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Isolation of verocytotoxin-producing *Escherichia coli* from milk filters in south-western Ontario

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SUMMARY

A total of 1012 milk filters collected from 498 dairy farms in south-western Ontario during three study periods (December 1985–March 1986) were tested for the presence of verocytotoxin-producing *Escherichia coli* (VTEC). VTEC were detected and isolated using a Vero cell assay. Supernatants from 20 of the milk filter cultures had verocytotoxic activity and 7 VTEC strains were isolated from these positive samples. The prevalence of VTEC in the samples in each of the three study periods were 0·44, 0·65 and 0·99% respectively. All seven VTEC strains isolated were sensitive to commonly used antimicrobial agents. The serotypes of these strains were O 26.H11, O43.H2, O 153.H25, O ?.H8, O ?.H19, O ?.non-motile, and Orough.H19. Two of these serotypes (O 153.H25 and O 26.H11) have previously been associated with disease in humans.

INTRODUCTION

Verocytotoxin-producing *Escherichia coli* (VTEC) have been implicated as causative agents of haemorrhagic colitis (Johnson, Lior & Bezanson, 1983; Riley *et al.* 1983) and haemolytic uraemic syndrome in humans (Karmali *et al.* 1983; Karmali *et al.* 1985; Neil, Agosti & Rosen, 1985). A number of outbreaks have been associated with VTEC serotype O 157.H7 (Riley *et al.* 1983; Johnson, Lior & Bezanson, 1983; Pai *et al.* 1984; Spika *et al.* 1986; Carter *et al.* 1987; Mai *et al.* 1987). Consumption of beef or raw milk has been suspected or confirmed as the most likely source of infection in many of the outbreaks that have been investigated (review by Karmali, 1989). In addition to O 157.H7 several serotypes of VTEC have been isolated from patients suffering from haemorrhagic colitis or haemolytic uraemic syndrome (Karmali *et al.* 1985; Bopp *et al.* 1987; Ish-Shalom *et al.* 1987; Scotland *et al.* 1988). A wide variety of VTEC serotypes, including

O 157.H7 and other serotypes associated with human disease have been isolated from cattle (Mohammed *et al.* 1985; Sherwood, Snodgrass & O'Brien, 1985; Martin *et al.* 1986; Mohammed, Peiris & Wijewanta, 1986; Moxley & Francis, 1986; Borczyk *et al.* 1987; Mainil *et al.* 1987; Orskov, Orskov & Villar, 1987, Schoonderwoerd *et al.* 1988), suggesting that these animals may be an important reservoir of VTEC for humans.

Dairy farms in Ontario use filters placed in the milk lines to trap debris in milk going from the milking machines to the bulk tanks. Culture of these filters has been shown to be a more sensitive screening technique for detection of salmonella in bulk milk supplies than the culture of raw milk samples (Styliadis & Barnum, 1984) and should be a suitable method for detecting other bacteria in milk. The purpose of this study was to determine the prevalence of VTEC strains in culture of milk filers and to determine if any similarities exist between the serotypes of VTEC recovered from milk filters and those that have been isolated from humans.

MATERIALS AND METHODS

Farm selection

The milk filters that were used in this study were originally collected for another research project whose main objective was to determine the prevalence of salmonella in bulk milk from south-western Ontario dairy farms (MacEwen *et al.* 1988). The owners of the participating dairy farms were asked permission to culture their milk filters for VTEC.

Details of dairy farm selection and milk filter acquisition have been reported elsewhere (McEwen *et al.* 1988). In brief, dairy farms were sampled using a two-stage sampling technique, with bulk milk transport companies as the primary sampling units ($n = 60$) and dairy farms served by these transport companies as the secondary sampling units ($n = 5221$). Eight primary sampling units (milk transporters) were randomly selected with probability proportional to size. Approximately 80 farms from each transporter (160 farms from each of two transporters selected twice) were asked to participate in the study. All participating dairy farms were sampled over two distinct sampling periods and a subset was sampled a third time. The milk filter collection period took place between December 1985 and March 1986.

Collection and culture of milk filters

Milk filters were collected and transported under refrigeration to the laboratory in 1·2 l sterile sealed plastic bags with resealable tops (Whirl-Pac, Arnold Farm-Vet Ltd., Guelph, Ontario). Upon arrival at the laboratory, the plastic bags were opened, and 40 ml of nutrient broth (Difco Laboratories, Detroit, Michigan) were added and gently mixed with the milk and filter present in the bag. The samples were incubated overnight at 37 °C. A 1 ml sample of the nutrient broth culture was mixed with 9 ml of MacConkey broth (Difco) and incubated overnight at 37 °C. This step was introduced to enhance the growth of *E. coli* and to reduce false positive reactions due to toxic substances produced by Gram-positive organisms which could mask the cytotoxic effects of VTEC in the Vero cell assay. The

MacConkey broth cultures were then subcultured (1/10 v/v) into brain heart infusion broth (BHIB, Difco) and incubated overnight at 37 °C. This step was required because MacConkey broth is toxic for Vero cells.

Verotoxin assays

The BHIB cultures were initially tested for verocytotoxic activity using the screening assay described by Gannon, Gyles & Friendship (1988). Briefly, a 50 μl sample of each culture was transferred to a flat-bottomed microtitre plate (NUNC) containing Vero cell monolayers. Following a 15 min incubation at 37 °C the cell monolayers were washed with sterile phospate-buffered saline and 200 μl of Eagle's minimal essential medium (EMEM, Gibco) was added to each well. Plates were incubated for 3 days at 37 °C in 5% CO_2 atmosphere and examined for cell death using an inverted microscope. Positive cultures were retested in the routine assay also described by Gannon, Gyles & Friendship (1988). Briefly, 200 μl of EMEM with 5% fetal calf serum was added to each well of a flat-bottomed 96-well microtitre plate. A 50 μl sample of culture supernatant was added to the first well, then 1:5 dilutions were made in each of the remaining wells of the column. A 100 μl aliquot of Vero cells (4×10^5 cells/ml) was added to each well and the plates were incubated and observed as in the screening assay. Confirmed positive cultures were streaked on MacConkey plates and five pools of approximately 20 colonies each were examined in the routine Vero cell assay. Positive pools were streaked on MacConkey plates and 20 single colonies were picked and tested in the Vero cell assay.

Biochemical and antimicrobial tests

Suspect VTEC colonies were identified as *E. coli* based on biochemical reaction as determined in the Repliscan system (Cathra International, St Paul, Minnesota). The biochemical reactions were evaluated on multiple inoculation plates containing glucose, sucrose, lactose, citrate, arabinose, inositol, mannose, adonitol, sorbitol, acetimide, lysine, ornithine, arginine, bile esculin, urea, ferric ammonium citrate and colistin. The antimicrobial resistance profile of each VTEC isolate was determined in the same system using breakpoint concentrations of amipicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, neomycin, polymyxin B, spectinomycin, sulfisoxazole, tetracycline and trimethoprim-sulfamethoxazole.

RESULTS

Culture supernatants from 20 of the milk filter cultures were cytotoxic for Vero cells. From these cultures seven VTEC strains were isolated. The recovery of VTEC from milk filters is summarized in Table 1. Four hundred and fifty-five milk filters that were collected from different dairy farms during the first study period (December 1985 to January 1986) were cultured. VTEC (serotypes O ?.H19, O ?.H8) were isolated from two of these milk filters, hence the prevalence of VTEC was approximately 0·44%. One hundred and fifty-four milk filters collected from Perth Country, Ontario during the second study period (February 1986) were cultured and VTEC was isolated from one of these filters (serotype O 153.H25).

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Table 1. *Prevalence of verocytotoxin-producing* E. coli *from dairy farm milk filters in Ontario*

Study period	Number cultured	Number positive	Prevalence (± approximate S.E.M.*)
December/ January	455	2	0·44 ± 0·0000
February	154	1	0·65 ± 0·0071
March	403	4	0·99 ± 0·0050
	1012	7	

* Standard error of the mean with clusters (transporters) of unequal size.

Table 2. *Serotypes and biochemical characteristics of verocytotoxin-producing* E. coli *from dairy farm milk filters in Ontario**

Serotype	Mug†	Sorbitol	Lactose	Rhamnose
O ?. H19	+	+	+	+
O ?. H8	+	+	+	+
O 153. H25	+	+	−	+
O rough. H19	−	−	+	+
O 26. H11	+	+	+	−
O ?. NM	+	+	+	+
O 43. H2	+	+	+	+

* Only those biochemical reactions where differences in the group existed are listed.

† +, fluorescence under U.V. light on media containing 4-methylumbelliferyl B-glucuronide (MUG) indicating the presence of B-glucuronidase. −, no fluorescence.

The prevalence of VTEC contamination of milk filters during this period was approximately 0·65%. Finally four milk filters from a total of 403 collected during the third study period (March 1986) were positive for VTEC (serotypes filter contamination during this study period was approximately 0·99%.

All of the verocytotoxin-producing strains isolated were identified as *E. coli*. Differences in biochemical reactions between the strains are listed in Table 2. All of the strains were sensitive to all the antimicrobials tested.

The milk filters from which VTEC were isolated in this study had all tested negative for the presence of salmonella (McEwen *et al.* 1988).

DISCUSSION

The estimates of prevalence of VTEC in the bulk milk filters cultured in this study are probably underestimates of the true prevalence of contamination of the bulk milk supplies with these bacteria. The Vero cell culture assay used in this study is highly sensitive. VTEC strains that produce 'high levels' of VT(s) can be detected when they constitute as little as 0·1% of a population of *E. coli*. Failure to isolate VTEC strains from most of the positive cultures was likely due to the presence of VTEC as a small percentage of the bacteria cultured. Only approximately 100 colonies from each positive sample were tested and it is not practical to test a larger number of colonies in the Vero cell assay. The use of DNA probes (Scotland *et al.* 1988) and immunoblotting techniques (Doyle *et al.* 1987)

have shown promise to improve the power of selection for VTEC in mixed cultures. The results of this study may be of use in planning research efforts using more sensitive isolation techniques to more accurately determine the prevalence of VTEC in bulk milk supplies. It is clear that, even if all samples that were positive in the tests of culture supernatant contained VTEC, the prevalence of these organisms in milk filters was very low.

Recently a great deal of attention has been paid to human illness associated with *E. coli* serotype O 157.H7. As a result, a number of studies have been conducted to detect this serotype in humans (Harris *et al.* 1985; Cahoon & Tompson, 1987; Pai *et al.* 1988), animals (Martin *et al.* 1986; Borczyk *et al.* 1987; Orskov, Orskov & Villar, 1987) and foods (Doyle & Schoeni, 1987). There is growing evidence however, that a variety of non-O 157.H7 VTEC strains play a role in haemorrhagic colitis and haemolytic uraemic syndrome in humans (Karmali *et al.* 1985; Bopp *et al.* 1987; Scotland *et al.* 1988; Tzipori *et al.* 1988). These facts require that prevalence surveys and the examination of food samples be conducted in a manner that would ensure that all VTEC serotypes and not just serotype O 157.H7 would be detected. A major advantage of using the Vero cell assay was its ability to detect all serotypes of VTEC. *Escherichia coli* O 157.H7 was not isolated from the milk filters, but two of the serotypes that were isolated (O 26.H11 and O 153.H25) have been associated with disease in humans (Smith & Linggood, 1971; Smith, Green & Parsell, 1983; Bopp, 1987; Levine *et al.* 1987; Scotland *et al.* 1988; Karmali, 1989). The pathogenicity for animals or humans of the other serotypes isolated in this study is not known.

Differences in biochemical reactions of VTEC strains isolated in this study constitute further evidence of the diversity of the VTEC group and indicate the limitations of biochemical reactions as a basis for screening for these pathogens. Detection of VTEC serotype O 157.H7 is often based on the inability of these strains to ferment sorbitol (Wells *et al.* 1983; Harris *et al.* 1985; March & Ratnam, 1986) and a negative beta-glucuronidase reaction (Doyle & Schoeni, 1984; Krishnan *et al.* 1987). In the present study these tests would only have detected one of the VTEC strains isolated (O rough.H19) and this strain would probably have been discarded due to its inability to agglutinate O 157 antisera.

These results demonstrate that VTEC serotypes that have been associated with human disease are present in raw milk. It cannot be determined from this study whether the initial source of these strains was from the cattle or from human contamination during milking. The role that milk and milk products play in the epidemiology of haemorrhagic colitis and haemolytic uraemic syndrome is not completely understood, however in two outbreaks (Duncan *et al.* 1987; Wells *et al.* 1987) consumption of raw milk was the likely source of infection. Konowalchuk, Spiers & Stavric (1977), in their initial report of the discovery of VTEC, found that two strains (O 68.H12; O 26.K60(36).H32) isolated from cheese were VT producers. These results indicate that further research is necessary to determine the extent of VTEC contamination of unpasteurized cheese or other milk products and that public health officials must continue to educate the public on the risks of consuming raw milk.

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