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Comparison between human and animal isolates of Shiga toxin-producing *Escherichia coli* O157 from Australia

N. FEGAN[1]* AND P. DESMARCHELIER[1]

[1] *Food Science Australia, PO Box 3312, Tingalpa DC, 4173, Australia*

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SUMMARY

There is very little human disease associated with enterohaemorrhagic *Escherichia coli* O157 in Australia even though these organisms are present in the animal population. A group of Australian isolates of *E. coli* O157:H7 and O157:H- from human and animal sources were tested for the presence of virulence markers and compared by *Xba*I DNA macrorestriction analysis using pulsed-field gel electrophoresis (PFGE). Each of 102 isolates tested contained the gene *eae* which encodes the *E. coli* attaching and effacing factor and all but one carried the enterohaemolysin gene, *ehxA*, found on the EHEC plasmid. The most common Shiga toxin gene carried was stx_{2c}, either alone (16%) or in combination with stx_1 (74%) or stx_2 (3%). PFGE grouped the isolates based on H serotype and some clusters were source specific. Australian *E. coli* O157:H7 and H- isolates from human, animal and meat sources carry all the virulence markers associated with EHEC disease in humans therefore other factors must be responsible for the low rates of human infection in Australia.

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* O157:H7 has been responsible for large outbreaks and sporadic cases of disease in countries such as Japan [1], the United States [2] and the United Kingdom [3]. Cattle are a reservoir for *E. coli* O157 and outbreaks of infection have been associated with bovine sources such as meat and dairy products [2, 4–7].

All *E. coli* O157:H7 isolates share particular virulence markers including the production of Shiga toxins, the ability to produce attaching and effacing lesions [8, 9] and an enterohaemolysin encoded on a large 60-MDa plasmid [10]. Although *E. coli* O157:H7 appears to be the predominant serotype found associated with foodborne infections, *E. coli* O157:H- isolates may also contain these virulence markers and cause disease in humans [11, 12].

While *E. coli* O157:H7 and H- isolates have been found in Australian cattle and sheep [13, 14], there are very few cases of human disease associated with either of these serotypes [15–17]. Other serotypes of enterohaemorrhagic *E. coli* (EHEC) are more common. Most Australian isolates of *E. coli* O157 from human cases and from food animals are non-motile, and as yet have not been associated with an outbreak of disease [15]. It is not known why the epidemiology of *E. coli* O157 disease is different in Australia. This may be due to a combination of host, bacterium or epidemiological features. This study was conducted to assess whether *E. coli* O157 isolates from Australian human and animal sources differed in their virulence markers or clonal groupings.

* Author for correspondence.

MATERIALS AND METHODS

Bacterial strains

A total of 102 (14 from human sources and 88 from animal and meat sources) *E. coli* O157 isolates were tested for the presence of genes encoding various virulence markers. None of the isolates fermented

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sorbitol within 24 h or produced β-glucuronidase, and all contained Shiga toxin genes. *E. coli* O157 isolated from humans were kindly provided by Dr John Bates (Queensland Health, Nathan, Queensland, Australia) and Professor Roy Robins-Browne (Department of Microbiology and Immunology, University of Melbourne, Victoria, Australia). All other isolates were obtained from animal faeces, animal carcasses and retail meat during studies conducted at this laboratory during 1992–7. Isolates were stored in Protect Bacterial Preservers (Technical Service Consultants, Lancashire, UK) and sub-cultured on nutrient agar (Oxoid, Basingstoke, UK) when bacterial growth was required. All *E. coli* isolates were confirmed as serotype O157 using the *E. coli* O157 Test Kit (Oxoid) and with a PCR specific for the *E. coli* O157 *rfb* locus encoding O157 antigen [18]. Motility of the *E. coli* O157 isolates was determined by successive passaging through motility media [19]. Isolates that were motile were serotyped for the H7 antigen using RIM® *E. coli* O157:H7 (Remel, Lenexa, KS, USA).

PCR for virulence genes

The presence of various virulence markers was determined by PCR amplification of crude cell lysates. Lysates were prepared by boiling a washed cell suspension for 10 min before pelleting the cellular debris through centrifugation. The DNA in supernatant fluids was used as templates in PCR reactions. The genes detected included: Shiga toxin genes, stx_1 and stx_2 [20] and stx_{2c} [21]; the gene encoding the H7 flagella antigen, $fliC_{H7}$ [22]; the *E. coli* attaching and effacing factor (*eae*) [23], and the enterohaemolysin on EHEC plasmid (*ehxA*) [24]. PCR amplifications were performed in 25 μl volumes using 2 μl of crude cell lysates except for the stx_{2c} PCR which was performed in 50 μl volumes using 5 μl of crude cell lysates. The PCR for detecting stx_1 and stx_2, *eae* and *ehxA* was performed in a DNA Thermal Cycler (Hybaid Ltd, Middlesex, UK) using *Taq* polymerase supplied by Geneworks (Adelaide, Australia). Other PCR amplifications were performed in either a GeneAmp PCR system 9600 or 9700 using AmpliTaq DNA polymerase (Perkin Elemer, Norwalk, CT, USA). All primers were manufactured by Life Technologies (Gaithersburg, MD, USA). Products of PCR amplifications were separated by electrophoresis on agarose gels and visualized by ethidium bromide staining. The amplification product of stx_{2c} PCR was digested using *Hae*III and *Fok*I (New England Biolabs, Beverly, MA, USA) [21] to distinguish between stx_{2c} and stx_2.

Pulsed-field gel electrophoresis (PFGE)

The *E. coli* O157 isolates from human and animal sources were compared by PFGE of *Xba*I digests [25]. Isolates were cultured overnight in 5 ml of nutrient broth (NB; Oxoid) at 37 °C, cells were pelleted, washed and resuspended to give an OD_{610} of 1·0. The plugs were prepared by mixing the cell suspensions with an equal volume of molten 2% agarose (BioRad, Hercules, CA, USA) prepared in 0·5 × Tris Borate EDTA Buffer (TBE) [26] and dispensed into 1·5 mm thick plug moulds (BioRad). The DNA in plugs was digested with *Xba*I (Progen Industries, Darra, Australia) according to the manufacturer's instructions. Restriction fragments were separated by electrophoresis on 1·2% agarose gels in 0·5 × TBE for 22 h in a CHEF-DR® III electrophoresis system (BioRad). The electrophoresis conditions consisted of a pulse time ramped at 5–50 sec and a voltage of 6 V/cm. Restriction patterns were analysed using Molecular Analyst Fingerprinting software version 1.6 (BioRad, Hercules, CA, USA). Phenograms were generated using band correlation with Jaccard similarity coefficient and clustering by unweighted pair group method using arithmetic averages (UPGMA).

RESULTS

The majority (78%) of the 102 *E. coli* O157 isolates were non-motile (O157:H-). The human isolates examined consisted of 2 (14%) *E. coli* O157:H7 and 12 (86%) *E. coli* O157:H-, with similar results for the isolates of animal origin (23% *E. coli* O157:H7 and 77% *E. coli* O157:H-). The isolates of *E. coli* O157 from the carcasses and faeces of sheep and lambs were all non-motile. *E. coli* O157:H7 isolates were found in the faeces and on the carcasses of both cattle and calves, and in retail meat. All *E. coli* O157 isolates, whether they expressed motility or not, contained the $fliC_{H7}$ gene encoding for H7 and carried *eae*; all but one were positive for *ehxA*.

The *E. coli* O157 isolates varied in the combination of Shiga toxin genes they carried, with 92% of the isolates containing stx_{2c}, either alone (16%), in combination with stx_1 (74%) or with stx_2 (3%) (Table 1). Only 8 (8%) of the Australian isolates did not carry stx_{2c}. None of the isolates tested carried stx_2 alone, and stx_2 was present in isolates either in

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Table 1. *Source and* stx *genotype of* E. coli *O157 isolates*

Source/serotype	No. of isolates	No. of *E. coli* O157 with *stx* genotype				
		stx_1	stx_{2c}	stx_1, stx_2	stx_1, stx_{2c}	stx_2, stx_{2c}
Human faeces						
O157:H7	2			1		1
O157:H-	12	2	1	1	7	1
Cattle faeces						
O157:H7	3		2		1	
O157:H-	9			1	8	
Beef carcasses						
O157:H7	10		6		4	
O157:H-	5			1	4	
Calf faeces						
O157:H7	2		1		1	
O157:H-	16				16	
Calf carcasses						
O157:H7	4				4	
O157:H-	23		3		20	
Sheep/lamb faeces						
O157:H-	6	1	1		4	
Lamb carcasses						
O157:H-	4			1	3	
Retail meat						
O157:H7	1					1
O157:H-	5		2		3	
Total	102	3 (3)*	16 (16)	5 (5)	75 (74)	3 (3)

* Numbers in parentheses are percentages.

combination with stx_1 or stx_{2c}. The combination of *stx* was also different when comparing H7 and H- isolates. The number of *E. coli* O157:H7 isolates carrying only stx_{2c} was significantly higher ($P < 0{\cdot}01$) than *E. coli* O157:H-, while the number of *E. coli* O157:H- isolates positive for stx_1 and stx_{2c} was significantly higher ($P < 0{\cdot}01$) than *E. coli* O157:H7. The three isolates that contained stx_1 alone were *E. coli* O157:H-.

A phenogram of the *Xba*I digests separated using PFGE is shown in Figure 1. There was some clustering in association with serotype as most *E. coli* O157:H7 were found in cluster B. All of the cluster B isolates were exclusively the H7 serotype and were of bovine origin, including retail meat and the faeces and carcasses of cattle and calves. The three other O157:H7 isolates were grouped in clusters A (1 isolate of human origin) and C (2 isolates, bovine and human). Human isolates were grouped in clusters A, C, H and I, with cluster I containing human isolates only. Ovine isolates were found in a range of clusters, including A, C, E–H, and although no cluster consisted entirely of sheep isolates, the majority of isolates in cluster C were from ovine sources. Cluster C also contained the largest number of human isolates. Cattle isolates were represented in all but one cluster (I), while clusters B, D, J and K consisted entirely of bovine isolates.

Epidemiologically related isolates (i.e. those from different animals housed on the same farm or from the carcasses of animals slaughtered on the same day; data not shown) were often closely related and grouped within the same cluster. Some unrelated isolates (from different sources and isolated at different times; data not shown) also showed high percentage similarities.

DISCUSSION

Most outbreaks of disease associated with enterohaemorrhagic *E. coli* have been attributed to *E. coli* O157:H7 [27]. The incidence of human disease associated with *E. coli* O157:H7 within Australia is low with many other serotypes and *E. coli* O157:H-

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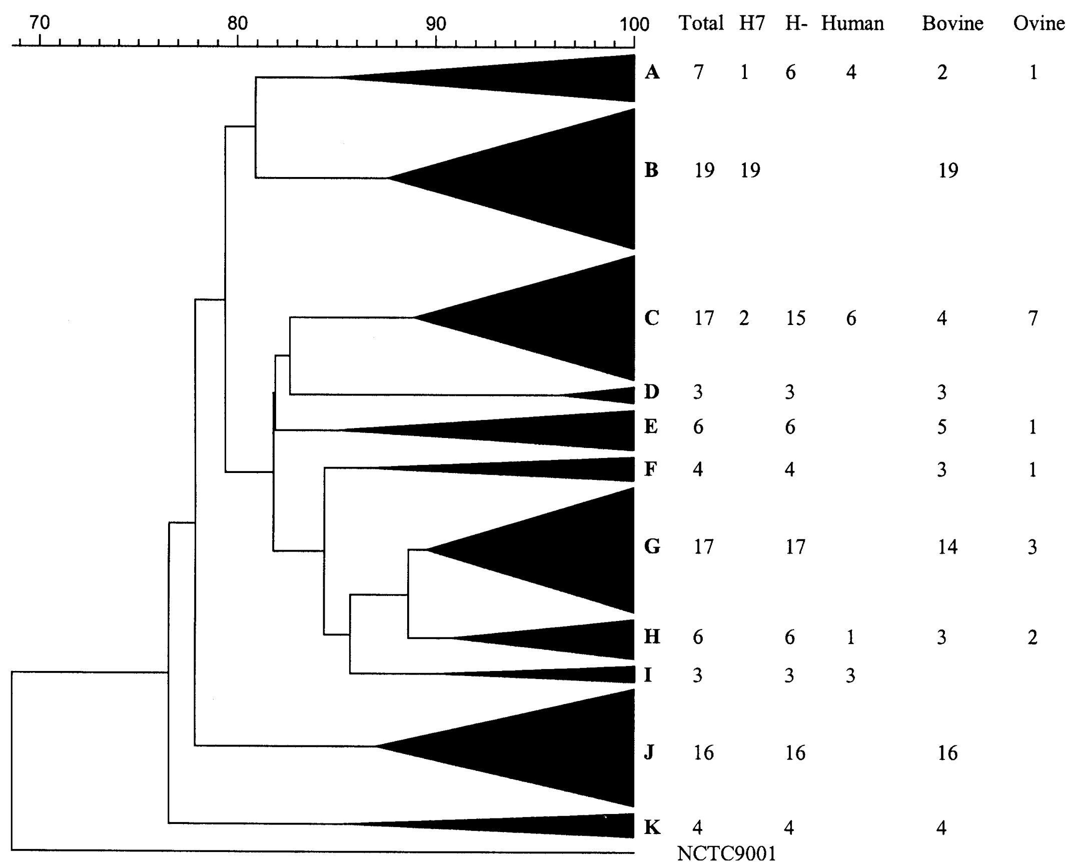

Fig. 1. Phenogram of *E. coli* O157 isolates. Cluster designations (A–K) and number of *E. coli* O157 isolates corresponding to serotypes and source are listed. *E. coli* NCTC 9001 was used as an unrelated control. Isolates from retail meat and carcasses are grouped into ovine or bovine sources as appropriate.

more commonly associated with Haemolytic Uraemic Syndrome (HUS) and bloody diarrhoea [15]. The majority of isolates found in Australia tend to be the non-motile serotype *E. coli* O157:H-. Isolates of *E. coli* O157:H- have been recovered from cases of human disease [12, 23, 28] and also from animal populations [29]. The proportion of isolates with the H7 serotype was low from both human (14%) and animal (22%) sources. In previous studies, Shiga toxin-producing *E. coli* O157:H- isolates have been found to carry the gene for H7 production [22, 30]. The non-motile isolates from this study all carried the $fliC_{H7}$ so it is possible that with further subculture, they would become motile or had lost other genetic components necessary for expression of motility.

There were differences in the types of *stx* found in the *E. coli* O157 isolates, though the significance of this is not clear. The *E. coli* O157:H- isolates more frequently carried stx_1 and stx_{2c} than the *E. coli* O157:H7 isolates, which more often carried stx_{2c} alone; stx_1 alone was found only in *E. coli* O157:H- isolates. These differences may reflect the clonal nature of the *E. coli* O157:H7 and O157:H- populations. It is interesting that so few of these isolates carry stx_2 as in the Northern Hemisphere, *E. coli* O157:H7 isolates with stx_2 appear to be more frequently found than isolates with stx_{2c} [31, 32]. However, more detailed investigations of the differences in populations of *E. coli* O157 from many different sources are required before the importance of these findings can be properly understood. The presence of stx_{2c} in more isolates than stx_2 in Australia does not account for the lower incidence of human disease as stx_{2c} was found to have the same level of toxicity as stx_2 in a mouse model [33]. Australian isolates did not differ from those of other countries in relation to the additional virulence markers, *eae* and *ehxA*, as all isolates (except for one which lacked *ehxA*) carried these markers.

PFGE analysis of *Xba*I digests of *E. coli* O157 isolates has been used successfully for studying relationships between *E. coli* O157 isolates [1, 25, 34–38]. The *E. coli* O157 isolates from Australia clustered partially based on serotype. Most *E. coli*

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O157:H7 isolates were found in one cluster (B), with only two other clusters containing *E. coli* O157:H7 isolates (A and C). This may also reflect the difference in the number of isolates from each serotype (22 H7 and 80 H-). The largest proportion of isolates in this study were from cattle and these showed the greatest diversity in *Xba*I patterns as strains from bovine origin were present in all but one cluster. Human isolates were grouped with bovine and ovine isolates in clusters A, C and H, but cluster I was composed exclusively of human isolates. Cluster A and C comprised the majority of human isolates (71%) and together with isolates from cluster I, indicate a possible clonal grouping of isolates that are able to infect humans. The presence of human isolates grouping with bovine isolates is supportive of transmission between cattle and humans [34, 39]. The presence of a large number of the ovine and human isolates in cluster C suggests that sheep are also an important reservoir for *E. coli* O157. This is also supported by other studies [40–42].

All *E. coli* O157:H7 and H- isolates from Australia, regardless of source, carry the virulence markers associated with human disease. It is possible that the low number of human cases of *E. coli* O157 infection in Australia may be due to factors other than those relating to the bacteria, including animal husbandry practices, food processing and handling practices, human behaviour and eating preferences. There may also be other virulence properties that have yet to be determined which may be necessary for *E. coli* O157 isolates to cause human disease. The low incidence of *E. coli* O157 associated disease in Australia may reflect the absence of such undetermined virulence factors in Australian *E. coli* O157 isolates.

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