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

D. Kobori¹ E.C. Rigobelo² C. Macedo³ J.M. Marin⁴ F.A. Avila⁵*

**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 (stx1 and stx2) and intimin (eae) genes. Twenty (64.5%) strains were detected by PCR to harbor the Shiga toxin genes (13 the stx1 gene, 3 the stx2 gene, and 4 the stx1-stx2 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%).

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**Key words**

Cattle – Dairy cow – *Escherichia coli* – Mastitis – Pathogenicity – PCR – Brazil.
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 48 h. 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, stx1, stx2, 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, and 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 stx1, stx2 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 10⁶ 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), cefalothin (CEF, 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 antisera (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 (stx1 and stx2) 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 stx1 gene, 3 (15.0%) possessed the stx2 gene, and 4 (20.0%) carried both stx1 and stx2 genes. Five (25.0%), 2 (10.0%), and 3 (15.0%) of the stx1, stx2 and stx1-stx2 strains, respectively, also harbored the eae gene (Table II). Three strains were non STEC and harbored only the eae gene.

**Table I**

<table>
<thead>
<tr>
<th>Serogroup</th>
<th>Num. of strains/Total samples</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>O55</td>
<td>08/31</td>
<td>25.80</td>
</tr>
<tr>
<td>O114</td>
<td>05/31</td>
<td>16.12</td>
</tr>
<tr>
<td>O26</td>
<td>04/31</td>
<td>12.90</td>
</tr>
<tr>
<td>O111</td>
<td>04/31</td>
<td>12.90</td>
</tr>
<tr>
<td>O86</td>
<td>03/31</td>
<td>9.67</td>
</tr>
<tr>
<td>O125</td>
<td>02/31</td>
<td>6.45</td>
</tr>
<tr>
<td>O119</td>
<td>02/31</td>
<td>6.45</td>
</tr>
<tr>
<td>O126</td>
<td>02/31</td>
<td>6.45</td>
</tr>
<tr>
<td>O142</td>
<td>01/31</td>
<td>3.22</td>
</tr>
</tbody>
</table>

**Table II**

<table>
<thead>
<tr>
<th>Num. of isolates</th>
<th>O serogroup (num. of isolates)</th>
<th>Virulence factor profile</th>
</tr>
</thead>
<tbody>
<tr>
<td>08</td>
<td>O55(4); O111(1); 119(2); O125(1)</td>
<td>stx1</td>
</tr>
<tr>
<td>01</td>
<td>O86(1)</td>
<td>stx2</td>
</tr>
<tr>
<td>05</td>
<td>O114(3); O86(2)</td>
<td>stx1, eae</td>
</tr>
<tr>
<td>02</td>
<td>O111(2)</td>
<td>stx2, eae</td>
</tr>
<tr>
<td>01</td>
<td>O142(1)</td>
<td>stx1, stx2</td>
</tr>
<tr>
<td>03</td>
<td>O55(2); O111(1)</td>
<td>stx1, stx2, eae</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>None</td>
</tr>
</tbody>
</table>
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.](image)

**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).

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 *stx1* gene in Brazil in contrast with a predominance of the *stx2* 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 *stx1*, but also with *stx2* and *stx1-stx2* 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 *stx1*-positive *eae*-positive isolates among the STEC isolated from diarrheic children in Sào 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...
Virulence of Escherichia coli in Bovine Mastitides

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, Lazar 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 antibiotics 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.

Acknowledgments

The authors thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for D. Kobori’s scholarship. This research was supported by grants from the Conselho Nacional de Desenvolvimento Científico e Tecnológico and FAPESP.

REFERENCES


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 (stx1 et stx2) 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 stx1, 3 le gène stx2 et 4 le gène stx1-stx2). 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).


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 (stx1 y stx2) e intimina (eae). Veinte cepas fueron detectadas mediante PCR con genes de toxina Shiga (13 con el gen stx1, 3 con el gen stx2 y 4 con los genes stx1-stx2). 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 antimicrobianos. La resistencia más frecuentemente observada fue a la novobiocina (100%), la lincomicina (96,8%), la penicilina (96,8%) y la eritromicina (90,3%). Todas las cepas examinadas mostraron resistencia al menos a un agente antimicrobiano y la resistencia múltiple a drogas fue muy común (96,8%).