ANTIMICROBIAL RESISTANCE IN BRAZILIAN ISOLATES OF SHIGA TOXIN-ENCODING *ESCHERICHIA COLI* FROM COWS WITH MASTITIS

RESISTÊNCIA A AGENTES ANTIMICROBIANOS EM CEPAS BRASILEIRAS DE ESCHERICHIA COLI CODIFICADORA DE SHIGA TOXINA ISOLADAS DE VACAS COM MASTITE

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SUMMARY

Antimicrobial resistance is a matter of growing concern in public and animal health. Public health can be threatened by the transfer of pathogens from animals to people via indirect contact through food or directly by contact with animals. The potential transfer of resistance determinants from animals to humans through *Escherichia coli* strains is an additional cause of concern. From February to November 2004, 670 samples of bovine mastitic milk were collected in two Brazilian states. The 231 *E. coli* strains isolated from the samples were screened for the presence of genes encoding Shiga toxin (*stx* 1 and *stx* 2) and intimin (*eae*). Twenty (8.6%) strains were shown by PCR to harbor the Shiga toxin genes (8 harbored the *stx* 1 gene, 12 the *stx* 2 gene and none both of them). Two (0.8%) strains were *eae* positive; however, they did not carry the *stx* 1 or *stx* 2. These strains were also screened for resistance to 12 antimicrobial agents. Predominant resistance was to amikacin (60.0%), gentamicin (50.0%), streptomycin (50.0%) and ampicillin (45.0%). Multidrug resistance was found among 7 isolates (35.0%). These results indicate that dairy cattle from the region surveyed may be a source of STEC potentially pathogenic for humans.

KEY-WORDS: *Escherichia coli.* STEC strains. Antimicrobial resistance. Bovines. Mastitis.

RESUMO

A existência de resistência aos agentes antimicrobianos entre as bactérias tem-se revelado um sério problema para a saúde publica e para a saúde animal. A saúde publica pode ser comprometida pela transferência de bactéria patogênica proveniente dos animais para as pessoas através de um contato indireto pelos alimentos ou através de um contato direto com os animais. A possível transferência de genes de resistência a antimicrobianos entre a microbiota animal e a microbiota humana através da bactéria *Escherichia coli* representa um motivo adicional de apreensão. De Fevereiro a Novembro de 2004 foram coletadas 670 amostras de leite obtido de bovinos que apresentavam mastite provenientes de dois estados brasileiros. As 231 cepas de *E. coli* isoladas destas amostras de leite foram analisadas para a verificação da presença dos genes codificadores de Shiga toxina (*stx* 1 e *stx* 2) e da intimina (*eae*). Através da técnica de PCR foram detectadas 20 cepas (8,6%) de *E. coli* carregando os genes de Shiga toxina (8 apresentavam o gene *stx* 1 e 12 apresentavam o gene *stx* 2, e nenhuma das cepas apresentava os dois genes). Duas cepas carregavam o gene *eae* (0,8%), no entanto elas não apresentavam o gene *stx* 1 nem *stx* 2. Estas linhagens também foram analisadas em relação a sua resistência frente a 12 agentes antimicrobianos. Resistência foi predominantemente determinada para amicacina (60,0%), gentamicina (50,0%), estreptomicina (50,0%) e ampicilina (45,0%). Resistência a múltiplas drogas foi determinada em 7 destas cepas. Estes resultados indicaram que o gado leiteiro das regiões avaliadas pode representar uma fonte de disseminação de cepas STEC potencialmente patogênicas para os seres humanos.

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INTRODUCTION

Economically, mastitis is considered the most important disease of dairy cattle and a major cause of financial loss to the dairy industry (HORTET & SEEGERS, 1998). Classically, mastitis pathogens have been divided into contagious and environmental organisms on the basis of their proclivity to cause persistent or transient opportunistic infections, respectively (WATTS, 1988). Environmental mastitis caused by coliform bacteria has increased in many countries and herds while at the same time contagious mastitis has been successfully controlled (BURVENICH et al., 2003). The majority of coliform bacteria characterized as *Escherichia coli* originates in the cow's fecally contaminated environment and infects the udder via the teat canal (EBEHART, 1984). Mastitis triggered by *E. coli* is usually sporadic and the clinical signs vary from very severe, even fatal forms, to mild mastitis, the cows only showing local udder signs (WENZ et al., 2001).

Shiga toxin-producing *E. coli* (STEC) strains are the most important among the recently emerged group of food borne microorganisms. They are a major cause of gastroenteritis that may be complicated by hemorrhagic colitis (HC) or the hemolytic uremic syndrome (HUS), a major cause of acute renal failure in children (BEUTIN et al., 2004). Domestic ruminants, especially cattle, sheep and goats, have been implicated as the main reservoirs of STEC strains causing human infections (ZSCHOCK et al., 2000). Transmission occurs through consumption of undercooked meat, non-pasteurized dairy products and vegetables contaminated by feces (CAPRIOLI et al., 2005). In developed countries serotype O157:H7 represents the major cause of human disease. However, there have been increasing reports of non-O157 STEC strains associated with gastrointestinal infections (LOCKARY et al., 2007). In addition to toxin production, another virulence-associated factor expressed by STEC is a protein called intimin. The protein is encoded by the chromosomal gene *eae* (NATARO & KAPER, 1998) and associated with attaching and effacing lesions and bacterial adherence to epithelial cells.

Food production of animal origin is currently dependent on the use of large amounts of veterinary drugs for disease control. This provides favorable conditions for selection, spread and persistence of antimicrobial-resistant bacteria. During the last decade there has been an increasing awareness of potential problems to human health caused by the selection of antibiotic-resistant organisms in food producing animals (AARESTRUP, 2005). Severe public health implications that may lead to treatment failures including death and prolonged illness could be the result of this enhanced resistance in addition to the associated costs.

The aim of the present study was to analyze STEC strains isolated from cows with mastitis in two Brazilian states to determine the presence of virulence genes (*stx* 1, *stx* 2 and *eae*) and their susceptibility to 12 antimicrobial drugs.

MATERIAL AND METHODS

Milk samples (670) from cows in two Brazilian states (Minas Gerais and Rio Grande do Sul) were aseptically collected from 37 dairy farms between February and November 2004. Teat ends were cleaned using 70.0 % alcohol-moistened swabs and allowed to dry. After discarding the first few streams, 2-4ml of the milk samples were collected into sterile 10 ml glass flasks, and submitted to the California Mastitis Test (CMT) (SCHALM & NOORLANDER, 1957) using a 1-5 scale (KLASTRUP, 1975). CMT-positive samples were refrigerated to about 4^{o}C and immediately delivered to the laboratory for plating on MacConkey Agar (Mac-Difco) and incubated for 24h at 37^{o}C. At least five colonies from each plate were selected for biochemical confirmation of the strains. *E. coli* was defined as oxidase negative, indole positive, Simon's citrate negative, urease negative and hydrogen sulfide negative (KONEMAN et al., 1997).

Bacterial strains (*E. coli* isolates) grown overnight in nutrient broth (Sigma Chemical Co, St Louis, USA) at 37 ^{o}C were tested for the presence of *stx* (*stx* 1 and *stx* 2) and *eae* genes using the polymerase chain reaction (PCR) protocol of China et al. (1996). DNA templates were prepared by pelleting 1 ml of culture enriched by centrifugation at 12000g. The cell pellet was resuspended in 250µl of sterile distilled water and boiled for 10 min at 100 ^{o}C, again centrifuged and their supernatants subjected to PCR performed in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). *Stx* 1, *stx* 2 and *eae* genes were detected using primers and PCR conditions in the above-mentioned protocol. The amplified DNA products were separated by electrophoresis on 1.5% agarose gel, stained with ethidium bromide and detected under ultraviolet light. Reference *E. coli* strains used as controls were EDL 933 (O157:H7, *stx*1, *stx* 2, *eae*) and DH5α (negative control). The STEC isolates were typed for the O serotype O157 using the O157 Latex Agglutination test kit (Oxoid, Basingstoke, Hampshire, UK). The EDL 933 strain was used as a positive control. Strains negative for agglutination were considered non-O157 strains.

Antimicrobial susceptibility tests were performed using the disk diffusion method recommended by the NATIONAL COMMITTEE FOR CLINICAL LABORATORY STANDARDS (NCCLS 1999, 2001). Drug-impregnated disks (CEFAR, São Paulo, BR) were placed on agar surfaces using a disk dispenser. The following twelve antimicrobial agents were tested: ampicillin (AMP,10µg); amoxicillin (AMO,10µg); tetracycline (TET, 30µg); amoxicillin/clavulanic acid (AMC,30µg); amikacin (AMK,30µg); cephalothin (CFL,30µg); ceftriaxone (CEF,30µg); gentamicin (GEN,5µg); streptomycin (STR,10µg); nalidixic acid (NAL,30µg); cotrimoxazole (COT, 25µg); ciprofloxacin (CIP,5µg).

RESULTS

Two hundred and thirty one *E. coli* strains were isolated from 51 milk samples positive for *E. coli* of

670 mastitc milk samples analyzed. All *E. coli* isolates were investigated by PCR, for the presence of genes encoding Shiga toxin (*stx* 1 and *stx* 2) and intimin (*eae*). As can be seen from Table 1, 22 (9.5 %) of the strains carried the *stx* and/or the *eae* genes. PCR showed that 8 (3.4 %) of STEC strains carried only the *stx* 1 gene, 12 (5.2 %) the *stx* 2 gene, and none carried both genes; two strains carried only the *eae* gene. All STEC strains isolated were tested by the O157 latex agglutination test kit, and no one O 157 isolate was detected. The patterns and phenotypes of antimicrobial resistance amongst these 20 STEC strains were also shown in Table 1 and Table 2. The highest resistance among the 20 STEC isolates was observed against amikacin (60.0%), followed by gentamicin (50.0%), streptomycin (50.0%) and ampicillin (45.0%). Low resistance was determined to cotrimoxazole (5.0%), amoxicillin (10.0%), amoxicillin/clavulanic acid (10.0%) and ciprofloxacin (10.0%).

Table 1 - Antimicrobial drug resistance and virulence gene profiles in STEC strains isolated from cows with mastitis in two Brazilian states, between February-November 2004.

Isolate	Virulence factor	Resistant phenotype
2	stx 2	*
5	stx 2	GEN, AMK**
11	stx 2	AMP, TET, GEN, STR, AMK
30	stx 1	TET, STR, AMK, NAL
32	stx 1	NAL, CFL
35	stx 1	COT, GEN
36	stx 1	AMP, AMO, GEN, AMK, NAL, CIP
38	stx 2	AMP, AMO, TET, GEN, STR, AMK
39	stx 2	CFL, GEN, STR, AMK
42	stx 2	*
51	stx 1	AMP, NAL
52	stx 1	AMP, TET, GEN, STR, AMK, CIP
80	stx 1	GEN
115	stx 2	AMC, TET, GEN, STR, AMK
117	stx 2	*
130	stx 1	STR, AMK
132	stx 2	AMP, AMC, CFL, TET, AMK
138	stx 2	AMP, GEN, STR
142	stx 2	AMP, STR, AMK
160	stx 2	AMP, STR, AMK
186	eae	*
192	eae	*

* susceptible to all antimicrobials ** Resistant phenotype: AMP- ampicillin, AMO- amoxicili, AMC- amoxicillin/clavulanic acid, AMK – amikacin, CFL- cephalothin, CEF- ceftriaxone, GEN- gentamicin, STR- streptomycin, NAL- nalidixic acid, COT- Cotrimoxazole, CIP- ciprofloxacin..

Table 2. Antimicrobial susceptibly testing of 20 STEC strains isolated from cows with mastitis in two Brazilian states, between February-November 2004.

Antimicrobial drug	Number of resistant strains* (%)	Number of sensitive strains (%)
Ampicillin	9 (45)	11 (55)
Amoxicillin	2 (10)	18 (90)
Amoxicillin/clavulanic acid	2 (10)	18 (90)
Cephalothin	3 (15)	17 (85)
Ceftriaxone	0 (0)	20 (100)
Tetracycline	6 (30)	14 (70)
Gentamicin	10 (50)	10 (50)
Streptomycin	10 (50)	10 (50)
Amikacin	12 (60)	8 (40)
Nalidixic acid	4 (20)	16 (80)
Ciprofloxacin	2 (10)	18 (90)
Cotrimoxazole	1 (5)	19 (95)

* Intermediate resistant strains were considered as resistant.

DISCUSSION

Cattle have long been regarded as the main reservoir of STEC strains (ZSCHOCK et al., 2000). While *E. coli* O157:H7 is the dominant STEC responsible for human infections in the United States and Canada, evidence suggests that non-O157 STEC may also cause human infections (JOHNSON et al., 2006). In Brazil, only few studies reported the isolation and characteristics of STEC in cattle (LEOMIL et al., 2003, IRINO et al., 2005, OLIVEIRA et al., 2008), all of them from healthy or diarrheic cattle. LIRA et al. (2004) reported the isolation of STEC strains from mastitic milk with a similar frequency of *stx* 2 gene, in agreement with the results reported in the present study.

The STEC strains seem to be pathogenic to humans only if they possess accessory virulence factors. The *eae* gene, responsible for attachment and effacement lesions is present in most human STEC strains belonging to enterohemorrhagic serotypes. In this study the *eae* gene was not detected in STEC isolates. The low prevalence of *eae*-positive STEC has been previously reported (ZSCHOK et al., 2000, IRINO et al., 2005). However, in this study the small percentage of *E. coli* strain (0.8 %) (Table 1) *eae*- positive did not harbor *stx* genes as confirmed by other authors who also detected *eae*-positive non-STEC strains (KOBORI et al., 2004). The pathogenicity of *eae*-positive non-STEC in calves is not clear, but Fischer et al. (1994) showed that an *eae*-positive verotoxigenic-negative strain was able to experimentally induce the attaching and effacing lesion.

The emergence of antimicrobial resistance among pathogens that impact animal health has been a growing concern in veterinary medicine (WHITE & MCDERMOT, 2001). *E. coli*, one of them is commonly found in the gastrointestinal tracts of humans and animals. Various selective pressures in their environment favor the development, persistence and dissemination of robust strains some of which may be resistant to antimicrobial agents. In this study antimicrobial resistance to several classes of antimicrobials was observed (including β-lactams, aminoglycosides, sulphonamides, quinolones, tetracyclines and cephalosporins). These findings are supported by other authors (MAIDHOF et al., 2002, LIRA et al., 2004) confirming that resistance to a broad variety of antimicrobials classes can occur in STEC. Among the 20 STEC isolates analyzed (35.0% of which were MDR) three showed resistance to six antimicrobials in agreement to other reports (MAIDHOF et al., 2002).

Results in the present study revealed that most STEC isolates were resistant to aminoglycosides including amikacin, gentamicin and streptomicin (Table 2) as reported by others (MORA et al., 2005). Tetracycline resistance was also found in 30.0% of isolates, which is consistent with Zhao et al. (2001) and Mora et al. (2005), who reported tetracycline resistance in 43.0% and 32.0% STEC isolates, respectively. In contrast, there are reports that STEC isolates from cows with mastitis are sensitive to gentamicin (LIRA et al., 2004).

Among quinolones, resistance was found in 2 (10%) and 4 (20.0%) STEC isolates, respectively for ciprofloxacin and nalidixic acid agreeing with Lira et al. (2004). Resistance of *E. coli* isolated from cows with mastitis to antimicrobials used in human medicine is consistent with Turnidge (2004) who reported that resistance to antimicrobials of human importance has been generated in some food-producing animals.

Because antimicrobial resistance is a threat to public health, animal health, and food safety more research is needed for a better understanding of the distribution of both pathogenic and non-pathogenic bacteria, their resistance profiles and their abilities to, and mechanisms for, transferring resistance. A strong control of antimicrobial drug commercialization and access to data related to resistance by the pathogens responsible for bovine mastitis is urgently needed in Brazil.

To conclude, the present study showed the presence of STEC strains in bovine mastitis, showing a high level of antimicrobial resistance and an elevated degree of multiresistance, what is a reason for concern due to the usual consumption of raw milk or raw milk products by the Brazilian population in the rural areas without a heat treatment for the milk.

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