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Analysis of *Escherichia coli* isolated from bovine mastitic milk[1]

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ABSTRACT.- Rangel P. & Marin J.M. 2009. **Analysis of *Escherichia coli* isolated from bovine mastitic milk.** *Pesquisa Veterinária Brasileira 29(5):363-368.* Departamento de Morfologia, Estomatologia e Fisiologia, Faculdade de Odontologia de Ribeirão Preto, Universidade de São Paulo, Avenida do Café s/n, Campus USP, Ribeirão Preto, SP 14040-904, Brazil. E-mail: jmmarin@forp.usp.br

Mastitis has been recognized for some time as the most costly disease in dairy herds. From February to November 2004, 670 samples of bovine mastitic milk from which 231 *Escherichia coli* strains were isolated, were collected from two Brazilian states. The strains were screened for the presence of Shiga toxin-producing (*stx* 1 and *stx* 2) and intimin (*eae*) genes. Twenty (8.6%) strains were detected by PCR to harbor the Shiga toxin genes (8 the *stx* 1 gene, 12 the *stx* 2 gene and none both of them). Two (0.8%) of the *Escherichia coli* strains studied were *eae* positive non Shiga toxin-producing. The strains were also examined for resistance to 12 antimicrobial agents. The predominantly observed resistance was to tetracycline (92.2%), streptomycin (90.4%), nalidixic acid (88.3%), amikacin (86.5%) and cephalothin (84.8%). Multidrug resistance was found among 152 isolates (65.8%).

INDEX TERMS: *Escherichia coli*, mastitis, antimicrobial agents, multidrug resistance, STEC, *eae* gene.

RESUMO.- [**Análise de *Escherichia coli* isolada de leite de vacas com mastite.**] A um longo tempo a mastite tem sido reconhecida como a doença que provoca as maiores perdas econômicas nos rebanhos leiteiros. De fevereiro a novembro de 2004, foram coletadas 670 amostras de leite mastítico de vacas, provenientes de dois estados brasileiros, das quais foram isoladas 231 cepas de *Escherichia coli*. Estas cepas foram analisadas para a detecção dos genes de produção de Shiga toxina (*stx* 1 e *stx* 2) e do gene da intimina (*eae*). Vinte cepas (8,6%) foram detectadas através de PCR como contendo os genes da Shiga toxina (8 *stx* 1, 12 *stx* 2 e nenhuma delas ambos os genes). Duas cepas (0,8%) de *E. coli* eram *eae* positivo não produtoras de Shiga toxina. As cepas de *E. coli* foram também examinadas para detectar a resistência a 12 agentes antimicrobianos. As resistências mais freqüentes foram para tetraciclina (92,2%), estreptomicina (90,4%), ácido nalidixico (88,3%), amicacina (86,5%) e cefalotina (84,8%). A resistência a múltiplas drogas foi encontrada em 152 cepas (65,8%).

TERMOS DE INDEXAÇÃO: *Escherichia coli*, mastite, agentes antimicrobianos, resistência a múltiplas drogas, STEC, gene *eae*.

INTRODUCTION

Mastitis remains a major cause of financial loss to the dairy industry and is considered to be economically the most important disease of dairy cattle (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).

Dairy cattle with acute mastitis, caused primarily by *Escherichia coli*, that is classified for this case, as an environmental pathogen, produces a wide range of symptoms, going from a mild disease showing only local

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inflammatory changes of the mammary gland, to a severe form presenting significant systemic signs including rumen stasis, dehydration, shock, and even death (Wenz et al. 2001). The host defense of the bovine mammary gland has been shown to be efficient in controlling and eliminating *E. coli* infection (Hill et al. 1979); however, this ability has been shown to be less effective during early lactation, due to deficiencies in neutrophil function and number (Shuster et al. 1996).

Numerous studies to identify virulence factors of *E. coli* isolated from cows with clinical mastitis have been conducted (Barrow & Hill 1989, Kaipainen et al. 2002). Typically, most of the genes found in mastitis strains did not possess any of the virulence genes evaluated (Wenz et al. 2006). The only virulence characteristic consistently associated with *E. coli* isolated from clinical mastitis cows, was serum resistance (Hill 1994).

Shiga toxin-producing *E. coli* (STEC) strains are considered to be the most important pathogens between a recently emerged group of food borne strains. This type of strain is a major cause of gastroenteritis and also can be responsible for hemorrhagic colitis (HC) or the hemolytic uremic syndrome (HUS), the major cause of acute renal failure in children (Karmali 1989, Paton & Paton 1998, Beutin et al. 2004). Domestic ruminants, especially cattle, sheep and goats, have suggested to be the principal reservoirs of STEC strains that cause human infections (Zschock et al. 2000, Chapman et al. 2001). Transmission would occurs through consumption of undercooked meat, non-pasteurized dairy products, vegetables and water contaminated by feces containing STEC strains (Paton & Paton 1998, Caprioli et al. 2005). The STEC strain most frequently associated with clinical disease in the United States and Europe is serotype O157:H7 (Nataro & Kaper 1998, Caprioli et al. 2005). Nevertheless, since non-O157 STEC strains are the most prevalent ones in animals and as food contaminant, humans are probably more exposed to these strains (Beutin et al. 2004, Blanco et al. 2004). In addition to toxin production, another virulence-associated factor expressed by STEC is a protein called intimin, associated with attaching and effacing lesions and bacterial adherence to epithelial cells; intimin is encoded by the chromosomal gene *eae* (Nataro & Kaper 1998).

An acceleration of the emergence of multidrug resistant pathogens has been occurring over the past 10 to 15 years (Shea 2003). Resistance of some bacteria to most of the antimicrobial agents, has become a growing problem in human medicine and in veterinary medicine (Levy 1998). Observations have suggested that the use of antibiotics in animal husbandry is a driving force for the development of antibiotic resistance of certain pathogenic bacterial species (Witte 1998). The use of antibiotics in animal agriculture has been a controversial issue due to the potential transfer of antibiotic resistance from animals to humans. This could have several public health implications that may cause treatment failure, including death and illness prolongation, as well as increasing in the associated costs (Kelly et al. 2004).

The aim of the present study was to determine the virulence genes (*stx* 1, *stx* 2 and *eae*) and the susceptibility to 12 antimicrobial drugs of 231 *E. coli* strains isolated from mastitic milk proceedings from two Brazilian states.

MATERIALS AND METHODS

Bacterial isolates. Milk samples (670) from cows from thirty-seven dairy farms at two Brazilian states (Minas Gerais and Rio Grande do Sul) were aseptically collected between February and November 2004. Teat ends were cleaned using 70.0% alcohol-moistened swabs and allowed drying. After discarding the first few streams, 2-4ml of the milk samples was 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 4ºC and immediately delivered to the laboratory for analysis. They were plated on MacConkey Agar (Mac-Difco) and incubated for 24h at 37ºC. At least five colonies from each plate were selected for analysis. Biochemical confirmation of the strains was performed and *E. coli* was defined as oxidase negative, indole positive, Simon's citrate negative, urease negative and hydrogen sulfide negative (Koneman et al. 1997).

Determination of *stx* genes. Bacterial strains (*Escherichia coli* isolates) grown overnight in nutrient broth (Sigma Chemical Co, St Louis, USA) at 37ºC were tested for the presence of *stx* genes (*stx* 1 and *stx* 2) using the polymerase chain reaction (PCR) protocol of Orden et al. (1998). DNA templates were prepared by pelleting 1 ml of culture enriched by centrifugation at 12000g. The cell pellet was resuspended into 250µl of sterile distilled water and boiled for 10min at 100ºC, again centrifuged and their supernatants subjected to PCR performed in an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany). *Stx* 1 and *stx* 2 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 DH5á (negative control).

Characterization of isolates. Isolates were confirmed as *stx*+ and tested for accessory virulence marker (*eae*) using the PCR protocol of China et al. (1996).

O157 latex agglutination. 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. Negative strains for agglutination were considered non-O157 strains.

Susceptibility testing. Antimicrobial disk susceptibility tests were performed using the disk diffusion method recommended by the National Committee for Clinical Laboratory Standards (NCCLS 1999). Drug-impregnated disks (Cefar, São Paulo, Brazil) were placed onto the surface of the agar using a disk dispenser. The following twelve antimicrobial agents were tested: ampicillin (AMP,10ìg); amoxicillin (AMO,10ìg), amoxicillin/clavulanic acid (AMC,30ìg); amikacin (AMK,30ìg); cephalothin (CFL,30ìg); ceftriaxone (CEF,30ìg); gentamicin (GEN,5ìg); streptomycin (STR,10ìg); nalidixic acid (NAL,30ìg); cotrimoxazole (SUT, 25ìg); ciprofloxacin (CIP,5ìg).

RESULTS

A total of 231 *Escherichia coli* strains were isolated from 51 mastitic milk samples positive for *Escherichia coli*, from

Table 1. Virulence genes profile of *Escherichia coli* isolates from mastitic milk obtained from two Brazilian states between February and November, 2004

Virulence factor profile	Number of isolates (%)	STEC
stx 1	8 (3.4)	+
stx 2	12 (5.2)	+
eae	2 (0.8)	Non STEC strains

Table 2. Antimicrobial susceptibility of 231 *Escherichia coli* strains isolated from mastitic milk from two Brazilian states from February to November 2004

Antimicrobial drugs	Number of resistant strains[a] (%)	Number of sensitive strains* (%)
Ampicillin	137 (58.3)	94 (40.7)
Amoxicillin	133 (57.5)	98 (42.4)
Amoxicillin+clavulanic acid	33 (14.3)	198 (85.7)
Cephalothin	196 (84.9)	35 (15.1)
Ceftriaxone	41 (17.7)	190 (82.2)
Tetracycline	213 (92.2)	18 (7.8)
Gentamicin	157 (67.9)	74 (32.0)
Streptomycin	209 (90.5)	22 (9.5)
Amikacin	200 (86.6)	31 (13.4)
Nalidixic acid	204 (88.3)	27 (11.7)
Ciprofloxacin	120 (51.9)	111 (48.1)
Cotrimoxazole	72 (31.1)	159 (68.8)

[a] Intermediate resistant strains were considered as resistant.

a total of 670 mastitc milk samples analyzed. All *E. coli* isolates were investigated by PCR, for the presence of Shiga-like toxin-producing genes (*stx* 1 and *stx* 2) and for the intimin (*eae*) gene. As can be seen from Table 1, 22 (9.5%) of the strains harbored 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 *stx*1 and *stx* 2 genes; two strains carried only the *eae* gene. All STEC strains isolated were tested by the O157 latex agglutination test kit, and no one O157 isolate was detected.

Among the 231 *E. coli* isolates tested, the highest resistance was observed against tetracycline (92.2%), followed by that to streptomycin (90.4%) and nalidixic acid (88.3%); lower resistance to amoxicillin/clavulanic acid and ceftriaxone were detected in 14.2% and in 17.7% of the isolates, respectively (Table 2). Sixteen isolates were

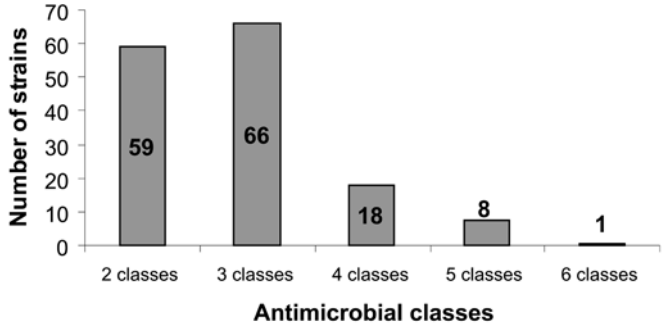

Fig.1. Distribution of multidrug resistance to 12 antimicrobial drugs among 152 strains of *Escherichia coli* isolated from bovine mastitic milk.

Table 3. Patterns and phenotypes of antimicrobial drug resistance of 27 strains of *Escherichia coli* resistant to four or more antimicrobial classes of agents, from mastitic bovine milk

Phenotype	Number of strains
AMP-CFL-TET-STR	1
AMP-TET-AMK-NAL	1
CFL-TET-STR-AMK-NAL	1
AMP-AMC-CFL-TET-AMK	1
AMP-CFL-TET-GEN-STR	1
AMP-TET-GEN-AMK-NAL	1
AMP-CFL-GEN-STR-AMK-CIP	1
SUT-TET-GEN-STR-AMK-NAL	1
AMP-CFL-GEN-AMK-NAL-CIP	1
AMP-AMO-CIP-GEN-AMK-NAL	1
AMP-TET-STR-AMK-NAL-CIP	1
AMO-TET-GEN-STR-AMK-CIP	1
AMP-CFL-TET-GEN-STR-AMK	1
AMO-TET-GEN-STR-AMK-NAL	1
AMO-TET-GEN-STR-AMK-CIP	1
AMC-CFL-TET-GEN-AMK-NAL	1
AMC-CFL-TET-GEN-STR-AMK-NAL	1
TET-GEN-STR-AMK-NAL-CIP-SUT	1
AMP-AMO-TET-GEN-STR-AMK-SUT	1
AMP-AMO-CFL-GEN-STR-AMK-SUT	1
AMP-AMO-CFL-TET-STR-AMK-SUT	1
AMP-AMO-CFL-TET-STR-AMK-CIP	1
AMO-AMC-CFL-TET-GEN-AMK-NAL	1
AMP-TET-GEN-STR-AMK-NAL-CIP-SUT	1
AMP-AMO-AMC-CFL-TET-STR-AMK-NAL	1
AMP-AMO-CFL-TET-GEN-STR-AMK-CIP	1

susceptible to all antimicrobial agents tested, and 152 isolates were resistant to two or more antimicrobial classes (Fig.1). The multidrug resistant phenotypes (resistance to more than 4 antimicrobial classes) for the 27 isolates of *E. coli,* are depicted on Table 3.

DISCUSSION

Mastitis has been recognized for some time as the most costly disease in dairy herds (Miller et al. 1993). Based on epidemiological studies, it has been hypothesized that cows are infected with *Escherichia coli* from their environment, as feces and straw (Lipman et al. 1995). It is well known that bacterial, hosts and environmental factors are interdependent and influence susceptibility to mastitis. Many studies performed during the last decade indicate that the severity of *E. coli* mastitis is mainly determined by host's factors, rather than by *E. coli* pathogenicity (Burvenich et al. 2003). Dogan et al. (2006) examined *E. coli* strains associated with intramammary infection and demonstrated the absence in them of either K99, F1845, LT, Sta, Stb, *stx* 1, *stx* 2, *cnf* 1, *cnf* 2, *eae, pap, sfa, afa, hly* and *ipa* H or other known genes associated with virulence and invasion.

Out of 279 *E. coli* isolates investigated by Linton & Howe (1979), 217 (77.1%) could be O-serogrouped, and 67 different O-serogroups were found. This indicates that *E. coli* mastitis is not caused by a limited number of specific pathogenic strains, but seems to be associated with environmental fecal contamination and be multifactorial.

Cattle have long been regarded as the principal reservoir of STEC strains including those belonging to serotype O157:H7 (Paton & Paton 1998). More than 200 different STEC serotypes have been isolated from cattle, some being more frequently isolated than others (Beutin et al. 2004, Caprioli et al. 2005). In Brazil, only a few studies have reported the isolation and the characteristics of STEC in cattle (Cerqueira et al. 1999, Leomil et al. 2003, Salvadori et al. 2003, Irino et al. 2005); all of them were from healthy or cattle presenting diarrhoea. Only Lira et al. (2004) have reported the isolation of STEC strains from mastitic milk, the carriage of *stx* gene (12.08%) and the distribution of 22.7% (*stx* 1) and 45.5% (*stx* 2) genes. This agrees with the results reported in the present study: *stx* carriage (8.6 %), was distributed between 40.0% (*stx* 1), and 60.0% (*stx* 2), with a predominance of *stx* 2 gene in both studies, in agreement with the results of Hornitzky et al. (2002) in Australia, Zschok et al. (2000), in Germany, and Irino et al. (2005), in Brazil.

Another objective of this study was to characterize STEC from dairy cows for their virulence markers and for possible relationships with known human pathogenic types of *Escherichia coli*. The *eae* gene is responsible for attachment and effacement lesions similar to those observed in enteropathogenic *E. coli* (Gannon et al. 1993). *Eae* genes are present in most human STEC strains belonging to enterohaemorrhagic serotypes. In the present study, the *eae* gene was not detected in the STEC isolates herein studied. The low prevalence of *eae*-positive STEC strains has been reported in several studies (Beutin et al. 1993, Zschok et al. 2000, Irino et al. 2005). However, in this study a small percentage of *E. coli* strain (0.8 %) (results not shown) were *eae* positive but none harbored *stx* genes. Other authors also detected *eae*-positive non-STEC strains (Mainil et al. 1993, Orden et al. 1998, 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. As suggested by Wieler et al. (1996) the *eae*-positive *E. coli* strains isolated from cattle may harbor genes that are structurally different from, but functionally identical, to the EPEC genes.

Prevalence and extent of antimicrobial resistance in a population is strongly correlated to antibiotic usage, since selection and dissemination of resistant bacteria are greatly increased by the pressure exerted by these drugs. As a consequence, resistance is most commonly found where there occurs heavy use of antibiotics and appreciable host to host contact; therefore, sites of intensive farming constitute a large reservoir of antibiotic- resistant bacteria (Murray 1992). In this situation, resistant microorganisms are very easily disseminated within units via fecal contact, promoting contamination of the water used by animals or environmental contamination of the soil (Teuber 2001). Among the 231 isolates tested in this work, *E. coli* antimicrobial drug susceptibilities were high especially for amoxicillin+ clavulanic acid (85.7%), ceftriaxone (82.2%) and cotrimoxazole (68.8%) (Table 2).

Zhao et al. (2001) examined 50 strains of *E. coli* Shiga toxin-producing (STEC), 29 O157 strains and 21 non-O157 strains; both groups showed high resistance level to ampicillin, tetracycline and streptomycin, in agreement with the present study (Table 2). The authors reported that 78.0% of the strains exhibited resistance to two or more antimicrobial agents; among them the resistant phenotype for streptomycin, tetracycline and sulfametoxazole was the most commonly observed. In the present study a high multidrug resistance (65.8%) was reported (Fig.1).

In Europe there exist two different situations related to antimicrobial utilization; some countries like Spain do not exert a rigid control over antimicrobial agents utilization, while other ones like Sweden and Denmark exerted such control. Orden et al. (2000) examined 195 *E. coli* strains isolated from diarrheic cattle in Spain and reported a high resistance to ampicillin, tetracycline, streptomycin and trimethropim, agreeing with the results presented in this work (Table 2); the authors also reported a great number of multiresistant strains (76.9%), 67.7% of them resistant to four or more drugs. When Lanz et al. (2003) in Sweden, examined the susceptibility of 581 *E. coli* strains to 16 antimicrobial drugs, they reported a discrete level of resistance to ampicillin (21.0%) followed by streptomycin (22.0%) and tetracycline (20.0%) and total susceptibility to the other antimicrobial drugs tested in 80.0% of the bacterial strains examined.

In Brazil, Langoni et al. (2000) reported a discrete level of resistance to tetracycline (13.0%) and ampicillin (12.0%) among *E. coli* isolates from bovine mastitis, while Amaral et al. (1996) also reported high levels of resistance to ampicillin (92.9%) and tetracycline (71.4%) of bovine mastitis isolates. The data reported in the present study agree with those reported by Baptista & Marin (2006) for isolates from mastitic and diarrheic cattle, showing an intermediate level between those reported above.

Although antibiotic resistance patterns may reflect the antibiotics used for mastitis prevention and treatment, some authors (Hillerton & Berry 2005) defended the idea that convincing evidence is still lacking for supporting this theory. Studies performed in the United States indicate that there is no correlation among increased resistance of and antimicrobials that are commonly used in dairy cattle for treatment of mastitis (Erskine et al. 2001, Makovec & Ruegg 2003). In Switzerland, there was no increased antibiotic resistance of mastitis pathogens during the last 20 years (Roesch et al. 2006), indicating different points of view about this theme. In Brazil a strong control of antimicrobial drugs commercialization and access to data related to resistance to antimicrobial drugs presented by the pathogens responsible for bovine mastitis would first be necessary before a conclusive answer about this matter is given.

Concluding, the present study demonstrated the presence, in a low number, of STEC strains among those

isolated from bovine's mastitis cases. Also it was observed a high level of antimicrobial resistance to many antibiotics and an elevated number of multiresistant strains among the *E. coli* strains examined.

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