant/total nº of isolates)
Ampicillin	29.9 (29/97)
Amoxicillin – clavulanic acid	33.0 (32/97)
Cefoxitin	40.2 (39/97)
Ceftriaxone	46.2 (45/97)
Cephalothin	14.4 (14/97)
Streptomycin	73.2 (71/97)
Kanamycin	70.1 (68/97)
Gentamicin	60.8 (59/97)
Amikacin	67.0 (65/97)
Tetracycline	32.0 (31/97)
Nalidixic acid	70.1 (68/97)

For *E. coli* strains harboring only *eae*, amikacin (77.8%), nalidixic acid (75.6%) and streptomycin (73.3%) were the antimicrobials with the highest resistance levels. For this group, the lowest antimicrobial resistance levels were due to cephalotin (15.6%), tetracycline (24.4%) and ampicillin (26.7%) (Table 4).

For the *E. coli* strains with no virulence factor, the highest antimicrobial resistance levels were to streptomycin (78.2%), followed by kanamycin and nalixic acid (74.1%), amikacin (64%). For the same group, the lowest antimicrobial resistance levels were to cephalotin (17.4%),

ampicillin (32.9%) and amoxicillin – clavulanic acid (36.0%) (Table 5).
Among the groups presenting only one virulence factor and the group presenting no virulence factor, there was no statistical difference (p>0.01) when comparing their antimicrobial resistance patterns.

Table 4. Frequency of resistance to antimicrobial agents among *E. coli* Shiga like toxin (STEC isolates harboring *eae* from dairy-cow herds (n=45).

Antimicrobial agent	Percentage of resistance (nº of resistant/total nº of isolates)
Ampicillin	26.7 (12/45)
Amoxicillin – clavulanic acid	28.9 (13/45)
Cefoxitin	44.4 (20/45)
Ceftriaxone	46.7 (21/45)
Cephalothin	15.6 (7/45)
Streptomycin	73.3 (33/45)
kanamycin	64.4 (29/45)
Gentamicin	48.9 (22/45)
Amikacin	77.8 (35/45)
Tetracycline	24.4 (11/45)
Nalidixic acid	75.6 (34/45)

Table 5. Frequency of resistance to antimicrobial agents among ordinary *E. coli* isolates without *stx*1, *stx*2 or *eae* from dairy-cow herds (n=115)

Antimicrobial agent	Percentage of resistance (nº of resistant/total nº of isolates)
Ampicillin	32.9 (38/115)
Amoxicillin – clavulanic acid	36.0 (41/115)
Cefoxitin	45.5 (52/115)
Ceftriaxone	41.2 (47/115)
Cephalothin	17.4 (20/115)
Streptomycin	78.2 (90/115)
Kanamycin	74.1 (85/115)
Gentamicin	68.9 (79/115)
Amikacin	64.0 (74/115)
Tetracycline	38.0 (44/115)
Nalidixic acid	74.1 (85/115)

Table 6 shows the frequency of serogroups of *E. coli* strains that harbour *stx1*. The serogroups detected were O119 (58.0%), O114 (17%), O111 (9.0%), O26, O126, O127, O55 (4.0%). For *E. coli* strains that carry *stx2*, the serogroups and frequencies were O9 (57.0%), O8 (32.0%), O101 (9.0%), O20 (2.0%). For *eae*+ *E. coli* strains, the serogroups and frequencies were O9 (34.0%), O8, O127 (22.0%), O119, O20 (11.0%) and for *E. coli* strains negative for the virulence genes, the serogroups and frequencies presented were O9 (19.0%), O119 (16.0%), O114 (17.0%), O86 (6.0%), O125 (5.0%), O126 (3.0%), O101, O26, O127, O128 (2.0%) and O20 (1.0%). The comparison between the presence of serogroups with the presence or absence of virulence genes did not show correlation.

Table 6. Frequency of serogroups of *E. coli* strains carrying *stx1*, *stx2*, *eae* genes or none of these genes

E. coli strains carriers of *stx1* gene (n=90)	
Serogroup	Frequency (%)
O119	58.0
O114	17.0
O111	9.0
O26 O126, O127, O55	4.0
E. coli strains carriers of *stx2* gene (n=97)	
O9	57.0
O8	32.0
O101	9.0
O20	2.0
E. coli strains carriers of *eae* gene (n=45)	
O9	34.0
O8, O127	22.0
O119, O20	11.0
E. coli strains with none of the virulence genes (n=115)	
O9	19.0
O119	16.0
O114	17.0
O86	6.0
O125	5.0
O126	3.0
O101, O26, O127, O128	2.0
O20	1.0

No statistically significant association was detected between any AMR phenotype and virulence genes and neither between virulence genes and serogroups in this *E coli* population isolated of dairy-cow herds. The antimicrobial resistance patterns between the isolates carriers of at least one virulence gene or lacking any virulence gene were not different. The results showed that, the presence or absence, and the type of virulence gene did not interfere with the antimicrobial resistance level and also did not favor the selection of resistance genes (Tables1-6). Gow & Waldner (2009) verified no association between antimicrobial resistance measured phenotypically or the presence of AMR genes and virulence genes (*stx1*, *stx2* and *eae*) in STEC.

The lack of association between AMR and *stx* virulence genes may be explained, at least in part, by the requirement for a specific receptor for phage attachment and also the limited amount of DNA incorporated into a phage head and by specific mechanism adopted by bacteriophages to incorporate the bacterial DNA in its genome (SCHWARTS et al., 2006). The mechanisms by which these virulence and resistance genes are transmitted from bacteria to bacteria are determinants for occurrence of these associations (ACHESON et al., 1998; MUNIESA, et al., 2004; NEELY et al., 1998).

Gow & Waldner (2006) analyzed 106 fecal generic *E. coli* isolates from calves in cow-calf herds and the most common virulence gene detected was *stx2* followed by *eae*. In our study the most common virulence gene detected was *stx1* followed by *stx2* (Table 1). Also, in our study, all antimicrobial resistance levels were measured via phenotype rather than genotype. This measurement can lead to some differences. On this account, the presence of unexpressed

genes would not be measured. However, Gow & Waldner (2009) measured the antimicrobial resistance level of their isolates phenotypically and also genotypically and did not find difference between both methods.

When the association between antimicrobial resistance and virulence factor occurs, as demonstrated with other genes in previous works (BOERLIN et al., 2005, NAGACHINTA et al., 2008), it becomes more beneficial for the spread of pathogenic bacteria. The association between these factors depends on four main determinants: the bacterial species involved, virulence and resistance mechanisms, the ecological niche, and the host (BECEIRO et al., 2013).

Pereira et al. (2011) analyzed 117 fecal samples from dairy calves divided into two groups, one group received growth promoting antimicrobials (GPA) through milk and another group (NGPA) did not. The results of this study showed that NGPA group had statistically significantly lower levels of antimicrobial resistance for most of the antimicrobials tested than GPA group. Also, there was no association between virulence factors and NGPA or NGA and neither between antimicrobial resistance and virulence factors. Likely, the use of antimicrobials selects some resistance genes, but not virulence genes. Even though, in this and previous studies, there was no association between virulence factors and antimicrobial resistance. This association could take place when the virulence factors and antimicrobial resistance genes are carried in a similar fashion. In this case, it is possible that they could be linked and then co-selected (MARTINEZ & BAQUEIRO, 2002). Calhau et al. (2013) found a profile of virulence and resistance more prevalent in *E. coli*. They suggest that these features may be responsible for making them concomitantly virulent and extremely resistant. Nagachinta et al. (2008) found an association between virulence factors and antimicrobial resistance in *E. coli* strains isolated from dairy cows. However, it is important to point out that these studies have analyzed others genes different than *stx1, stx2* and *eae*.

Concerning the presence of serogroups, even though there had been the prevalence of O9 between *E. coli* strains carriers of *stx2* and *eae*, there was also the prevalence of the same serogroup between the strains with no virulence factor, suggesting that there was no association between serogroups and virulence factors in this study.

The results did not show relationship between virulence factor and antimicrobial resistance when this association occurs the antimicrobial resistance might be selected through the antimicrobial use and thereby the virulence factor would be selected as well. Probably the mechanism of transfer from one bacterium to another does not permit the association with antimicrobial resistance genes.

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