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Prevalence of the *eae*A gene in verotoxigenic *Escherichia coli* strains from dairy cattle in Southwest Ontario

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SUMMARY

This study determined the prevalence of the *eae*A gene and its relationship to serotype and type of verotoxin produced in a collection of 432 verotoxigenic *Escherichia coli* (VTEC) obtained from the faeces of healthy cows and calves in a systematic random survey involving 80 dairy farms in Southwest Ontario. A PCR amplification procedure involving primer pairs which target the conserved central region of the O157:H7 *eae*A gene showed that 151 (35·2%) strains were positive for the *eae*A gene. All isolates (9-21 for each O group) of O groups 5, 26, 69, 84, 103, 111, 145 and 157 were positive, whereas all isolates (7-34 for each O group) of O groups 113, 132, and 153 and serotype O156:NM (38 isolates) were negative for *eae*A. Seventy-three percent of 130 isolates of *eae*A-positive serotypes produced VT1 only compared with 20% of 253 isolates of *eae*A-negative serotypes. We conclude that there is a strong association between certain O groups and the *eae*A gene, that serotypes of *eae*A-positive and *eae*A-negative VTEC implicated in human and cattle disease are present at high frequency in the faeces of healthy cattle, that VT1 is more frequently associated with *eae*A-positive than with *eae*A-negative serogroups, and that the *eae*A gene is more frequently found in VTEC from calves compared with VTEC from adult cattle.

INTRODUCTION

Verotoxigenic *Escherichia coli* (VTEC) are characterized by their ability to produce at least one cytotoxic protein that is active on Vero cells and is referred to as verotoxin (VT) or Shiga-like toxin (SLT) [1, 2]. VTEC have been associated with a wide spectrum of human diseases, including diarrhoea, haemorrhagic colitis (HC) and the haemolytic uremic syndrome (HUS) [3–7]. Those VTEC serotypes which are able to induce haemorrhagic colitis are called enterohaemorrhagic *E. coli* (EHEC). Only a few serotypes, including O157:H7, O157:NM, O26:H11 and O111:NM have been clearly demonstrated to be EHEC and many serotypes isolated from humans with disease are of uncertain status. Nonetheless it is useful to use the term EHEC for referring to disease-producing VTEC.

Serotype O157:H7 is the most prevalent serotype implicated in outbreaks of VTEC-mediated disease in humans [1, 5, 7, 8]. VTEC isolates of serotypes O5:NM, O8:H9, O26:H11 and O111:NM have been associated with natural disease in calves 2–8 weeks of

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age and have been used in experimental reproduction of dysentery in calves [9–12]. VTEC of serotypes O26:H11 and O111:NM are frequently implicated in diseases in humans as well as calves [8, 10, 13–15], whereas VTEC of serotype O5:NM are frequent in cattle and less frequent in humans and VTEC of serotype O8:H9 have not been reported from humans [16]. Host species specificity is most evident with serotype O157:H7 which causes disease in humans but not in cattle. Serotypes of VTEC implicated in human disease have all been recovered from the faeces of cattle [16, 17] and foods contaminated with bovine faeces appear to be important sources of VTEC associated with human diseases [15, 18–20].

Certain VTEC cause attaching and effacing (AE) lesions in the large intestine similar to those caused by enteropathogenic *E. coli* (EPEC) in the small intestine and both types of *E. coli* are referred to as 'attaching and effacing *E. coli*' (AEEC) [21–27]. In EPEC, the chromosomal gene *eae*A encodes a 94-kDa outer membrane protein (intimin) which is necessary for intimate attachment to epithelial cells *in vitro* and *in vivo* [26, 28, 29]. A gene probe derived from the EPEC *eae*A nucleotide sequence recognizes a similar locus in certain VTEC strains [29].

Recently, two groups have cloned and sequenced the *eae*A gene from VTEC strains and have compared the sequences for the *eae*A gene from VTEC and EPEC strains [30, 31]. They have shown striking homology (97%) in the N-terminal and central regions, with less homology (59%) at the C-terminal region, which determines receptor specificity and is probably responsible for colonization of different regions of the intestine by these types of *E. coli* [30, 31]. Intimin is thought to bind membrane receptors which anchor the polymerized actin lying immediately underneath the adherent bacterium [32]. Recently it has been reported that the product of the VTEC *eae*A gene is a 97 kDa surface-exposed protein, which appears to play a key role in pathogenesis of the AE lesion [33].

Serotypes of VTEC implicated in bovine disease produce the same verotoxins as those which cause human disease [2] and factors required for colonization of the intestine are likely to be critical in determining virulence and host species specificity. The EaeA protein (intimin) is one factor which appears to be involved in the intimate adherence of pathogenic VTEC to the intestinal mucosa. In the present study, we examined a collection of bovine VTEC obtained in a systematic random survey to determine the prevalence of the *eae*A gene and its association with serotype, type of VT produced, and previous implication in disease of humans and cattle.

MATERIALS AND METHODS

Sampling

Eighty dairy farms from 12 counties in southern Ontario were selected by a formal random sampling procedure. A single rectal swab was taken from all healthy calves less than 3 months of age and from a random sample of the healthy milking herd, consisting of 25% of the herd or a minimum of 10 cows. This plan resulted in samples from 592 calves and 886 cows. Faecal swabs were transported on ice to the laboratory. The faecal swab was added to 9 ml of MacConkey broth (Difco, Detroit, MI) and incubated overnight at 37 °C. Next morning 500 μl of MacConkey broth culture were added to 5 ml of brain heart infusion broth (BHI) (Difco) and incubated for 24 h. The BHI culture was centrifuged at 12000 *g* in a microfuge and the supernatant used in a Vero cell assay (VCA) for detection of VT [34].

Attempts were made to isolate individual VT-producing colonies from samples that were positive in the screening test of BHI culture supernatant. The MacConkey broth cultures were streaked onto MacConkey agar plates which were incubated at 37 °C overnight. Five individual colonies and a sweep of colonies from the initial streak area were then tested for VT production, using the VCA. When only the colony sweep was positive, further testing was done to identify positive colonies. The specificity of the cytotoxicity on Vero cells was confirmed by polymerase chain reaction (PCR) amplification to determine whether the isolates possessed the genes for VT1, VT2 or both VT1 and VT2 [35].

Polymerase chain reaction

Primers C1 (5′-TCGTCACAGTTGCAGGCCTGG-T-3′ [803-824]) and C2 (5′-CGAAGTCTTATCCGC-CGTAAAGT-3′ [1912-1890]) were used in the PCR reaction to amplify a 1·1 kg DNA fragment (base pair 803-1912). These primers, derived from the conserved central region of the *eae*A gene of *E. coli* O157:H7 [29], were prepared in the Health of Animals Laboratory, Guelph. A 1 ml volume of overnight culture of the bacterium in Brain Heart Infusion broth (Difco, Detroit, MI) was centrifuged and the pellet

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washed in FA buffer (Difco, Detroit, MI) then suspended in 500 μml double distilled water. The bacterial suspension was boiled for 10 min then cooled on ice for 2 min. The supernatant was used as template DNA. Amplification reactions of 25 μl consisted of 1 mM 1X PCR buffer, 1·24 μl Taq polymerase (GeneAmp, Perkin Elmer Cetus, Norwalk, CT), 20 pmol. of each primer and 5 μl template DNA. Amplification was performed in a GeneAmp PCR system 9600 (Perkin Elmer Cetus) for 35 cycles as follows: 94 °C for 1 min, 55 °C for 1 min at 72 °C for 1·5 min. A negative control which contained all components of the reaction mixture, with the exception of template DNA was included in each set of samples tested. Two O26:H11 and two O157:H7 VTEC were used as positive controls. All samples were tested with the C1 and C2 primers. After amplification, 10 μl of the reaction mixture was analyzed by agarose gel electrophoresis.

Specificity of the amplification produce was confirmed by two methods. First, the amplification products from 30 isolates, selected at random, were digested with restriction endonucleases *Cla*I and *Ban*I, which cleave the 1109 bp product to yield fragments of 567 and 542 bp and 674 and 435 bp, respectively. The digested DNA was analyzed by agarose gel electrophoresis. Secondly, the 1109 bp product from the O157:H7 positive control strain was digoxigenin-PCR labelled (Boehringer Mannheim) and used as a probe in hybridization studies under stringent conditions according to the manufacturer's instructions to confirm the identity of the amplification products derived from the 30 isolates.

Serotyping

All isolates were serotypes at both the National Laboratory for Enteric Pathogens, Laboratory Centre for Disease Control, Ottawa, and the Agriculture Canada Health of Animals Laboratory, Guelph.

RESULTS AND DISCUSSION

A total of 608 VTEC were isolated. These consisted of 422 unique isolates and 186 isolates which were second colonies picked from positive samples. This latter group served as internal controls and are not included in the data presented. The results with the second colony were always the same as with the first colony from the same animal.

PCR amplification indicated that 145 (34%) of the 422 VTEC isolates possessed the *eae*A gene (Tables 1, 2). For 26 serotypes, all isolates were positive for the *eae*A gene (Table 1). Some serotypes (section B) were represented by low numbers, but were recorded so that their identity may become a part of the literature on serotypes which occur at low frequency. Of the 145 *eae*A-positive isolates (Tables 1, 2), 21 could not be typed precisely (Table 2). Among the 277 *eae*A-negative isolates (Tables 2, 3), 108 could not be typed completely. They include 80 O untypable isolates (section C, Table 3), which belong to 14 H antigen types, with more than half of these being H21.

To our knowledge this is the first study in which the prevalence of the *eae*A gene has been examined in VTEC obtained in a randomly collected manner from

Table 1. *Serotypes of verotoxigenic* E. coli *characterized as* eae*A-positive**

Serotype	Number tested	VT1	VT2	VT1 and VT2
A				
O5:NM†	14 (1)‡	10 (1)	0	4
O26:H11	17 (1)	16 (1)	1	0
O69:H11	5	4	1	0
O84:NM	8	8	0	0
O98:H25	4 (2)	4 (2)	0	0
O103:H2	19 (3)	19 (3)	0	0
O111:H8	4	2	0	2
O111:NM	7 (1)	7 (1)	0	0
O145:NM	12 (4)	4	8 (4)	0
O156:H25	3	3	0	0
O157:H7	13 (4)	1	4 (2)	8 (2)
B				
O5:H11	1	1	0	0
O18:H11	1	1	0	0
O26:NM	1	1	0	0
O49:NM	1	0	1	0
O74:NM	1 (1)	1 (1)	0	0
O76:H25	1	1	0	0
O80:NM	2	0	2	0
O84:Hunty§	1	1	0	0
O98:NM	2	1	1	0
O103:NM	1	1	0	0
O118:H16	2	2	0	0
O118:NM	1	1	0	0
O119:H25	1	1	0	0
O145:H8	1	1	0	0
O172:NM	1	0	1	0
Total	124 (17)	91 (9)	19 (6)	14 (2)

* All isolates that were tested were positive for the *eae*A gene.
† NM, non-motile.
‡ Numbers in parentheses are numbers of isolates from cows.
§ unty, untypable.

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Table 2. *Verotoxigenic* E. coli *characterized as* eae*A-variable*

Group of strains	Number tested	*eae*A +ve	VT1	VT2	VT1 and VT2
O119:NM*	6	1	6 (1)†	0	0
OR:NM‡	4 (**1**)	1	1 (1)	2 (**1**)	1
O?:H25§	11 (**8**)	1 (**1**)	4 (1) (**4**)	3 (**1**)	4 (**3**)
O?:NM	28 (**8**)	18 (**6**)	4 (1)	23 (16) (**7**)	1 (1) (**1**)
Total	**49** (**17**)**	**21** (**7**)	**15** (4) (**4**)	**28** (16) (**9**)	**6** (1) (**4**)

* NM, non-motile.
† Number in parentheses refers to *eae*A-positive isolates.
‡ OR, rough isolates.
§ O?, O antigen not in international scheme.
** Bold number in parentheses refers to number of isolates from cows.

a cattle population. Beutin and colleagues [36] recently reported that none of 33 VTEC isolates from healthy cattle was positive for *eae*A, but the collection of isolates was small and had not been obtained in a randomized manner. Seventeen of the 33 isolates were untypable but those that were typed were all different from those listed in Table 1. Three isolates from sheep that were found to be *eae*A-positive were of serotype O119:H25 (see Table 1). Interestingly, Beutin and colleagues [36] tested 52 O5:NM isolates (26 from sheep and 26 from goats) and found them all to be *eae*A-negative by the colony blot hybridization procedure. All 14 isolates of this serotype from healthy cattle that were tested by the PCR technique in the present study were positive.

The age of animal carrier may be related to prevalence of the *eae*A gene among VTEC isolates. It appears that the 33 isolates investigated by Beutin and colleagues [36] were from adult animals. In the present study, there were 24 *eae*A-positive isolates among 133 isolates (18%) from adult cattle, compared with 121 *eae*A-positive isolates among 289 (42%) isolates from calves (Table 4).

In contrast to the findings with VTEC from healthy cattle, Mainil and colleagues [37] found that 75% of 80 VTEC from calves with diarrhoea were *eae*A-positive by gene probe. The VTEC consisted of 70 isolates among 268 *E. coli* from Belgian calves with diarrhoea and 10 which had been received from researchers in the United States, Germany, the United Kingdom and Canada. Similarly, Barrett and colleagues [17] observed that 83% (29 of 35) of VTEC from calves with diarrhoea were *eae*A-positive, whereas only 49% (18 of 37) of VTEC from apparently healthy calves were *eae*A-positive. The high prevalence of *eae*A among VTEC from calves with diarrhoea [17, 37], the low prevalence of *eae*A among VTEC from healthy cattle [17, 36] and the higher prevalence of VTEC from healthy calves compared with healthy cows in this study suggest that *eae*A may be a factor in the colonization of the calf intestine by bovine VTEC.

Strains of serotype O157:H7 may affect the apparent association between *eae*A and pathogenicity for calves. In the study by Barrett and colleagues [17] 9 of the 18 *eae*A-positive isolates carried by healthy cattle were O157:H7 strains, which are associated with human but not bovine disease. In the present study, all O157:H7 strains (9 from healthy calves and 4 from healthy cows) were *eae*A positive (Table 1). It is interesting that isolates of this serotype constituted only 3% of the VTEC isolates, yet this serotype is the predominant serotype implicated in human disease. This finding suggests that this serotype may possess attributes other than *eae*A which favour its colonization of the human intestine and/or transmission among humans.

Among human VTEC, a high percentage of strains associated with disease carry the *eae*A gene [38, 39]. An exception was the finding by Wilshaw and colleagues [40] that there were only 13 *eae*A-positive isolates among 45 VTEC strains of human origin, that represented 17 O serogroups and were mostly from cases of HUS and HC. These results undoubtedly reflect the fact that the authors did not include strains of O groups 157 and 26 in their study, because it is well established that these possess the *eae*A gene.

Except for O groups 76 and 156, presence or absence of the *eae*A gene was related to O group. Thus, within each of Tables 1 and 3 it is common to find one O group with several H antigens or without the H antigen. There was a clear association of the

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*eae*A gene with certain serotypes, including O5:NM, O26:H11, O69:H11, O103:H2, O111:NM, O145:NM and O157:H7 (Table 1), which have all been associated with disease in calves or humans. Other researchers [17, 29, 39, 40] have also found that the predominant O serogroups among typed *eae*A-positive strains were O5, O26, O111 and O157. Serotypes O80:NM and O84:NM have not been associated with disease, but all strains of these serotypes were positive for the *eae*A gene.

For 49 bovine VTEC (Table 2) the link between serotype and the *eae*A gene could not be made because the strains could not be assigned to a serotype. Non-motile strains of the same O group may represent more than one H type which have lost the ability to express flagella and or O? strains may represent strains of a single H type with different O antigenic origins. Possibly, a combination of serotyping with biotyping may be a simple method for distinguishing among groups of strains of the same O serogroup. Electrophoretic typing, although less simple, may be even more valuable in making these distinctions.

Certain VTEC serogroups which are associated with HC and HUS in humans lack the *eae*A gene. These include O91:HNM, O113:H21, O117:H4, O153:H25. Possibly, another gene fulfils the function of the *eae*A gene for these serotypes. A recent report [41] that VTEC O113:H21 isolated from human diarrhoea can efface microvilli in rabbit cecum is consistent with this hypothesis. It is more difficult to assess the role of the *eae*A gene in virulence of bovine VTEC, because the reported serotypes of *eae*A-negative cattle pathogens (O?:H16, O?:H19, O?:H21) lack identified O antigens and their relationship to isolates with the same designation in this study is uncertain.

There appeared to be an association of the *eae*A gene with type of VT produced (Tables 1, 2, 4). Prevalence of VT1 and of *eae*A was higher in VTEC from calves compared with those from cows (Table 4). Among 124 strains of serotypes characterized as *eae*A-positive, 73% produced VT1 only (Table 1), whereas among the *eae*A-negative serotypes only 20% produced VT1 only (Table 3). If one considers the established calf pathogenic serotypes (O5:NM, O26:H11, O103:H2 and O111:NM), then 52 of 57 isolates (91%) produced VT1 (Table 1). This finding is consistent with the report by Wieler and colleagues [42] who showed an association of VT1 with diarrhoeic compared with healthy calves and with the study by Mainil and colleagues who reported that,

Table 3. *Serotypes of verotoxigenic* E. coli *characterized as* eae*A-negative**

Serotype	Number tested	VT1	VT2	VT1 and VT2
A				
O2:H29	3 (1)†	0	3 (1)	0
O8:H19	5 (5)	0	5 (5)	0
O22:H8	13 (12)	3 (3)	1 (1)	9 (8)
O38:H21	4 (4)	1 (1)	2 (2)	1 (1)
O113:H4	17 (2)	7	1	9 (2)
O113:H21	10 (6)	0	6 (3)	4 (3)
O113:NM‡	7	0	2	5
O116:H21	3 (3)	1 (1)	1 (1)	1 (1)
O132:NM	10 (1)	1	9 (1)	0
O153:H25	12 (11)	3 (3)	5 (4)	4 (4)
O153:H31	7	7	0	0
O156:NM	38 (4)	0	33 (3)	5 (1)
B				
O2:H5	1	1	0	0
O2:H27	1 (1)	0	1 (1)	0
O2:NM	2 (1)	1 (1)	1	0
O6:H34	2 (1)	0	2 (1)	0
O7:H4	1	0	1	0
O8:H35	1	0	1	0
O22:H2	1 (1)	0	1 (1)	0
O39:H49	2 (2)	1 (1)	0	1 (1)
O46:H38	2 (2)	2 (2)	0	0
O46:NM	2 (2)	1 (1)	0	1 (1)
O76:H19	1 (1)	1 (1)	0	0
O76:Hunty§	2	2	0	0
O85:NM	1	1	0	0
O88:H25	1 (1)	0	1 (1)	0
O91:H7	1 (1)	0	0	1 (1)
O112:H2	1 (1)	1 (1)	0	0
O113:H7	1 (1)	0	1 (1)	0
O115:H8	1 (1)	1 (1)	0	0
O115:H18	2 (1)	0	0	2 (1)
O117:H4	2	0	0	2
O117:NM	1	0	0	1
O121:H7	1 (1)	1 (1)	0	0
O136:H12	1 (1)	1 (1)	0	0
O136:NM	1	1	0	0
O139:H19	1 (1)	0	1 (1)	0
O146:H8	1 (1)	0	0	1 (1)
O156:H7	1 (1)	1 (1)	0	0
O156:H8	1	0	1	0
O163:H2	1 (1)	0	1 (1)	0
O163:H19	1 (1)	0	0	1 (1)
O163:NM	1 (1)	0	0	1 (1)
O171:H2	1	0	1	0
C				
O Untypable	80 (25)	10 (7)	50 (10)	18 (8)
Total	249 (99)	50 (26)	132 (38)	67 (35)

* All strains that were tested were negative for the *eae*A gene.

† Numbers in parentheses are numbers of isolates from cows.

‡ NM, non-motile.

§ unty, untypable.

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Table 4. *Distribution of verotoxins and* eae*A gene in VTEC from cows and calves*

	Number tested	*eae*A +ve	VT+	VT1	VT2	VT1 and VT2
Cows	886	24 (18)	133 (15)*	39 (29)	53 (40)	41 (31)
Calves	592	121 (42)	289 (49)	117 (41)	126 (44)	46 (16)
Total	1478	145 (34)	422 (29)	156 (37)	179 (42)	87 (21)

* Numbers in parentheses indicate percentages.

among 60 *eae*A-positive bovine VTEC from diseased animals, 93% produced VT1 as the only VT [37]. This pattern is different from that for O145:NM and O157:H7 (Table 1), two important human pathogens implicated in outbreaks of disease. Further studies are in progress to characterize these isolates with respect to their interaction with bovine colon to determine whether it may be possible to differentiate bovine and human pathogenic VTEC.

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