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Virulence Properties and Serotypes of Shiga Toxin-Producing *Escherichia coli* from Healthy Australian Slaughter-Age Sheep

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A group of 1,623 ovine fecal samples recovered from 65 geographically distinct mutton sheep and prime lamb properties across New South Wales, Australia, were screened for the presence of Shiga toxin-producing *Escherichia coli* (STEC) virulence factors (*stx*$_1$, *stx*$_2$, *eaeA*, and *ehxA*). A subset was cultured for STEC isolates containing associated virulence factors (*eaeA* and/or *ehxA*), which were isolated from 17 of 20 (85%) and 19 of 20 (95%) tested prime lamb and mutton sheep properties, respectively. STEC isolates containing *stx*$_1$, *stx*$_2$, and *ehxA* were most commonly isolated (19 of 40 flocks; 47.5%), and this profile was observed for 10 different serotypes. Among 90 STEC isolates studied, the most common serotypes were O91:H$^-$ (22 isolates [24.4%]), O5:H$^-$ (16 isolates [17.8%]), O128:H2 (11 isolates [12.2%]), O123:H$^-$ (8 isolates [8.9%]), and O85:H49 (5 isolates [5.6%]). Two isolates (2.2%) were typed as O157:H$^-$. A total of 78 of 90 STEC isolates (86.7%) expressed Shiga toxin in Vero cell culture and 75 of 84 *ehxA*-positive isolates (89.3%) expressed enterohemolysin on washed sheep blood agar. *eaeA* was observed in 11 of 90 (12.2%) ovine STEC isolates, including serotypes O5:H$^-$, O84:H$^-$, O85:H49, O123:H$^-$ O136:H40, and O157:H$^-$. Although only 2 of 90 isolates were typed as O157:H$^-$, the predominant serotypes recovered during this study have been recovered from human patients with clinical disease, albeit rarely.

Although isolates belonging to serogroup O157 are regarded as the most clinically significant Shiga toxin-producing *Escherichia coli* (STEC) strains, the number of non-O157 serotypes recovered from episodes of hemorrhagic colitis and hemolytic-uremic syndrome (HUS) continues to increase. Currently over 160 serotypes of *E. coli* have been isolated from human sources (5, 6, 13, 14, 25). Ruminants, in particular (6, 7, 16, 24), but also other domestic animals, including pigs, poultry, cats, and dogs (1, 6, 8), are natural reservoirs of STEC. Although more than 200 different STEC serotypes have been isolated from cattle (reference 13 and references therein), few studies have extensively examined the presence of STEC in sheep. Existing studies have been performed on comparatively small numbers of sheep or have focused intensively on a single flock or only examined the presence of O157 serotypes (3, 12, 16, 17). Kudva et al. (17) investigated the presence of *E. coli* O157:H7 in a single flock over a 16-month period and described the presence of non-O157 STEC isolates of serotypes O128:NM, O5:NM, O6:H49, O88:NM, and O91:NM, with various combinations of the virulence-associated genes *stx*$_1$, *stx*$_2$, and *eaeA*. In Australia, one of the largest sheep-producing countries in the world, recent studies focusing primarily on the southeastern parts of Queensland have suggested that the prevalence of *stx* in fecal cultures ranges from 69 to 88% (12, 22).

In this study, we investigated the presence of STEC in fecal enrichment broths derived from 65 geographically diverse flocks of slaughter-age sheep (mutton sheep and prime lambs) in New South Wales, Australia, using a multiplex PCR which detects *stx*$_1$, *stx*$_2$, *eaeA*, and *ehxA* (11) and vancomycin-cefixime-cefsulodin blood agar (BVCCA) (18). Mutton sheep and prime lambs represent two different genetic lines of meat-producing animal and comprise sheep of different slaughter ages and production systems, parameters which have been reported to influence STEC colonization in ruminant species (26). Our aim was to isolate STEC strains that contained at least one other virulence factor (*eaeA* and/or *ehxA*). These STEC isolates were serotyped and examined for the ability to express Shiga toxins using Vero cell cultures.

Commercial properties from 29 prime lamb and 36 mutton sheep flocks were selected. Feces were collected from healthy animals due for slaughter within a month of collection, or where this was not possible, the animals selected represented slaughter-age animals. Grazing animals were fresh off the pasture and were sampled within 4 h of yarding. The mutton sheep were predominantly Merino, being culled for age, with ages ranging from 1 to 8 years (average, 5 years). The prime lambs were predominantly crossbred meat breeds or crossbred meat breed × Merino, with ages ranging from 4 to 12 months. Typically, feces from 25 sheep per flock were collected by rectal palpation and placed into sterile 50-ml plastic containers and stored at 4°C during transit from the property to one of three New South Wales Agriculture Veterinary Laboratories. Multiplex PCR methodologies were standardized among the three laboratories. Fecal broth cultures were prepared by inoculating 50 mg of feces into 10 ml of modified EC broth and

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incubated at 37°C for 18 to 20 h as described previously (11). For the preparation of DNA for PCR, a 15-μl aliquot of the overnight fecal culture or a single colony of *E. coli* was diluted in 1 ml of sterile water in a microcentrifuge tube, and bacteria were pelleted by centrifugation at 11,000 rpm for 1 min in a Biofuge *pico* (Heraeus, Hanau, Germany). DNA was extracted for PCR analyses using Instagene matrix as recommended by the manufacturer (Bio-Rad, Richmond, Calif.) and 2 μl was used for PCR.

Fecal enrichment broths from 20 mutton sheep and 20 prime lamb flocks which were positive in a multiplex PCR for Shiga toxin(s) and *ehxA* (with or without *eaeA*) (11) were plated (100 μl) onto BVCCA (25) at a dilution which would yield approximately 100 to 150 colonies per plate (10-mm diameter). The basis for selecting each of the 20 prime lamb and 20 mutton sheep flocks for the isolation of STEC was viability upon plating on BVCCA. Long transportation times and inappropriate storage conditions rendered the fecal enrichment broths from 6 of 26 prime lamb and 14 of 34 mutton sheep properties nonviable. Up to 10 colonies per fecal sample showing a narrow zone of hemolysis were subcultured, and DNA from a single colony was used in a second multiplex PCR (20) to determine the stx_1, stx_2, *eaeA*, and *ehxA* profile. This PCR targeted different but conserved regions of each gene compared to the multiplex PCR used to screen fecal enrichment broths (11). Fecal samples which showed a *stx* and *eaeA* profile but were negative for *ehxA* were diluted and plated onto MacConkey's agar. Ten colonies were chosen at random for multiplex PCR. Amplified DNA fragments were resolved by gel electrophoresis using 2% (wt/vol) agarose. Gels were stained with 0.5 μg of ethidium bromide/ml, visualized with UV illumination, and imaged using a GelDoc 1000 image analysis station (Bio-Rad). Colonies shown to possess the required STEC profile were confirmed as being *E. coli* based on routine biochemical tests. For serotyping analyses, STEC isolates were selected based on differences in virulence gene profiles and geographic location, with less emphasis on isolates recovered from the same animal with the same virulence factor profile. This ensured that STEC isolates containing all combinations of virulence genes were serotyped. These STEC isolates were serotyped using O antisera, from O1 to O173, and H antisera, from H1 to H56, as previously described (3, 4, 10). The strains were also tested on sorbitol MacConkey agar and washed sheep blood agar (WSBA) (2) to test for the ability to ferment sorbitol and to express enterohemolysin. STEC isolates were tested for the ability to produce Shiga toxin(s) using the Vero cell assay based on the method of Konowalchuk et al. (15).

Ninety percent (26 of 29 flocks) of the prime lamb and 92% (33 of 36 flocks) of the mutton sheep properties had at least one fecal multiplex PCR profile showing *stx* plus *eaeA* and/or *ehxA*. From 25 fecal samples tested for each mutton sheep and prime lamb flock the number of such STEC-positive broths ranged from 0 to 17 of 25 and 0 to 20 of 25, respectively (Tables 1 and 2). Multiplex PCR of fecal enrichment broths derived from 36 mutton sheep flocks most commonly detected stx_1 (651 of 904 [72%]), followed by *ehxA* (346 of 904 [38.3%]) and *eaeA* (94 of 904 [10.4%]); we observed only a few sheep excreting *E. coli* with stx_2 (37 of 904 [4.1%]). Fecal broths from mutton sheep containing populations of STEC with different combinations of virulence factors most commonly contained stx_1 and *ehxA* (219 of 904 [24.2%]). Other combinations were observed with much lower frequency (<4%) (Table 1). Similarly, among 29 prime lamb flocks, stx_1 (421 of 719 [58.6%]) was the most common virulence factor, followed by *ehxA* (235 of 719 [32.7%]). *eaeA* and stx_2 were observed for 117 of 719 (16.3%) and 28 of 719 (3.9%) fecal enrichment broths, respectively. Fecal broths containing populations of *E. coli* with mixed combinations of virulence factors showed that the three most common profiles were stx_1 and *ehxA* (115 of 719 [16%]), stx_1, *ehxA*, and *eaeA* (49 of 719 [6.8%]), and stx_1 and *eaeA* (37 of 719 [5.1%]) (Table 2).

STEC isolates containing associated virulence factors were isolated from 85% (17 of 20 flocks) of prime lamb and 95% (19

TABLE 1. Presence of virulence factors in 904 mutton sheep fecal samples from 36 flocks[a]

Virulence factor(s)	No. of samples with factor	Mean (SD) no. of samples per flock
None	169	4.694 (4.477)
eaeA	6	0.167 (0.845)
eaeA, stx_2	2	0.056 (0.333)
eaeA, stx_1	35	0.972 (1.682)
eaeA, *ehxA*	4	0.111 (0.398)
eaeA, stx_2, stx_1	1	0.0278 (0.167)
eaeA, stx_2, stx_1, *ehxA*	10	0.278 (0.701)
stx_2	3	0.083 (0.280)
stx_2, stx_1	6	0.167 (0.378)
stx_2, *ehxA*	1	0.0278 (0.167)
stx_2, stx_1, *ehxA*	14	0.389 (1.022)
stx_1	336	9.333 (5.514)
stx_1, *ehxA*	219	6.083 (5.067)
ehxA	62	1.722 (3.502)
eaeA, stx_1, *ehxA*	36	1.000 (1.568)
eaeA, stx_2, *ehxA*	0	0 (0)
Total	904	25.11 (0.575)

[a] Feces were collected from 25 animals per flock, except four flocks from which 28, 26 (two flocks), and 24 samples were collected.

TABLE 2. Presence of virulence factors in 719 prime lamb fecal samples from 29 flocks[a]

Virulence factor(s)	No. of samples with factor	Mean (SD) no. of samples per flock
None	231	7.966 (5.834)
eaeA	10	0.345 (0.614)
eaeA, stx_2	1	0.034 (0.186)
eaeA, stx_1	37	1.276 (1.461)
eaeA, *ehxA*	11	0.379 (0.679)
eaeA, stx_2, stx_1	0	0 (0)
eaeA, stx_2, stx_1 *ehxA*	8	0.276 (0.702)
stx_2	1	0.034 (0.186)
stx_2, stx_1	7	0.241 (0.831)
stx_2, *ehxA*	2	0.069 (0.258)
stx_2, stx_1, *ehxA*	8	0.276 (0.841)
stx_1	197	6.793 (4.109)
stx_1, *ehxA*	115	3.966 (3.018)
ehxA	41	1.414 (2.693)
eaeA, stx_1	49	1.690 (2.407)
eaeA, stx_2, *ehxA*	1	0 (0)
Total	719	24.79 (1.114)

[a] Feces were collected from 25 animals per flock, except one flock from which 19 samples were collected.

TABLE 3. Association between serotype and virulence factor profiles among 90 STEC isolates

Serotype	No. of isolates	Virulence factor profile (no.)	WSBA result[c]	Vero cell assay result	Sorbitol fermentation	Property type (no.)[a]
O4:H25	1	*ehxA* stx_1 stx_2	+	−	+	PL (1)
O5:H^{-b}	16	*ehxA* stx_1 stx_2 (3)	+	+	+	MS (6), PL (2)
		ehxA stx_1 (12)	+	+	+	
		eaeA stx_1	+	+	+	
O8:Hnt	2	*ehxA* stx_2	+	+	+	PL (1), MS (1)
			−	−	+	
O8:H49	1	*ehxA* stx_2	−	−	+	MS (1)
(O8):K25:H16	2	*ehxA* stx_1 stx_2 (2)	+	+	+	MS (1)
O75:H$^-$	4	*ehxA* stx_1 stx_2 (2)	+	+	−	MS (2)
		ehxA stx_1 (2)	+	+	−	
O75:H40	2	*ehxA* stx_1 stx_2	−	+	−	MS (1), PL (1)
			+	+	−	
O84:H$^-$	2	*ehxA* stx_1	+	+	+	MS (1), PL (1)
		ehxA eaeA stx_1	+	+	+	
O84:H16	1	*ehxA* stx_1 stx_2	−	−	+	MS (1)
O85:H49	5	*eaeA* stx_1 (3)	−	−	+	MS (2)
		eaeA ehxA stx_2	−	−	+	
		ehxA stx_1	−	−	+	
O91:H^{-b}	22	*ehxA* stx_1 (9)	+	+	+	MS (5), PL (1)
		(8)	+	+	−	
		(1)	−	−	+	
		ehxA stx_1 stx_2 (2)	+	+	−	
		(2)	+	+	+	
O91:H28	1	*ehxA* stx_1 stx_2	−	−	+	PL (1)
O123:H$^-$	8	*ehxA eaeA* stx_2 stx_1	+	+	−	MS (1), PL (4)
			+	+	+	
		ehxA stx_1 (3)	+	+	+	
		ehxA stx_1 stx_2 (3)	+	+	+	
O128:H2[b]	11	*ehxA* stx_1 (8)	+	+	+	MS (3), PL (2)
		ehxA stx_1 stx_2 (3)	+	+	+	
O136:H40	1	*eaeA* stx_1	−	+	+	MS (1)
O157:H^{-b}	2	*ehxA* stx_1 stx_2 *eaeA*	+	+	−	MS (2)
		eaeA stx_2	+	+	−	
O166:H49	1	*ehxA* stx_1	−	−	+	MS (1)
OX3:HR	1	*ehxA* stx_1	+	+	+	MS (1)
OX3:H2	1	*ehxA* stx_1	+	+	+	MS (1)
Ont:H4	6	*ehxA* stx_1 (5)	+	+	+	MS (3), PL (1)
			+	+	−	

[a] PL, prime lamb; MS, mutton sheep.
[b] Serotype associated with human disease.
[c] A positive result indicates the ability to express enterohemolysin.

of 20 flocks) of mutton sheep fecal samples. Table 3 provides a summary of virulence factor combinations observed in multiplex PCR analyses of STEC isolates. STEC isolates with different combinations of virulence factors were observed within and between flocks. The most prevalent combinations of factors in mutton sheep were stx_1 and *ehxA* (8 of 20 properties) and stx_1, stx_2, and *ehxA* (7 of 20 properties). For prime lambs the most prevalent factors were stx_1, stx_2, and *ehxA* (12 of 20 properties) and stx_1 and *ehxA* (4 of 20 properties). Fecal broths containing stx_2 were not commonly observed, which was later shown to be a limitation of this multiplex PCR (see below).

Ninety STEC isolates with different combinations of associated virulence factors and from geographically unrelated farms were serotyped. Thirteen different O types and eight different H types were found, and 6 of 90 (6.7%) isolates could not be O typed (Table 3). All of these had the same H4 antigen. The most common serotypes encountered were O91:H$^-$ (22 isolates [24.4%]), O5:H$^-$ (16 isolates [17.8%]), O128:H2 (11 isolates [12.2%]), O123:H$^-$ (8 isolates [8.9%]), and O85:H49 (5 isolates [5.6%]). Two isolates (2.2%) were typed as O157:H$^-$. It was not uncommon to find more than one STEC isolate with a different serotype within a single flock, and some flocks contained as many as five serotypes (data not shown). Eleven of the 90 (12.2%) STEC isolates contained *eaeA* and five of these (serotypes O123:H$^-$ [2 isolates], O157:H$^-$, O84:H$^-$, and O85:H26) also contained *stx* and *ehxA*.

STEC isolates with associated virulence factors were recovered from 85 to 95% of ovine properties, and the majority (84 of 90 [93.3%]) of the isolates contained *ehxA*. Of the 84 *ehxA*-positive STEC isolates 75 (89.3%) were found to express enterohemolysin on WSBA. Of interest was the observation that 20 (22.2%) of the 90 isolates did not ferment sorbitol. This inability to ferment sorbitol was restricted to certain serotypes, with all six O75 strains (whether H$^-$ or H8) being sorbitol negative on sorbitol MacConkey agar. Ten of 22 O91:H$^-$ strains, 1 of 8 O123:H$^-$ strains, both O157:H$^-$ strains, and 1 of 6 Ont:H4 strains was sorbitol negative. Vero culture assays confirmed that the majority (78 of 90 [86.7%]) of STEC isolates were capable of expressing Shiga toxin(s). Toxin titers ranged from 10^{-1} to 10^{-7}.

The majority of STEC isolates associated with human disease typically contain at least one of the virulence factors intimin or enterohemolysin, but occasionally disease has been associated with STEC strains which lack both of these virulence factors (1, 9, 13, 14, 19, 21). Our approach was to isolate STEC from healthy, slaughter-age sheep (65 flocks) grazing throughout the eastern half of New South Wales and to determine the serotypes. The serotype remains the most consistent factor in identifying STEC isolates likely to be associated with human disease (13). Among the 90 complex STEC isolates recovered in our study, 13 different O types, some with multiple H types, were observed. Serotypes O128:H2, O123:H$^-$, O157:H$^-$, O5:H$^-$, and O91:H$^-$ were the most predominant (65.5%) serotypes isolated in our study, and with the exception of O123:H$^-$, these serotypes have been recovered from (or were strongly implicated in) patients with serious diseases (e.g., HUS and hemorrhagic colitis). Beutin et al. (6) readily recovered serotypes O5:H$^-$, O91:H$^-$, O128:H2, OX3:H8, and O123:H10 from sheep (and occasionally goats, but not cattle), and serotypes O119:H25, O146:H8, O171:H12, and O136:H20 were also isolated, but with less frequency. Beutin et al. (7) showed that STEC serotypes O125:H$^-$/H26/H14, O128:H2, and O146:H21 were primarily recovered from sheep, but not cattle, in longitudinal studies of these two animal species, and no *eaeA* sequences were observed among the 67 ovine isolates studied.

A review of the world literature, recording well over 1,000 reports of isolations of non-O157 STEC strains (http://www.microbionet.com.au), shows that the ovine serotypes reported in our study (Table 3) have been commonly reported from sheep and sheep meat in other parts of the world. It is further noteworthy that parallel studies we carried out on cattle feces rarely isolated serotypes O5:H$^-$, O91:H$^-$, O123:H$^-$, and O128:H2 (M. A. Hornitzky et al., unpublished results), suggesting that particular STEC serotypes preferably inhabit different ruminant species. Although several studies reported bovine STEC isolates with serotype O5:H$^-$ (13, 14, 24), this serotype has not been observed in Australian cattle. We are currently characterizing fecal *E. coli* isolated from cattle and sheep simultaneously grazing the same pastures in an effort to determine if these serotypes can colonize and establish infection in both species.

In a study involving 101 healthy Australian sheep, Bettelheim et al. (3) described the presence of STEC serotypes O163:H19, O5:H$^-$, and O91:H$^-$, which were previously reported to be associated with human disease, including HUS. In a study of sheep and lambs from 14 farms primarily located in southern Queensland, 117 STEC isolates (45% of 144 sheep feces samples) were recovered (12). Most isolates (64%) contained both stx_1 and stx_2 and 26% contained *ehxA*. Only 2.6% contained *eaeA* and these isolates belonged to the serotype O157:H$^-$; the serotype of the majority of the non-O157 STEC isolates was not reported. Further, in a study by Fegan and Desmarchelier (12), fecal enrichment broths were tested for the presence of *stx* by PCR but were not screened for *eaeA* and *ehxA*. In our study, we detected *eaeA* in a total of 10.4 and 16.3% of mutton sheep and prime lamb fecal enrichment broths by multiplex PCR. STEC isolates containing *eaeA* and belonging to serotypes O123:H$^-$, O136:H40, O157:H$^-$, O5:H$^-$, O84:H$^-$, and O85:H49 were identified and represented 12.2% (11 of 90) of the 90 isolates serotyped. While it is plausible to speculate that screening methodologies may bias for the isolation of different populations of STEC, other factors such as seasonal variation, animal age, diet, and genetic background are likely to also affect the recovery rates of serologically diverse STEC and should be considered when comparing results from different studies.

Previous studies (9, 13) suggest that *eaeA* may be the single most important accessory factor correlated with severe disease. However, it should be noted that in a recent case of HUS associated with a strain of STEC serotype O5:H$^-$(23), this strain lacked *eaeA* and was shown to give virtually identical pulsed-field gel electrophoresis patterns as ovine isolates of the same serotype which were epidemiologically unrelated to the case. Many cases of HUS still go undiagnosed because laboratories only use methods which will isolate strains of the O157:H7/H$^-$ clone and thus may miss such STEC serotypes. While methods such as colony hybridization in association with hydrophobic grid membrane filtration may improve the recovery rates of less prevalent populations of potentially virulent *E. coli* (e.g., STEC isolates containing *eaeA*), these approaches are labor intensive, time-consuming, and logistically may not be easily accommodated in future studies. Alternative methodologies yet to be developed are required to facilitate the rapid isolation of *eaeA*-containing *E. coli*.

stx_2-positive STEC isolates (34 of 90 [37.8%]) were readily recovered in our study. These data were not anticipated from the results of fecal enrichment broth multiplex PCR and suggested that the PCR failed to amplify some stx_2 subtypes that appeared to predominate in sheep. Previously we reported that our fecal enrichment broth multiplex PCR amplified stx_2 from O111:H8, O157:H7, O113:H21, and O111:H$^-$ serotypes (11). stx_2 subtyping studies recently undertaken in our laboratory confirmed that the fecal multiplex PCR amplified stx_2 from most variants but was unable to amplify the majority of stx_2 variants present in the predominant serotypes isolated from sheep in this study (20a). These observations also explain our inability to detect some STEC isolates containing stx_2 in a previous preliminary investigation (11). The use of a second multiplex PCR (20) to confirm virulence factor profiles provides an additional cross-check and is recommended for laboratories using multiplex PCR to recover STEC during fecal screening, particularly from atypical sources.

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