Journal of Applied Microbiology 2004, **97**, 861–866 doi:10.1111/j.1365-2672.2004.02384.x

The incidence of Shiga toxin-producing *Escherichia coli* in cattle with mastitis in Brazil

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2003/1169: received 19 December 2003, revised 19 May 2004 and accepted 26 May 2004

ABSTRACT

W.M. LIRA, C. MACEDO AND J.M. MARIN. 2004.

Aims: To determine the prevalence and molecular characteristics of Shiga toxin-producing *Escherichia coli* (STEC) isolates from bovine mastitic milk in Brazil.

Methods and Results: A total of 2144 milk samples from dairy cattle showing mastitis were screened for the presence of *E. coli*. A total of 182 *E. coli* isolates were selected and examined. All were subjected to dot blot analysis using the CVD419 probe for the detection of the enterohaemolysin (*hly*) gene, and to a multiplex PCR for the detection of *stx*1, *stx*2 and *eaeA* genes. STEC were isolated from 22 (12·08%) milk samples. All the STEC isolates were tested for sensibility to 10 antimicrobials; the resistances most commonly observed were to cephalothin (86·3%), tetracycline (63·6%) and doxycycline (63·6%).

Conclusion: STEC isolates were found in bovine mastitic milk in Brazil.

Significance and Impact of the Study: STEC isolates from mastitic milk were potentially pathogenic for human in that they belonged to serogroups associated with diarrhoea and haemolytic-uraemic syndrome, some of them were *stx*2, *eaeA* and *hly* positive.

Keywords: dairy cattle, *Escherichia coli*, mastitis, Shiga toxin-producing *E. coli*.

INTRODUCTION

Mastitis, caused by *Escherichia coli* accounts for considerable economic loss in dairy herds as indicated by lower production and quality of milk, cost of treatment, and occasionally the death of an animal (Sanchez-Carlo *et al.* 1984). The reason for the importance of *E. coli* mastitis is its increasing incidence and severe symptoms (Fang and Pyorala 1996).

Escherichia coli is a normal inhabitant of the animal intestinal tract, but particular *E. coli* strains such as the Shiga toxin-producing *E. coli* (STEC) (Schoonderwoerd *et al.* 1988) are associated with diarrhoea in calves and humans. STEC may cause a broad disease spectrum in humans, ranging from mild diarrhoea to severe diseases, like haemorrhagic colitis (HC) and the haemolytic-uraemic syndrome (HUS) (Karmali 1989; Griffin and Tauxe 1991). STEC strains able to induce HC and HUS, are called enterohaemorrhagic *E. coli* (EHEC) (Levine 1987). Consumption of beef or raw milk has been suspected or confirmed as being the most likely sources of infection in outbreaks which have occurred during the last decade mainly in Canada, the USA, the UK and Japan (Paton and Paton 1998). *E. coli* strain that produced a potent toxin cytotoxic for vero (green monkey kidney) cells were first reported by Konowalchuk *et al.* (1977). The toxin was appropriately named verotoxin (VT), and the group of *E. coli* that produced VT (VT1 and VT2) became known as the verotoxin-producing *E. coli*. As the structure of VT and Shiga toxin produced by *Shigella dysenteriae*-1 are similar, VT became known as Shiga-like toxin (SLTEC or STEC) (O'Brien and Holmes 1987). In an attempt to avoid confusion Calderwood *et al.* (1996) have proposed a rationalization of

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the nomenclature with the adoption of the term STEC that may produce two types of toxins (Stx1 and Stx2).

A wide range of STEC serogroups can cause human disease, although the most commonly isolated serogroup from patients infected with STEC, notably O26, O103, O111, O145 and O157 (Schmidt *et al.* 1999; Willshaw *et al.* 2001) are those harbouring the locus of enterocyte effacement (LEE) (Frankel *et al.* 1998). One of the genes located on the LEE is *eaeA* (*E. coli* attaching and effacing), which encodes intimin, an outer membrane protein involved in intimate attachment of the bacteria to intestinal host cells (Frankel *et al.* 1998). A factor that may also affect the virulence of STEC strains is the enterohaemolysin (*hly*) also called EHEC-haemolysin (EHEC-HlyA), which is encoded by the *hlyA* gene (Schmidt *et al.* 1995).

Although O157:H7 serotype is the dominant STEC in many parts of the world now it is recognized that STEC strains belong to a very broad range of O:H serotypes (Paton and Paton 1998). However, as STEC non-O157 are more prevalent in animals and as contaminants of food, humans are probably more exposed to these strains, and some of them have been associated with severe illness in humans (Boerlin *et al.* 1999; Blanco *et al.* 2003).

The aim of the present study was to investigate *E. coli* isolates from bovine mastitic milk for the presence of specific virulence markers. Antimicrobial resistance testing was carried out to further characterize some of the isolates.

MATERIALS AND METHODS

Bacterial strains

Milk samples from cows with mastitis were obtained asseptically by veterinarians with laboratory training. Approximately 5 ml of milk were collected in sterile glass bottles, stored on ice and transported to the laboratory for culture. Clinical and subclinical mastitis were identified by the California Mastitis Test (Schalm and Noorlander 1957) and clinical examination, and samples were collected in both cases. Samples were cultured in MacConkey (MAC) medium. Agar plates were incubated at 37°C and bacterial growth was evaluated after 24 and 48 h respectively. Gram-negative micro-organisms 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* (Buchanan and Gibbon 1994). Reference *E. coli* strains used as controls were EDL933 (O157:H7, *stx*1, *stx*2, *eae*, *hly*) and DH5α (negative control) (Chart *et al.* 2000).

Detection of O-serogroups

Escherichia coli isolated in MAC were biochemically confirmed and submitted to slide agglutination tests using polyvalent and monovalent sera against serogroups O26, O55, O86, O111, O114, O119, O126, O127, O128, O142, O158. Commercially available antisera from Probac do Brasil (São Paulo) were used.

DNA isolation

The strains were grown in Luria broth and plasmids were extracted by an alkaline lysis method (Sambrook *et al.* 1989).

DNA probe

A specific probe was used for the detection of the enterohaemolysin gene (*hly*), a 3·4 kb *Hin*dIII fragment of the pCVD419 (Levine *et al.* 1987). The plasmid was prepared, purified and digested with the appropriate restriction endonucleases (Amersham Biosciences, Sao Paulo, Brazil) and the appropriate restriction fragment was recovered as described by Faruque *et al.* (1992).

Dot blot analysis

For dot blot, 10 μl of plasmid DNA were mixed with 20 μl of 3 M NaCl-0·5 M NaOH and applied to a Hybond-N membrane previously soaked in 1·5 M NaCl-0·25 M NaOH (Bio-dot manifold, Bio-Rad, Hercules, CA, USA). DNA–DNA hybridization was performed by the traditional method (Sambrook *et al.* 1989) at 65°C for hybridization and washing.

Detection of *stx*1, *stx*2 and *eae* sequences by PCR

Bacterial strains grown overnight in nutrient broth (Sigma Chemical Co, St Louis, MO, USA) at 37°C, were pelleted by centrifugation at 1200 *g* for 10 min. The pellet was resuspendend in 250 μl of sterile distilled water, and the bacteria lysed by boiling for 10 min. The lysate was centrifuged as before and 200 μl of the supernatant were used directly as the template for the PCR (Wani *et al.* 2003).

A total of 182 *E. coli* isolates were subjected to PCR, performed in an Eppendorf Mastercycler (Ependorf AG, Hamburg, Germany). *stx*1, *stx*2 and *eae* genes were detected using the primers and PCR conditions described by China *et al.* (1996). The amplified DNA products were separated by electrophoresis on a 1·5% agarose gel, stained with ethidium bromide and detected under ultraviolet light.

Antimicrobial susceptibility tests

Antimicrobial susceptibility testing of bacteria was carried out by the disk diffusion method using commercial diks (Laborclin, São Paulo, Brazil), according to the guidelines of the National Committee for Clinical Laboratory Standards

(2000). Antimicrobial agents tested, loaded on the disks were the following: trimethoprim (TRI, 25 μg), gentamicin (GEN, 10 μg), cefotaxime (30 μg), amoxicillin-clavulanic acid (AMC, 20/10 μg), netilmicin (30 μg), tetracycline (TET, 30 μg), cephalothin (CPL, 30 μg), doxycycline (DOX, 30 μg), cefazolin (CFZ, 30 μg), nalidixic acid (NAL, 30 μg).

RESULTS

From March 1997 to August 1998, 2144 samples of mastitic milk were collected in seven Brazilian states. Among the 182 *E. coli* isolates analysed, the somatic antigen (serogroup) was determined in 141 isolates. A total of 77·4% of the isolates belonged to 12 O serogroups (O26, O55, O86, O111, O114, O119, O125, O126, O127, O128, O142 and O158) and 41 isolates belonged to other nonidentified serogroups, as reported elsewhere (Correa and Marin 2002).

All isolates were submitted to multiplex PCR, to detect *stx*1, *stx*2 and *eae* genes. STEC were isolated from 22 (12·08%) milk samples. The *stx* genotype, *eae* and *hly* genes are summarized in Table 1. Eleven (50%) of the 22 STEC strains carried the *eae* gene, predominantly associated with *stx*2 gene. Five (22·7%) of the STEC isolates were positive only for *stx*1, *stx*2 gene alone was detected in 10 (45·5%) isolates, *stx*1 and *stx*2 gene in combination was present in seven (31·8%) isolates. Sixteen of the STEC isolates (72·7%) showed positive hybridization with the *hly* gene (CVD419 probe) and were also predominantly associated with the *stx*2 gene (Table 1). When the STEC isolates were tested against 10 antimicrobial agents, resistance to CPL was the most commonly observed (86·3%), followed by TET (63·6%), DOX (63·6%) and less frequently by NAL (18·1%), TRI (18·1%) and CFZ (9·1%) respectively (Fig. 1).

Table 1 Virulence gene profile of STEC isolates from mastitic milk in Brazil

	Genotype			
Isolate (serogroup)	CVD419 (hly)	*stx*1	*stx*2	eae
933* (O157)	+	+	+	+
263 (O111)	–	+	+	–
304·1† (O119)	–	–	+	–
304·3† (O119)	–	+	–	–
339 (O26)	+	+	+	–
352 (O119)	+	+	+	+
535·1† (O55)	–	+	+	+
535·2† (O55)	+	+	–	+
536·1 (O111)	–	+	+	–
563·1† (O26)	+	–	+	+
563·2†(O26)	+	–	+	–
564 (O55)	+	+	+	–
568·2 (O111)	+	–	+	–
585·1† (O119)	+	–	+	+
585·2† (O119)	+	–	+	+
585·3† (O119)	+	–	+	+
812 (O26)	+	–	+	+
895 (O55)	–	–	+	–
912·1† (O26)	+	+	+	+
912·2† (O26)	+	+	–	+
919 (O111)	+	+	–	–
962·1 (O111)	+	+	–	–
979 (O26)	+	–	+	+

*Control strain *E. coli* EDL 933.

†Isolates from different quarters from the same cow.

DISCUSSION

In the present study, 182 *E. coli* isolates were selected and examined from 2144 milk samples from dairy cows with mastitis. A total of 77·4% of the isolates belonged to 12 serogroups (Correa and Marin 2002). Several authors have reported a wide range of serogroups in cattle (Wells *et al.* 1991; Wieler *et al.* 1996; Holland *et al.* 1999; Wani *et al.* 2003), some of them were found only in bovine and others like O26, O103, O111 and O157 have been found in bovine and humans (Paton and Paton 1998; Bettelheim 2000).

Cattle have long been regarded as the principal reservoir of STEC strains, including those belonging to serotype O157:H7 (Paton and Paton 1998). Beutin and Muller (1998) examined 41 *E. coli* isolates obtained in 1965 from calves in Germany, and verified that the isolates producing

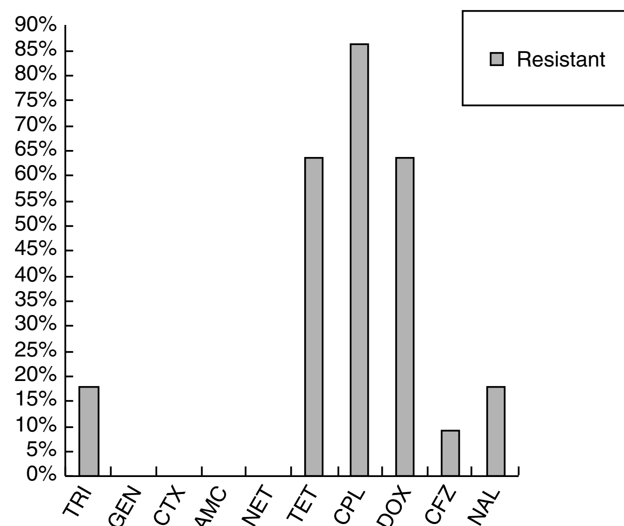

Fig. 1 Antimicrobial resistance patterns in 22 STEC strains from mastitic milk in Brazil. TRI, trimethoprim; GEN, gentamicin; CTX, cefotaxime; AMC, amoxicillin-clavulanic acid; NET, netilmicin; TET, tetracycline; CPL, cephalothin; DOX, doxycycline; CFZ, cefazolin; NAL, nalidixic acid

Stx and STEC-associated virulence markers were already present in cattle population more than 30 years ago. Studies carried out in different countries have shown that 10–80% of cattle may carry STEC (Wells *et al.* 1991; Beutin *et al.* 1993; Blanco *et al.* 1996; Cobbold and Desmarchelier 2000). More than 100 different STEC serotypes have been isolated from cattle, although some serotypes are more frequently isolated than others (Beutin *et al.* 1993). Isolation of STEC from healthy cattle was previously reported in Brazil (Cerqueira *et al.* 1999). In the present study STEC was found in 12·08% (22 isolates), in agreement with results of other authors (Orden *et al.* 1998; Cobbold and Desmarchelier 2000; Wani *et al.* 2003). In this study, the *stx*2 gene was more prevalent than the *stx*1 gene. This result contrasts with reports showing that most STEC strains from cattle harbour the *stx*1 gene (Sandhu *et al.* 1996; Wieler *et al.* 1996; Orden *et al.* 1998; Urdahl *et al.* 2003) but agrees with Hornitzky *et al.* (2002) who also found a predominance of the *stx*2 gene in STEC strains of Australian cattle. Almost the same distribution of *stx*2, *stx*1 + *stx*2, *stx*1 genes found in this study was reported by Zschock *et al.* (2000) in faecal samples from lactating cows in Germany. In cattle there is often a continual turnover of serotypes of *E. coli*, with many being isolated only sporadically from a herd (Hinton *et al.* 1985). Midgley *et al.* (1999) studied the dynamics of STEC strains in feedlot cattle in Australia during a period of 117 days. The percentage of *stx*+ samples fluctuated between 4 and 68% with the incidence being dependent on sampling day. Jenkins *et al.* (2002a) reported an 8-month study of a population of STEC strains in a Scottish cattle herd, they verified that the distribution of *stx* genes detected from faecal samples change during this period. In May, July and September *stx*2 gene was the most commonly detected while isolates showing *stx*1 + *stx*2 genes were only 6%, whereas *stx*1 + *stx*2 genes were most frequently detected in the STEC strains isolated in December (52%). The percentage of *stx* genes detected any time could be variable.

The STEC strains seem to be pathogenic for humans only if they possess accessory virulence factors. Another objective of this study was to characterize STEC from dairy cows for their virulence markers and thereby for possible relationships with the known human pathogenic types of *E. coli*. The *eae* gene is responsible for attachment and effacement lesions similar to those in enteropathogenic *E. coli* (Gannon *et al.* 1993). In most human STEC strains belonging to enterohaemorrhagic serotypes, *eae* genes are present. In this study, 11 (50%) of the STEC isolates were positive for *eae* gene sequence. The low prevalence of *eae*-positive STEC has been reported in many studies (Beutin *et al.* 1993; Blanco *et al.* 1997; Zschock *et al.* 2000; Jenkins *et al.* 2002b), *eae* gene-positive STEC were found more frequently in cattle with diarrhoea (Blanco *et al.* 1997). However, in agreement with the percentage of *eae*-positive gene reported in this study, Osek (2002) reported a high frequency (55·8%) of *eae*-positive STEC isolates from healthy cattle in Poland. Despite the claim that the *eae* gene is a major marker of virulence, there is evidence that STEC strains causing severe human disease do not necessarily contain the *eae* gene (Dytoc *et al.* 1994).

The incidence of enterohaemolysin (*hly* gene) in non-O157 STEC can be inferred by hybridization with the CVD419 probe. Barret *et al.* (1992) found that 28 of 28 *E. coli* O157, 16 of 19 human non-O157, but only 13 of 26 animal STEC were CVD419 probe positive and Willshaw *et al.* (1992) found that 29 of 48 non-O157 STEC were CVD419 probe positive. In this study 16 (72·7%) of the STEC isolates showed hybridization with the CVD419 probe, this result agrees with results reported by others (Leung *et al.* 2001; Jenkins *et al.* 2002b). A comparison of *eae*-positive and *eae*-negative STEC isolates in this study showed a strong linkage between *eae* and *hly* (91%) what agree with the results of Sandhu *et al.* (1996).

The emergence and dissemination of antimicrobial resistance among STEC strains may have potential negative clinical implications, although the diarrhoeal phase of illnesses associated with STEC strains is usually self-limiting and the role of early antimicrobial therapy in the prevention of HUS is still unclear (Griffin 1995). Antimicrobial-resistant STEC strains could possibly possess selective advantages over other bacteria colonizing the gastrointestinal tracts of animals that are treated with antibiotics (therapeutically or subtherapeutically). Resistant STEC strains could then become the predominant *E. coli* present under antibiotic selective pressures. This could result in STEC population increases and perhaps greater shedding, which could lead to greater contamination of animal food products with STEC (Zhao *et al.* 2001).

A total of 50 isolates of STEC including 29 O157:H7 and 21 non-O157 STEC strains from humans, animals and food were analysed for antimicrobial susceptibilities (Zhao *et al.* 2001). The authors reported high antimicrobial resistance to sulfamethoxazole (48%), streptomycin (43%), TET (43%) and ampicillin (33%). The reported results, agrees with those found in this study, the resistance to TET (63·6%) and also susceptibility to AMC, GEN and NAL.

In the present study, resistance to at least one of a series of antimicrobial agents tested was found in 100% of the isolates examined. Khan *et al.* (2002) reported resistance to one or more antibiotics in 49·2% of the STEC strains in India, with some of the strains exhibiting multidrug-resistance. Antimicrobial resistant bacteria from animals may colonize human populations via the food chain, it is therefore possible that resistant bacteria may be readily transferred to humans from animals used as food sources.

To conclude, in this work, we have identified the presence of STEC strains harbouring *stx*1, *stx*2, *eae* and *hly* gene in mastitic bovine milk.

ACKNOWLEDGEMENTS

The authors thank Dr Tânia A. Tardelli Gomes, Department of Microbiology, Immunology and Parasitology, Escola Paulista de Medicina, São Paulo, Brazil for the plasmid pCVD419, Vitafort Co Ltda/Dr Maria da Graça Portantiolo Correa for milk samples. This work is part of a thesis submitted by Walclecio Morais Lira to the Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal-UNESP in partial fulfilment of the requirements for the Master degree.

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