

## Serum Antibody Responses of Cattle following Experimental Infection with *Escherichia coli* O157:H7

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**Oral inoculation of calves and steers with 10<sup>10</sup> CFU of *Escherichia coli* O157:H7 induced prompt and sustained increases in serum antibodies to O157 lipopolysaccharide. Neutralizing antibodies to verotoxin 1 also increased rapidly in most steers but more gradually in calves. None of the animals developed neutralizing antibodies to verotoxin 2. These serological responses were not correlated with elimination of infection in calves or steers or protection of calves against reinfection with the same strain.**

Verotoxin (VT)-producing strains of *Escherichia coli* (VTEC) are an important cause of human hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) (14, 19, 20, 31). Known also as Shiga-like toxins, VTs are thought to cause the vascular endothelial damage in HC and HUS (19, 27). They are classified as VT1 or VT2, a more heterogeneous group with recognized variants (26). VTEC producing one or both toxins have been implicated in human disease (19); however, *E. coli* serogroup O157 strains producing VT2 or both VT1 and VT2 predominate in VTEC-associated HC and HUS (19, 36, 37).

Following outbreaks of HC and HUS associated with contaminated beef and dairy products (19, 41, 42), cattle have been identified as an important reservoir of *E. coli* O157:H7 and other VTEC (2, 4, 7, 15, 24, 28, 40, 43, 45, 46). A recent study (9) of cattle inoculated orally with *E. coli* O157:H7 confirmed that bovine infection with this organism is subclinical and revealed that fecal shedding could persist for up to 27 weeks in calves and 14 weeks in steers. The study also showed that calves were readily reinfected with the homologous strain following a second similar dose. Here we report the serological responses to VT1, VT2, and the O157 lipopolysaccharide (LPS) in a subset of animals from this study.

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**Animal inoculations and sera.** The organism used for animal inoculation was *E. coli* O157:H7 strain 3081 (9, 38). This strain is resistant to kanamycin and ampicillin and hybridizes with nucleic acid probes for VT1 and VT2 genes (26), the *eaec* gene (16), and CVD419 (23). A culture filtrate of the stock strain contained approximately 62,500 median cytotoxic doses of VT1 and VT2 per ml, determined by titration in a Vero cell cytotoxicity assay (see below) in the presence of specific antisera.

Sera were obtained at intervals from calves and adult cattle inoculated with *E. coli* O157:H7 strain 3081 and housed at the U.S. Department of Agriculture facilities (groups 1 to 3; Table 1) and from six randomly selected healthy uninoculated calves on Ontario dairy farms (group 4; Table 1). The four calves in group 1 were inoculated orally with 10<sup>10</sup> CFU of strain 3081 at 8 to 12 weeks of age (week 0) and received a second similar

dose at week 22 (calves M38 and M41) or week 33 (calves 832 and 835). The same dose was given once to nine 3-year-old steers (group 2). Group 3 steers were 1 year old when they were inoculated once with 10<sup>7</sup> CFU of strain 3081. Sera were collected from uninoculated calves (group 4) when they were 7, 12, and 18 weeks old. All sera were stored at -20°C and heated at 56°C for 30 min prior to testing.

Microbiological and pathological findings for inoculated cattle have been detailed elsewhere (9). Except for transient diarrhea in group 1 calves 1 day after inoculation, all animals remained healthy. *E. coli* O157:H7 strain 3081 was isolated from all group 1 calves for 7 weeks, from two calves (835 and 832) for 14 weeks, and from calf 832 until week 20. The level of fecal shedding was greater than 10<sup>5</sup> CFU/g during the first week and then declined. Group 2 steers were consistently culture positive during the first week after inoculation, although they shed significantly fewer organisms than did the calves. Two of the nine steers (219 and 678) were culture positive at week 7, and steer 678 was culture positive for 14 weeks. Among the five steers inoculated with 10<sup>7</sup> CFU of strain 3081 (group 3), two shed a few organisms for 1 day. Following reinoculation of group 1 calves at 8 to 10 months of age with 10<sup>10</sup> CFU of the same strain, their pattern of shedding resembled that of steers inoculated only once (group 2).

**O157Ab.** Antibodies to *E. coli* O157 LPS in serum (O157Ab) were titrated in an indirect enzyme-linked immunosorbent assay (ELISA) in microtiter plates. The ELISA was similar to that described previously (5), except that the antigen was highly purified *E. coli* O157:H7 LPS (11) (a gift from K. Dodds) diluted to 1 µg/ml in phosphate-buffered saline (PBS; 0.01 M phosphate, 0.3 M sodium chloride [pH 7.2]), the blocking solution was 1% gelatin in PBS, bound antibodies were detected with peroxidase-labelled goat anti-bovine immunoglobulin G (IgG) with heavy and light chain reactivity (Jackson Immunoresearch Laboratories, West Grove, Pa.), and the substrate and chromagen were hydrogen peroxide and the diammonium salt of 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), respectively, read at a wavelength of 405 nm (optical density at 405 nm [OD<sub>405</sub>]). Controls for each plate included O157Ab positive and negative bovine serum samples. Each serum sample was tested in duplicate at least twice, and the results were recorded as the mean OD values of replicate tests.

Preinoculation calf sera tested in the ELISA had low to moderate levels of O157Ab, which declined rapidly on dilution. To compare antibody responses, endpoint O157Ab titers

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TABLE 1. Reciprocal ELISA titers of O157Ab in calves and steers after oral inoculation with *E. coli* O157:H7 strain 3081

Group (dose) <sup>a</sup>	Animal no.	Reciprocal O157Ab titer at indicated week after inoculation									
		0	3	5	11	19	22	25	28	33	38
Group 1 calves (10 <sup>10</sup> CFU)	832	40	640	640	640		640	NT <sup>b</sup>	1,280	1,280 <sup>c</sup>	5,120
	835	40	320	640	640		320	NT	320	1,280 <sup>c</sup>	10,240
	M38	160	2,560	2,560	1,280		640 <sup>c</sup>	5,120			
	M41	20	10,240	NT	1,280		1,280 <sup>c</sup>	5,120			
Group 2 steers (10 <sup>10</sup> CFU)	219	640	2,560			1,280					
	J192	640	5,120			1,280					
	676	2,560	10,240			5,120					
	677	2,560	10,240			10,240					
	678	640	10,240			2,560					
	679	640	20,480			5,120					
	688	1,280	20,480			5,120					
	689	1,280	20,480			2,560					
	704	1,280	40,960			10,240					
Group 3 steers (10 <sup>7</sup> CFU)	SNT	160	320								
	Z32	160	160								
	Y20	320	320								
	Y39	640	640								
	43	320	320								
Group 4 calves (none)	2067	160		160	320						
	2068	320		80	40						
	HC63	80		160	160						
	JK55	320		160	320						
	HB7	320		80	160						
	HB8	320		160	80						

<sup>a</sup> An inoculum of *E. coli* O157:H7 strain 3081 was given orally. Group 1 calves were reinoculated at week 22 or 33.

<sup>b</sup> NT, not tested.

<sup>c</sup> Last titer before reinoculation with 10<sup>10</sup> CFU of *E. coli* O157:H7 strain 3081.

were determined as the highest dilution giving an OD value greater than the mean plus three standard deviations of the OD value of a 1:320 dilution of preinoculation sera from group 1 calves. Titers of O157Ab in these calves increased at least eightfold by week 3, remained elevated for more than 20 weeks, and increased further after reinoculation (Table 1). The steers had higher preinoculation levels of O157Ab than the calves and also showed strong and sustained increases in antibody titers after inoculation (Table 1). In contrast, inoculation of five steers with 10<sup>7</sup> CFU of *E. coli* O157:H7 strain 3081 induced little or no change in their O157Ab titers. In uninoculated calves (group 4), the titers between weeks 0 and 11 remained relatively constant or declined, suggesting that these antibodies were initially of maternal origin.

While the O157Ab in calf sera at week 0 may have been derived from colostrum, the reactivity of preinoculation sera from the adult cattle more likely reflected previous exposure to *E. coli* O157:H7, other *E. coli* of serogroup O157 that infect cattle (17, 44), or organisms with antigenically related O-polysaccharide or core-associated LPS antigens (5, 25, 29, 32, 39). To investigate this possibility, sera were tested by immunoblotting. Purified LPS (60 µg) was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (6, 22) in a 73-mm-wide preparatory gel (12% acrylamide). The resolved bands were transferred to nitrocellulose, from which strips were cut and reacted as described previously (6) with 1:100 dilutions of sera and the above-described peroxidase conjugate. Most preinoculation steer sera reacted more strongly with low-molecular-weight, core-associated antigens than with the characteristic O-polysaccharide "ladder." Staining of the O-polysaccharide and core-associated antigens became more intense

with sera collected three or more weeks postinfection from group 1 and 2 animals but not with sera from steers inoculated with the lower dose (group 3) or uninoculated calves (group 4) (data not shown). These findings indicate that reactivity detected in the ELISA at low serum dilutions may have been induced by organisms other than *E. coli* O157 and that the specificity of the ELISA might be improved by using purified O-polysaccharide rather than whole LPS as the antigen.

The prompt O157Ab response in animals inoculated with the higher dose of strain 3081 was similar to that detected previously by bacterial agglutination in cattle inoculated with an unspecified strain of *E. coli* O157 (8, 35). Unlike the sustained response detected by the ELISA, the agglutinating antibody response declined after 6 weeks and was not influenced by reinoculation with the same strain (8, 35). Agglutination reactions were largely eliminated by treatment of sera with 2-mercaptoethanol (8), indicating that the reaction was mediated by IgM antibodies (10). The ELISA, on the other hand, was designed with a conjugate to detect both IgG and IgM antibodies, and the sustained response to O157 LPS detected by the ELISA was probably due to IgG antibodies (13).

**Antibodies to VTs.** Titers of neutralizing antibodies to VT1 (VT1NAb) and VT2 (VT2NAb) were determined against approximately 10 50% cytotoxic doses of each toxin. Stock VT1 and VT2 were prepared from *E. coli* H30 (21) and C600-933w (34), respectively, and were titrated by a Vero cell cytotoxicity assay (12), with modifications in the final reading. Briefly, microplate wells were read microscopically after 48 h of incubation and then washed thoroughly with tap water. Residual cells were stained for 20 min with 100 µl of 0.025% crystal violet in 10% buffered formalin. After being washed, the

TABLE 2. Reciprocal titers of VT1NAb in calves and steers after oral inoculation with *E. coli* O157:H7 strain 3081

Group (dose) <sup>a</sup>	Animal no.	Reciprocal VT1NAb titer at indicated week after inoculation									
		0	3	5	11	19	22	25	28	33	38
Group 1 calves (10 <sup>10</sup> CFU)	832	<2	<2	8	16		64	NT <sup>b</sup>	64	8 <sup>c</sup>	256
	835	16	8	16	32		32	NT	32	16 <sup>c</sup>	512
	M38	4	<2	8	4		<2 <sup>c</sup>	512			
	M41	<2	<2	NT	<2		<2 <sup>c</sup>	8			
Group 2 steers (10 <sup>10</sup> CFU)	219	128	256			256					
	J192	8	64			64					
	676	<2	32			64					
	677	<2	16			8					
	678	<2	<2			<2					
	679	<2	128			256					
	688	8	512			256					
	689	4	1,024			128					
704	<2	<2			<2						
Group 3 steers (10 <sup>7</sup> CFU)	SNT	64	64								
	Z32	64	32								
	Y20	128	128								
	Y39	64	64								
	43	32	32								
Group 4 calves (none)	2067	64		16	512						
	2068	64		16	32						
	HC63	32		16	128						
	JK55	128		64	128						
	HB7	<2		<2	<2						
	HB8	256		256	64						

<sup>a</sup> An inoculum of *E. coli* O157:H7 strain 3081 was given orally. Group 1 calves were reinoculated at week 22 or 33.

<sup>b</sup> NT, not tested.

<sup>c</sup> Last titer before reinoculation with 10<sup>10</sup> CFU of *E. coli* O157:H7 strain 3081.

stained cells were solubilized with 100  $\mu$ l of 0.3% acetic acid in 0.5% sodium dodecyl sulfate, and the plates were read in a microplate reader at a wavelength of 630 nm. Titers were the highest twofold dilutions with greater than 50% cytotoxicity, with the OD<sub>630</sub> value of toxin-free wells taken as 0% cytotoxicity. Neutralizing antibody titers were determined by the same assay, except that duplicate twofold dilutions of test or control sera (50  $\mu$ l) were preincubated with 10 50% cytotoxic doses of VT1 or VT2 in 50  $\mu$ l of medium for 1 h at 37°C before the addition of freshly trypsinized Vero cells. Neutralization endpoints were the highest serum dilutions with less than 50% cytotoxicity. Fetal bovine serum used for the Vero cell cytotoxicity assay was heat inactivated and screened for VT1NAb and VT2NAb before use.

More than 40% of animals in each group had VT1NAb at week 0 (Table 2), as might be anticipated from the high prevalence of these antibodies in adult cattle (3, 30). Titers in inoculated calves generally declined or remained relatively constant until week 5 and then rose gradually. However, two inoculated calves (M38 and M41) had low titers until after reinoculation. This pattern is consistent with the presence of declining maternal antibodies, which may have delayed seroconversion. The similar pattern in the uninoculated calves (Table 2) was most likely due to infection with non-O157 VTEC, since none of these calves seroconverted to O157 LPS. In light of these findings, the VT1NAb responses of inoculated calves also may have been due to concurrent infections with non-O157 VTEC, which would not have been detected by the selective culture methods used to recover strain 3081 (9). In contrast, the VT1NAb responses of the steers given 10<sup>10</sup> CFU of strain 3081 (group 2) appeared more closely linked to inoc-

ulation. Titers in seven of the nine steers increased severalfold by week 3, perhaps reflecting prior sensitization to VT1 as well as the absence of the suppressive effect of maternal antibody. Two animals in this group did not have detectable VT1NAb responses during the study. Steers inoculated with the lower dose of strain 3081 (group 3) did not show the same VT1NAb response as steers given the higher dose (Table 2).

None of the animals had detectable VT2NAb levels during the study, even though *E. coli* O157:H7 strain 3081 produced both VT1 and VT2. This finding was consistent with the low prevalence of VT2NAb in cattle (3, 30) and with suggestions that VT2, unlike VT1, rarely induces specific neutralizing antibodies in cattle (3, 30), pigs (12), or humans (1, 13).

**Cross-reactivity with *Brucella abortus*.** In light of previously noted cross-reactivity of O157Ab with antigens of *B. abortus* (18, 25, 35), sera from group 1 and 2 animals were tested by the standard buffered antigen plate agglutination and complement fixation tests for bovine brucellosis used by Agriculture and Agri-Food Canada (33). Sera from calves M38 and M41 on weeks 0 and 25 and from steers 678 and 679 on weeks 0 and 3 were also tested by the SeroCard *B. abortus* antibody test used in the United States. None of these serum samples tested positive by the complement fixation or SeroCard tests, although 10 of the 57 serum samples reacted in the less-specific plate agglutination test, the strongest being those collected at week 3 from steers 688, 689, and 704.

This study has shown that calves and adult cattle infected with VTEC mounted strong serum antibody responses to antigens of O157 LPS lasting more than 5 months. The persistence of this response, as well as the antibody response to core-associated LPS antigens of related organisms, probably

accounts for the high rate of seropositivity to O157 LPS among adult cattle prior to inoculation. None of the animals developed VT2NAb, and although most developed VT1NAb, prior or concurrent exposure to non-O157 VTEC may have contributed to this response and to preinoculation levels of VT1NAb. Despite this evidence for a strong and rapid immune response to *E. coli* O157:H7, many animals remained infected for prolonged periods. Furthermore, the immune response failed to prevent reinfection with the homologous strain.

These findings have implications for both the epidemiology and control of *E. coli* O157:H7. Firstly, the ability of *E. coli* O157:H7 to persist in and reinfect animals in the face of a strong immune response is likely to contribute to the introduction and persistence of infection in herds. Secondly, failure of the immune response to prevent reinfection must raise the question of the efficacy of future vaccines as a means of controlling VTEC infection. However, the challenge dose was higher than would be anticipated under natural exposure, a factor that may be important since primary exposure to a lower dose ( $10^7$  CFU) resulted in only transient infection in two of five steers. Other factors such as host susceptibility, differences in the infectivity and pathogenicity of field strains, diet, and management factors such as age at weaning, housing, and manure handling may also influence the outcome of exposure to *E. coli* O157:H7.

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