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Prevalence of verocytotoxigenic *Escherichia coli* in ground beef, pork, and chicken in southwestern Ontario

S. C. READ[1], C. L. GYLES[2], R. C. CLARKE[1], H. LIOR[3] AND S. McEWEN[2]

[1] *Agriculture Canada, Health of Animals Laboratory, 110 Stone Road West, Guelph, Ontario, Canada N1G 3W4*

[2] *Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada N1G 2W1*

[3] *National Enteric Reference Centre, Laboratory Centre for Disease Control, Ottawa, Ontario, Canada K1A 0L2*

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SUMMARY

Samples of ground beef (225), pork (235) and chicken (200) were randomly selected from meat processing plants in the southwestern Ontario area. Supernatants of broth cultures of the samples were tested for verocytotoxins using a Vero cell assay. Neutralization of cytotoxic activity using antisera specific for three types of verocytotoxin (Verotoxin 1, Verotoxin 2 and Shiga-like toxin II) was performed on positive samples. Isolation of verocytotoxigenic *Escherichia coli* (VTEC) was attempted from positive samples. VTEC were confirmed as *E. coli* biochemically, tested for drug resistance, and serotyped. Based on neutralization studies, the prevalance of VTEC in beef and pork was at least 36·4% and 10·6%, respectively. This is much higher than has been reported from a survey of retail meats in which a method designed to detect only *E. coli* O 157.H7 was used. Isolations of VTEC were made from 10·4% of the beef samples and 3·8% of the pork samples. No VTEC were recovered from the chicken samples. The majority of VTEC isolates were susceptible to commonly used antimicrobial agents. A number of the serotypes of the VTEC isolates recovered have been associated with human disease; however, no VTEC of serotype O 157.H7 were isolated.

INTRODUCTION

Verocytotoxigenic *Escherichia coli* (VTEC) have been associated with a spectrum of disease in humans ranging from subclinical infections to mild diarrhoea, haemorrhagic colitis, haemolytic uraemic syndrome, and thrombotic thrombocytopenic purpura (reviewed by Karmali, 1989) [1]. A family of bacterial proteins toxic for Vero (African green monkey kidney) cells, and called verocytotoxins (VT) [2] or Shiga-like toxins (SLT) [3], has been proposed as one of the virulence factors produced by these *E. coli* [4–7]. Epidemiological investigations of outbreaks of human disease have implicated undercooked ground beef and unpasteurized milk as sources of infection [1]. A wide variety of VTEC serotypes, many of which have been associated with human disease, have

been isolated from cattle [8–14] and swine [14–18] suggesting that these animals may be an important reservoir of VTEC for humans.

Published methodologies for the detection of VTEC in meats have concentrated on isolation of a single VTEC serotype, *E. coli* O 157.H7 [19–21]. Although *E. coli* O 157.H7 has been the serotype most commonly isolated from human outbreaks, numerous other serotypes have been identified from clinical cases [1]. The standard method for identification of VTEC is the cytotoxicity of culture supernatants for Vero cells [2]. A single prevalence survey of VTEC in retail meats and poultry [20] focused on the isolation of *E. coli* O 157.H7. This serotype was isolated from 3·7% of ground beef, 1·5% of pork, 1·5% of poultry, and 2·0% of lamb samples. In a preliminary survey of VTEC in retail ground beef using the Vero cell assay, a prevalence of 18·0% was found (R. C. Clarke, unpublished findings). The objective of the current study was to determine the prevalence of VTEC in ground beef, ground pork and mechanically separated chicken from processing plants in the southwestern Ontario area by using the Vero cell assay and neutralization of cytotoxicity by specific antisera. Biochemical profiles and antibiotic resistance of VTEC isolates were also studied.

MATERIALS AND METHODS

Sample collection

Federally inspected meat-processing plants in the southwestern Ontario area were invited to participate in this study. Five beef, five pork, and two chicken processing plants agreed to participate. Two hundred and twenty-five ground beef, 235 ground pork, and 200 mechanically separated chicken samples were taken. The number of samples obtained from each plant was weighted according to the production of the product on a weekly basis. Samples were collected in lots of 20 on a systematic random basis, every 15 min, throughout the day and sampling days were randomly distributed over a 10-week period in the summer of 1988. Approximately 75 g of meat was taken aseptically at each sampling time. Samples were packaged individually, refrigerated for transportation to the laboratory and processed immediately upon arrival.

Vero cell assay

For initial testing, 25 g from each meat sample was added to 225 ml of nutrient broth (Difco Laboratories, Detroit, Michigan), processed in a stomacher for 1 min, and incubated overnight at 37 °C. Subsequent sample processing followed the method of Clarke and colleagues [29]. Briefly, 1 ml of nutrient broth culture was added to 9 ml of MacConkey broth (Difco) and incubated overnight at 37 °C. One hundred μl of the MacConkey broth culture was used to inoculate 1 ml of Brain Heart Infusion (BHI) broth (Difco), which was then incubated for 6 h at 37 °C. BHI broth cultures were centrifuged at 12000 g for 1 min. Fifty μl of the supernatant was used in the Vero cell assay as described by Gannon, Gyles and Friendship [15]. Samples with greater than 50% cytotoxicity in the 1/5 dilution were considered positive and investigated further for isolation of VTEC and neutralization of verocytotoxin activity.

Verocytotoxin neutralization assays

The initial BHI broth cultures of positive samples were stored at 4 °C until neutralization tests could be performed. To determine if cell death was due to the action of verocytotoxin, neutralizations of cytotoxicity by specific antisera were performed on the supernatants of the isolates and the initial BHI broth cultures, according to the method of Scotland and colleagues [22]. In the past, it was assumed that VT2 and SLT II were the same, but there are discrepancies in the literature on the pI and molecular weight of the subunits of toxin described as VT2 or SLT II [23, 24]. Recent studies by Head and co-workers [25] have demonstrated a difference between VT2 and SLT II based on neutralization assays. SLT II is completely neutralized by anti-VT2 but VT2 is only partially neutralized by anti-SLT II. These results have been confirmed in our laboratory (R. C. Clarke, unpublished findings). For this reason, the supernatants were tested initially with a combined antiserum (prepared by H. Lior) of anti-VT1 (H19) and anti-SLT II (K12 pEB1). Cytotoxicity of supernatants not neutralized by this antiserum combination were retested with a combined antiserum of anti-VT1 and anti-VT2. The VT2 antiserum in this combination (kindly provided by M. Karmali, Hospital for Sick Children, Toronto) was made against the VT2 of strain E32511.

VTEC Isolation

For positive samples, the initial BHI and MacConkey broths were streaked onto MacConkey agar (Difco) plates and incubated overnight at 37 °C. Twenty isolated colonies and four sweeps (each approximately 20 colonies) were taken from the plate inoculated with the BHI broth culture and 20 isolates and 2 sweeps from the plate inoculated with the MacConkey broth culture. These sweeps and isolates were individually inoculated into BHI broth and tested for cytotoxicity using the Vero cell assay as described above. If the sweeps were positive, the BHI broth from the sweep was streaked onto MacConkey agar, incubated overnight at 37 °C, and 20 isolates from this plate tested in the Vero cell assay. Serotyping of isolates was performed at the Laboratory Centre for Disease Control in Ottawa, Ontario.

Biochemical tests

Isolates were confirmed as *E. coli* using the GNI card of the automated microbial identification system, Vitek (Vitek Systems, Hazelwood, Missouri, USA). Biochemical tests included utilization of lysine, ornithine, arginine, malonate, tryptophan, acetamide, sodium thiosulphate, plant indican, urea, and citrate; glucose fermentation in the presence of DP-300 and *p*-coumaric; susceptibility to polymyxin B; hydrolysis of esculin and *o*-nitrophenyl-β,D-galactopyranoside; oxidation of glucose, lactose, maltose, mannitol, and xylose; and fermentation of raffinose, sorbitol, sucrose, inositol, adonitol, rhamnose, L-arabinose, and glucose. Isolates were also tested for the presence of β-glucuronidase using media containing 4-methylumbelliferyl-β-glucuronide (MUG).

Antimicrobial tests

Antimicrobial resistance patterns of *E. coli* isolates were determined using the Repliscan system (Cathra International, St Paul, Minnesota, USA) for the

following drugs at breakpoint minimal inhibitory concentrations: ampicillin, carbenicillin, cephalothin, chloramphenicol, gentamicin, kanamycin, neomycin, polymixin B, sulphasoxazole, tetracycline, trimethoprim-sulpha, spectinomycin, nalidixic acid, nitrofurantoin, streptomycin and amikacin.

RESULTS

A summary of the results of the Vero cell assay, neutralization tests, and VTEC isolations for each meat processing plant is listed in Tables 1 and 2.

Vero cell assay

Ninety-two (40·8 %) of the ground beef samples, 36 (15·3 %) of the ground pork samples and none of the chicken samples were positive in the Vero cell assay.

Neutralization of verocytotoxic activity

Of the initial BHI broth culture supernatants, 10 beef and 9 pork samples lost their cytotoxic activity upon storage, so neutralization tests could not be completed on these samples. The cytotoxicity of the remaining samples was neutralized by the combined antiserum containing anti-VT1 and anti-SLT II with the following exceptions. The cytotoxicity of two beef and three pork samples was neutralized by the combined antiserum containing anti-VT2 that had not been neutralized by the combined antiserum containing anti-SLT-II. The cytotoxicity of two of the pork cultures was not neutralized by either of the combined antisera. Thus, at least 36·4 % of the beef samples and 10·6 % of the pork samples had evidence of VTEC based on neutralization studies.

The cytotoxicity of supernatants of all subsequently obtained beef and pork isolates was neutralized by the combined VT1 and SLT II antiserum except one beef isolate (O 15.H27) and one pork isolate (O 6.H34). The cytotoxicity of these two cultures was neutralized by the mixture of anti-VT1 and anti-VT2 only. Also, the cytotoxicity of two pork isolates (O?.H9 and O?.H2) was not neutralized by either combination of antisera.

The neutralization results for the 24 beef isolates were identical to those of the initial BHI broth cultures except in two cases. In one of these cases, the cytotoxicity of the isolate was neutralized by anti-VT2, whereas the cytotoxicity of the initial BHI broth culture had been neutralized by anti-SLT II. In the second case, the reverse occurred.

The neutralization data for 3 of the 9 pork isolates corresponded with that of the initial BHI broth culture supernatants. For another three isolates, the initial culture supernatants lost their cytotoxic activity so the data could not be correlated. For one isolate, whose cytotoxicity was not neutralized by either of the combined antisera, the initial BHI broth culture supernatant was neutralized by anti-SLT II. For the remaining two isolates, the neutralization results were identical to those obtained with the two exceptional beef samples.

VTEC isolates from meats

Twenty-four isolations of VTEC were made from the beef samples (10·6 % of the total sampled). From the pork samples, a total of nine isolations of VTEC were

Table 1. *Recovery of VTEC from beef plants*

Plant	Total samples*	Number (%) positive†	Number (%) neutralized‡	Number (%) isolates
A	140	62 (44·0)	58 (41·4)	18 (12·9)
B	40	12 (30·0)	11 (27·5)	3 (7·5)
C	5	0	0	0
D	20	12 (60·0)	8 (40·0)	1 (5·0)
E	20	6 (30·0)	5 (25·0)	2 (10·0)
Totals	225	92 (40·8)	82 (36·4)	24 (10·6)

* Samples were collected in lots of 20 per plant per day except at plant C where production was low.

† Detected using the Vero cell assay.

‡ Determined by neutralization of cytotoxicity using specific antiserum. Cytotoxicity of 10 beef sample supernatants was lost upon storage, so neutralization tests could not be completed on these samples.

Table 2. *Recovery of VTEC from pork plants*

Plant	Total samples*	Number (%) positive†	Number (%) neutralized‡	Number (%) isolates
B	60	8 (13·0)	4 (6·6)	1 (1·7)
D	80	15 (19·0)	10 (12·5)	4 (5·0)
F	40	2 (5·0)	2 (5·0)	2 (5·0)
G	40	6 (15·0)	4 (10·0)	2 (5·0)
H	15	5 (33·0)	5 (33·0)	0
Totals	235	36 (15·3)	25 (10·6)	9 (3·8)

* Samples were collected in lots of 20 per plant per day except at plant H where production was low.

† Detected using the Vero cell assay.

‡ Determined by neutralization of cytotoxicity using specific antiserum. Cytotoxicity of nine pork sample supernatants was lost upon storage, so neutralization tests could not be completed on these samples.

made (3·8% of the total sampled). Up to three different serotypes were isolated from a single lot, sampled during a single day. In some cases a single serotype was repeatedly isolated (up to four times) from an individual lot. Serotypes of the isolates from the beef and pork samples are listed in Table 3.

Biochemical profiles of VTEC isolates

The biochemical profiles of the isolates were unremarkable with the following exceptions: two beef isolates were adonitol positive (both serotype O 117.H4); one isolate (O 113.H21) was sorbitol negative. Three of the pork isolates were MUG negative (serotypes: O?.H2; O?.H19; and O?.H9).

Antimicrobial susceptibility of VTEC isolates

One beef isolate (O 117.H4) was resistant to ampicillin, carbenicillin, tetracycline and streptomycin. A second beef isolate (O 117.H4) was resistant to sulphasoxazole, tetracycline and streptomycin. Resistance to tetracycline was

Table 3. *Serotypes of VTEC isolates*

Beef (% of isolates)	Pork (% of isolates)
O 113.H21 (6)*	O 82.H8 (2)*
O 139.H19 (4)	O ?.H19 (2)
O 117.H4 (3)*	O 2.H29 (1)
O ?.H19 (2)	O ?.H9 (1)
O 15.H27 (2)	O 6.H34 (1)
O 153.H25 (2)*	O ?.H2 (1)
O 22.H8 (2)	O ?.NM (1)
O 6.H34 (1)	
O 8.H19 (1)	
O 128.H35 (1)	

* Serotypes of VTEC that have been isolated from humans in Canada (H. Lior, personal communication).

expressed by a third beef isolate (O 113.H21). A single pork isolate (O ?.H9) was resistant to sulphasoxazole. The other 29 strains of *E. coli* isolated were susceptible to all antimicrobials tested.

DISCUSSION

We found the prevalence of VTEC, as determined by toxin neutralization studies, to be at least 36·4 and 10·6 % in ground beef and ground pork, respectively and 0 % in mechanically separated chicken. The prevalence estimates of VTEC in ground beef and pork are much higher than those reported in a previous study which identified only VTEC of serotype O 157.H7 [20]. Our study may overestimate the prevalence in meats of VTEC that are pathogenic for humans. First, although several of the VTEC serotypes isolated in this study have been associated with human disease, there were numerous serotypes identified whose pathogenicity for humans is unknown. Secondly, VTE (the VT associated with oedema disease of pigs) may have been present in some of our samples. Due to immunological cross reaction between VTE and VT2 [26, C. L. Gyles, unpublished findings], our neutralization assays would not distinguish samples containing VTE. To date, VTE has not been associated with disease in humans. In retrospect, testing the samples with the HeLa cell assay in addition to the Vero cell assay might have identified those samples which contained VTE as the only VT.

The cytotoxicity of two pork isolates was not neutralized by either combination of antisera. Whether these are a different antigenic type of the recognized verocytotoxins or a distinct toxin, remains to be elucidated.

For the beef samples, the neutralization results of the isolates corresponded very well with those of the original BHI broth cultures. The results with the pork samples were more variable. The inconsistencies may be explained by the possibility of more than one strain present in the original BHI broth culture. This is substantiated by the presence of up to three different serotypes within the same lot. The production of more than one antigenic type of VT by a single strain may also lead to inconsistent neutralization results [27].

In contrast to the work of Doyle and Schoeni [20] we found no evidence of

VTEC in our chicken samples. This is consistent with other work conducted in this laboratory where 500 chicken cloacal swabs [28] and 200 chicken carcass rinses (unpublished findings) were negative in the Vero cell assay. This discrepancy may be due to regional differences in the prevalence of VTEC in chickens, or perhaps cross-contamination of samples by humans or other meat products, since Doyle's samples were taken at the retail level.

Many outbreaks of VTEC infection have been associated with the consumption of inadequately cooked hamburger meat [1]. This association has been supported by the prevalance survey of Doyle and Schoeni [20] and is further sustained by the present study. Pork was shown by the same workers to be a source of *E. coli* O 157.H7. Our work has extended this observation to include another serotype of VTEC of importance in human disease.

Most of the VTEC serotypes isolated from beef in this study have been previously isolated from cattle in this region [9, 29, R. C. Clarke, unpublished findings]. This is the first report of serotypes O 139.H19, O 117.H4, and O 128.H35 from beef samples, although these serogroups have been previously reported from Sri Lanka [12]. This is the first report of VTEC serotypes O 82.H8 and O 6.H34 from porcine origin.

In the present study, no VTEC of serotype O 157.H7 were isolated, even though it is the most common VTEC serotype isolated from humans [1]. Unlike sorbitol–MacConkey screening methods, the Vero cell assay does not select for a particular serotype. Therefore, it reflects a more accurate picture of the VTEC population in the samples. The absence of *E. coli* O 157.H7 may be due to regional disparities in its prevalence. Other studies on VTEC from animal sources have also failed to show a predominance of this serotype [9–18]. *E. coli* O 157.H7 may have virulence factors which give it an advantage, over other VTEC, for the production of disease in humans.

No pattern of VTEC serotypes was evident in the recovery of isolates. Multiple serotypes were recovered from a single lot and the same serotype isolated repeatedly from a lot. This is consistent with data from other studies on the recovery of VTEC from cattle [9, 12].

The recovery of VTEC isolates from positive samples was low. The difficulty of VTEC isolation following the Vero cell assay has been discussed by Clarke and colleagues [29] and, along with its requirements for tissue culture, make it unsuitable as a routine diagnostic test. A method to isolate *E. coli* O 157.H7 from foods by biochemical characteristics has been proposed by Szabo, Todd and Jean [19]. This method is based on the inability of these strains to ferment sorbitol and a negative beta-glucuronidase reaction with subsequent serological confirmation. This screening procedure has resulted in the false identification of *E. hermanii* as *E. coli* O 157.H7 [30]. In the present study, this method would not have detected any of the VTEC strains isolated, due to the failure to fulfil all three criteria simultaneously. Furthermore, in a national survey conducted by Agriculture Canada based on this method (unpublished findings), 406 samples from imported boxed meats (beef, pork, sheep and chicken) were all negative and 748 samples from slaughter pork carcasses yielded a prevalence of only 0·13%. Clearly, this method underestimates the prevalence of VTEC in foods.

Doyle and Schoeni [20] used a hydrophobic grid membrane filter-immunoblot

procedure in their prevalence survey. They found a high number of false positives due to lack of specificity of the antiserum. In addition, the method is complex and labour-intensive and therefore inappropriate for routine diagnostic testing. The monoclonal antibody used by Todd and colleagues [21] in a hydrophobic grid membrane filter-enzyme-labelled antibody procedure was found to cross react with group N salmonella. All of these methods for foods are limited to the detection of VTEC of serotype O 157.H7.

Other methods for the detection of VTEC have been described in research settings. These include a colony blot assay using monoclonal antibodies against SLT [31], cloned DNA probes for VT1 and VT2 [22, 32], a cloned DNA probe for a 60 megadalton plamid encoding a fimbrial antigen from enterohaemorrhagic *E. coli* [33], oligonucleotide probes for SLT I and SLT II [34], a sandwich enzyme-linked immunosorbent assay (ELISA) for SLT I and SLT II [35], and a receptor-based ELISA assay for SLT I [36]. These techniques have all shown promise for the routine detection of VTEC in a diagnostic setting; however, they have yet to be adapted to food samples.

The majority of isolates were susceptible to all antibiotics tested. However, it is interesting to note that all of the resistant beef isolates were of serotypes that have been associated with human disease.

In conclusion, these results demonstrate that VTEC serotypes, other than O 157.H7, that have been associated with human disease are present in ground beef and pork but not chicken. Methods designed to detect only this serotype will underestimate the true prevalence of VTEC of significance to humans. Further research is required to identify a feasible diagnostic test for isolation of VTEC from foods and to determine the pathogenicity of serotypes isolated from foods which have not been associated with disease in humans. Public health officials must continue to educate the public on the handling of raw meats and the risks associated with consumption of undercooked meats.

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