Virulence Properties of Shiga Toxin-Producing *Escherichia coli* Isolated from Cases of Bovine Mastitis in Brazil

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Key words

Cattle – Dairy cow – *Escherichia coli* – Mastitis – Pathogenicity – PCR – Brazil.

Summary

Out of 528 milk samples obtained from dairy cows with mastitis, 31 (5.8%) had *Escherichia coli* strains, causative agent of mastitis. These strains were screened for the presence of Shiga toxin-producing (*stx*1 and *stx*2) and intimin (*eae*) genes. Twenty (64.5%) strains were detected by PCR to harbor the Shiga toxin genes (13 the *stx*1 gene, 3 the *stx*2 gene, and 4 the *stx*1-*stx*2 genes). Three (9.6%) of the *E. coli* strains studied were *eae* positive non Shiga toxin-producing. The *E. coli* strains were also examined for resistance to 15 antimicrobial agents. The most commonly observed resistance was to novobiocin (100%), lincomycin (96.8%), penicillin (96.8%) and erythromycin (90.3%). All the strains tested showed resistance to at least one antimicrobial agent and multidrug resistance was very common (96.8%).

■ INTRODUCTION

Mastitis is a major problem in dairy farms and *E. coli* mastitis especially is a major disease in cows (1) because of the latter increasing incidence and severe symptoms (16). The concept of *E. coli* mastitis is that the organisms live in the environment and contaminate the teats probably as a result of fecal contamination (20).

Shiga toxin-producing *E. coli* (STEC) isolates are an important group of food-borne pathogens that can cause severe gastrointestinal diseases in humans and complications such as the hemolytic uremic syndrome (HUS). The most often reported STEC serotype causing disease in humans worldwide is O157:H7, although many others have been described (31, 34, 42).

Domestic ruminants, especially cattle, sheep and goats, have been implicated as the principal reservoirs of STEC strains that cause human infections (8, 9, 49). The pathogenicity of these bacteria is mainly mediated by Shiga toxins (Stx1, Stx2) encoded by *stx*1 and *stx*2 genes, and the products of the locus of enterocyte-effacement pathogenicity island, with the *eae* gene (intimin) involved in the attaching and effacing (AE) lesion in the intestinal mucosa (34). STEC strains that possess the *eae* gene may be able to induce AE if they possess other genes necessary for initial adherence (46). The role of *eae* positive non STEC strains in cattle diseases is uncertain, but these strains may be similar to enteropathogenic *E. coli* (EPEC) in humans.

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The aim of the present study was to establish the serogroups and virulence genes of STEC strains isolated from bovine mastitic milk in Brazil. Drug resistance was carried out as further characterization of the isolates.

■ MATERIALS AND METHODS

Bacterial strains

Milk samples from mastitic cows were obtained aseptically in different dairy farms of Ribeirão Preto region, São Paulo State, Brazil, from February 2003 to November 2003. Approximately 5 ml of milk were collected in sterile glass bottles, stored in a cool box and transported to the laboratory for culture. Clinical and subclinical mastitides were identified by the California mastitis test (CMT) and clinical examination, and samples were collected in both cases. Samples were cultured in MacConkey (MAC) agar. Agar plates were incubated at 37°C and bacterial growth was evaluated after 24 and 48h. Gram-negative microorganisms were isolated from MAC agar and determined at the species level using cytochrome oxidase, triple sugar iron agar, urea and indole tests as putatively *E. coli* (6). Only one isolate for each animal was included. Reference *E. coli* strains used as controls were EDL 933 (O157: H7, *stx*1, *stx*2, *eae*); DH5α was used as a negative control (10, 34).

Serogrouping

The *E. coli* isolates were identified by slide and tube agglutination tests (15) using polyvalent and monovalent sera against serogroups O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142, and O158 (Probac do Brasil, São Paulo).

Extraction of bacterial DNA

Bacterial strains were grown in nutrient broth at 37°C overnight. Organisms from 1.5 ml growth were pelleted by centrifugation at 1200 *g* for 10 min. The bacterial pellet was resuspended in 250 µl sterile distilled water. The bacteria were lysed by boiling for 10 min. The lysate was centrifuged again as before and 200 µl of the supernatant were used directly as template for polymerase chain reaction (PCR) (44).

Examination of STEC isolates by PCR

A total of 31 *E. coli* isolates were subjected to PCR that was performed with a Mastercycler Eppendorf. The presence of *stx*1, *stx*2 and *eae* genes were detected as described by China et al. (11); PCR primers and conditions were those described by the authors. The amplified DNA products were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide and detected under ultraviolet light (37).

Antimicrobial susceptibility tests

Antimicrobial susceptibility testing of bacteria was done by the disk diffusion method using commercial disks (Cecon, Centro de Controle e Produtos para Diagnostico, São Paulo, Brazil) according to the guidelines of the National Committee for Clinical Laboratory Standards (32). Adjusted inoculation of bacteria (ca5 x 10^4 CFU) were inoculated on Mueller-Hinton agar and incubated for 18 h at 35°C. Strains were considered resistant or sensitive by measuring the diameter of the growth inhibition zone; interpretation of the results was done as recommended by NCCLS (32). The antimicrobial agents tested, the loads of the disks and the resistance breakpoint were as follows: ampicillin (AMP, 10 µg ≤ 13 mm), cephalothin (CEP, 30 µg ≤ 14 mm), chloramphenicol (CLO, 30 µg ≤ 12 mm), erythromycin (ERI, 30 µg ≤ 13 mm), gentamycin (GEN,10 µg ≤ 12 mm), kanamycin (KAN, 10 µg ≤ 13 mm), lincomycin (LIN, 50 µg ≤ 16 mm), nalidixic acid (NAL, 10 µg ≤ 13 mm), neomycin (NEO, 25 µg ≤ 12 mm), nitrofurantoin (NIT, 30 µg ≤ 14 mm), novobiocin (NOV, 30 µg ≤ 14 mm), penicillin (PEN, 30 µg ≤ 14 mm), streptomycin (STR, 30 µg ≤ 11 mm), tetracycline (TET, 30 µg ≤ 14 mm), trimethoprim-sulfadiazine (TMP, 25 µg ≤ 10 mm).

■ RESULTS

A total of 31 *E. coli* strains were isolated from 528 cows with mastitis. All the strains were submitted to an agglutination test to determine the serogroup with specific antiserum (Table I). Nine different serogroups were identified, and serogroups O55 (8 strains) and O114 (5 strains) were those the most often identified.

The strains were investigated for the presence of Shiga-like toxin-producing genes (*stx*1 and *stx*2) and for the presence of the intimin (*eae*) gene by PCR. As can be seen in Table II, 20 (64.5%) of the strains were STEC. PCR showed that 13 (65.0%) of STEC strains carried only the *stx*1 gene, 3 (15.0%) possessed the *stx*2 gene, and 4 (20.0%) carried both *stx*1 and *stx*2 genes. Five (25.0%), 2 (10.0%), 3 (15.0%) of the *stx*1, *stx*2 and *stx*1-*stx*2 strains, respectively, also harbored the *eae* gene (Table II). Three strains were non STEC and harbored only the *eae* gene.

Table I

Serogroup analysis of 31 isolates of *Escherichia coli* collected from mastitic milk in Brazil during 2003

Serogroup	Num. of strains/Total samples	%
O55	08/31	25.80
O114	05/31	16.12
O26	04/31	12.90
O111	04/31	12.90
O86	03/31	9.67
O125	02/31	6.45
O119	02/31	6.45
O126	02/31	6.45
O142	01/31	3.22

Table II

Virulence gene profile of *Escherichia coli* isolates from mastitic milk in Brazil

Num. of isolates	O serogroup (num. of isolates)	Virulence factor profile
08	O55(4); O111(1); 119(2); O125(1)	*stx*1
01	O86(1)	*stx*2
05	O114(3); O86(2)	*stx*1, *eae*
02	O111(2)	*stx*2, *eae*
01	O142(1)	*stx*1, *stx*2
03	O55(2); O111(1)	*stx*1, *stx*2, *eae*
11	–	None

The *E. coli* strains were tested for resistance to 15 antimicrobial agents. They were resistant most commonly to novobiocin (100%), lincomycin (96.8%), penicillin (96.8%) and erythromycin (90.3%) (Figure 1). All the strains tested showed resistance to at least one antimicrobial agent, but none showed resistance to all of them. Multidrug resistance, defined as being resistant to two or more classes of antibiotics, was very common and 96.8% of the strains showed resistance to three antimicrobial agents.

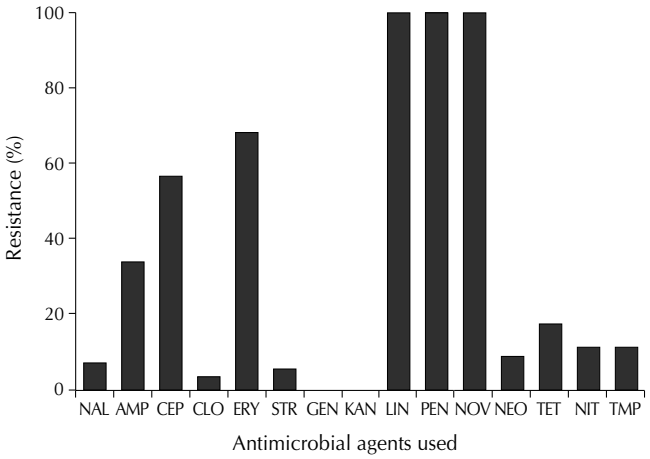

Figure 1: *Antimicrobial agents resistance patterns of* Escherichia coli *strains isolated from mastitic milk in Brazil. NAL: nalidixic acid; AMP: ampicillin; CEP: cephalothin; CLO: chloramphenicol; ERY: erythromycin; STR: streptomycin; GEN: gentamycin; KAN: kanamycin; LIN: lincomycin; PEN: penicillin; NOV: novobiocin; NEO: neomycin; TET: tetracycline; NIT: nitrofurantoin; TMP: trimethoprim-sulfadiazine.*

■ DISCUSSION

In the present study 31 *E. coli* strains were isolated from cows with mastitis. Nine different serogroups were found. Many authors have reported a wide range of serogroups in cattle (24, 45, 46). However, so far the serogroups associated with bovines have been essentially different from human serogroups, although sometimes serogroups such as O26, O111 and O119 were also isolated from healthy and diarrheic calves (24, 28, 36, 39).

In this study 100% of the *E. coli* isolates belonged to classical EPEC serogroups and among them O26, O55, O111, O119 represented around 58.0% of the isolates. This result was similar to those previously reported by Corrêa and Marin (13) and Saridakis et al. (39). The isolation of a great number of strains with serogroups O111 and O119 was a cause for concern because they have long been recognized as the most important EPEC serogroups associated with children diarrhea in Brazil (7, 19). Recently, serogroup O111 has been recognized as the most important human STEC serogroup in Brazil (21, 43). However, further serotyping work and molecular characterization are needed to confirm these isolates to be EPEC serotypes, as well as to compare genotypically the strains of animal and human origins. On the other hand, the present results were in contrast with those from a study in another Brazilian region in which 84.6% of the STEC isolates from healthy dairy cattle did not react with any antiserum obtained from a set with the most frequently isolated serotypes from human diarrhea diseases (30).

E. coli that produces Shiga toxins has emerged as a cause of serious human gastrointestinal disease and HUS (34). The most common serotype of STEC implicated worldwide is O157:H7 (34). In addition to this serotype a large number of other STEC strains have been isolated from humans (14) and animals (42).

In Germany during 1989 and 1996 STEC was detected in 6.0% of diarrheic calves (46). Blanco et al. (4, 5) in Spain found STEC in 9.0 to 12.0% of diarrheic calves, but STEC strains have also been isolated from healthy animals (2, 3). In Brazil STEC was detected in 12.0 to 16.1% of diarrheic calves (28, 36). However, Moreira et al. (30) reported 49.0% of STEC strains isolated from healthy dairy cattle. Regions with a high prevalence of STEC in cattle usually have high rates of STEC-associated human infections (34). Thus, it is remarkable that despite the relatively high prevalence of STEC in cattle, the occurrence of STEC-associated human infections in Brazil is uncommon (35, 43).

Table II shows that STEC isolates were observed with different combinations of virulence genes; these results were in agreement with those reported by Wieler et al. (46) in Germany, Sandhu et al. (38) in Canada, Orden et al. (33) in Spain, and Leomil et al. (28) in Brazil. Guth et al. (22) also reported a predominance of the *stx*1 gene in Brazil in contrast with a predominance of the *stx*2 gene in Argentina among the STEC non-O157 strains isolated from animals and food in both countries.

Some investigators have underlined the strong association between the carriage of the *eae* gene and the capacity of STEC strains to cause severe disease in humans, especially HUS (23). However, the association of *eae* and *stx* genes in STEC isolates from diarrheic calves for pathogenesis is controversial. Wieler et al. (46) determined that the prevalence of both virulence factors in STEC was 70.0%, while Cobbold and Desmarchelier (12) described only 0.8% positive *eae* among STEC isolates. Guth et al. (22) reported that the *eae* gene was infrequently identified among non-O157 STEC strains isolated from cattle in Argentina and Brazil, while Salvadori et al. (36) and Leomil et al. (28) reported a frequency of *eae* carriage of 21.2 and 41.0%, respectively, among the STEC isolates from calves. In the present study the *eae* gene was found predominantly together with *stx*1, but also with *stx*2 and *stx*1-*stx*2 isolates. Fifty percent of the STEC isolates possessed the *eae* gene, which was in agreement with the results reported by Leomil et al. (28) in diarrheic and non-diarrheic cattle in Brazil. It is also important because Vaz et al. (43) showed the predominance of *stx*1-positive *eae*-positive isolates among the human STEC isolated from diarrheic children in São Paulo, Brazil.

Three (9.6%) of the *E. coli* isolates included in this study were *eae*-positive non STEC. Other authors also reported the detection of *eae*-positive non STEC strains (29, 33). The pathogenicity of *eae*-positive non STEC in calves is not clear but Fischer et al. (17) showed that an *eae*-positive verotoxigenic-negative strain (serogroup O26) was able to induce experimentally the attaching and effacing lesion. As suggested by Wieler et al. (46) the *eae*-positive *E. coli* strains isolated from cattle may harbor genes that are structurally different from the EPEC genes but functionally identical.

STEC strains isolated from humans and animals have developed antibiotic resistance and many are resistant to multiple antimicrobials commonly used in human and veterinary medicine (18, 40). Schroeder et al. (41) reported the susceptibilities to 14 antimicrobial agents of 408 *E. coli* strains of serogroups O26, O103, O111, O128 and O145 isolated from cattle in the USA. They found 50.0, 47.0, 46.0 and 15.0% of resistance to streptomycin, tetracycline, sulfamethoxazole and ampicillin, respectively, among the isolates, and multidrug resistance was

commonly found among them. A total of 100% of the examined isolates in the present work showed antimicrobial resistance to one or more of the antimicrobial agents. Novobiocin, lincomycin, penicillin, and erythromycin showed the highest rates of resistance with 100, 96.8, 96.8, and 90.3%, respectively (Figure 1). Also 96.8% of the isolates showed resistance to three antimicrobial agents. The rates were quite high; however, Lazaro et al. (27) isolated *E. coli* strains from diarrheic cattle in Rio de Janeiro, Brazil, and reported the isolation of EPEC serogroups and enterotoxigenic *E. coli* among the isolates. They also found high rates of antibiotic resistance among them, with 85.0, 65.0, and 60.0% of resistance to tetracycline, streptomycin, and ampicillin, respectively. They also reported 80.0% of the isolates showing multidrug resistance.

For more than four decades, it has been a common practice on farms to use antimicrobial agents for disease prevention and growth promotion of animals. The widespread use of antimicrobial agents may have promoted the increasing frequency of STEC strains multidrug resistance in bovines (48). Indirect selection for multiresistant strains will contribute to the increase of emerging antimicrobial resistant pathogens and facilitate the spread of resistance by plasmids (41) or by integrons (26) to other bacteria. Ceftiofur is the sole extended-spectrum cephalosporin approved for use in food animals in the USA, and it is not approved for use in human clinical medicine (25). However, Schroeder et al. (41) reported two *E. coli* isolates from humans which showed resistance to ceftiofur, which suggests the transfer of resistant *E. coli* from food animal to humans. Also Winokur et al. (47) described the transfer of plasmid between *E. coli* and *Salmonella* isolates.

■ CONCLUSION

In this work, the authors have identified the presence of STEC isolates harboring *stx*1, *stx*2 and *eae* genes in milk samples from cattle with mastitis, and isolates harboring only *eae* gene were also found. The *E. coli* isolates showed a high level of resistance to antimicrobial agents and multidrug resistance was extremely common. A continued surveillance of *E. coli* isolates from animals and the development of adequate prevention strategies to diminish the spread of multiresistant bacteria and/or the mobile resistance elements are needed for public health reasons.

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Résumé

Kobori D., Rigobelo E.C., Macedo C., Marin J.M., Avila F.A. Caractéristiques de la virulence d'*Escherichia coli*, productrice de la toxine type Shiga, isolée à partir de cas de mammite bovine au Brésil

Sur 528 échantillons de lait provenant de vaches atteintes de mammites, 31 (5,8 p. 100) avaient des souches d'*Escherichia coli*, agent responsable de mammites. Ces souches ont été analysées afin d'évaluer la présence de gènes producteurs de toxine type Shiga (*stx*1 et *stx*2) et d'intimine (*eae*). Par la technique de la PCR, 20 (64,5 p. 100) des souches ont présenté les gènes de toxine type Shiga (13 le gène *stx*1, 3 le gène *stx*2 et 4 le gène *stx*1-*stx*2). Trois (9,65 p. 100) des souches *d'E. coli* étudiées ont été *eae* positives non-productrices de la toxine type Shiga. Les souches d'*E. coli* ont également été examinées pour tester leur résistance à 15 agents antimicrobiens. La résistance la plus forte a été observée pour la novobiocine (100 p. 100), la lincomycine (96,8 p. 100), la pénicilline (96,8 p. 100) et l'érythromycine (90,3 p. 100). Toutes les souches étudiées ont montré une résistance à un agent antimicrobien au moins et une résistance multiple à plusieurs antibiotiques a été très fréquente (96,8 p. 100).

Mots-clés : Bovin – Vache laitière – *Escherichia coli* – Mammite – Pouvoir pathogène – PCR – Brésil.

Resumen

Kobori D., Rigobelo E.C., Macedo C., Marin J.M., Avila F.A. Propiedades de virulencia de la *Escherichia coli* productora de toxina Shiga aislada de vacas de leche con mastitis en Brasil

De las 528 muestras de leche obtenidas a partir de vacas de leche con mastitis, 31 (5,8%) presentaron cepas de *Escherichia coli*, agente causal de la mastitis. Estas cepas fueron estudiadas para la presencia de genes productores de la toxina Shiga (*stx*1 y *stx*2) e intimina (*eae*). Veinte cepas fueron detectadas mediante PCR con genes de toxina Shiga (13 con el gen *stx*1, 3 con el gen *stx*2 y 4 con los genes *stx*1-*stx*2). Tres (9,6%) de las cepas de *E. coli* estudiadas fueron *eae* positivas no productoras de la toxina Shiga. Las cepas de *E. coli* también fueron examinadas para la resistencia a 15 agentes anti microbianos. La resistencia más frecuentemente observada fue a la novobiocina (100%), lincomicina (96,8%), penicilina (96,8%) y eritromicina (90,3%). Todas las cepas examinadas mostraron resistencia al menos a un agente anti microbiano y la resistencia múltiple a drogas fue muy común (96,8%).

Palabras clave: Ganado bovino – Vaca lechera – *Escherichia coli* – Mastitis – Patogenicidad – PCR – Brasil.