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Relationship between Virulence Gene Profiles of Atypical Enteropathogenic *Escherichia coli* and Shiga Toxin-Producing *E. coli* Isolates from Cattle and Sheep in New Zealand▽

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Virulence gene profiles of atypical enteropathogenic *Escherichia coli* (aEPEC) and Shiga toxin-producing *E. coli* (STEC) from cattle, sheep, and humans were examined to determine the relationship between pathotypes. Shared virulence factors (intimin, EHEC hemolysin, serine protease, and a type II secretion system) were identified, suggesting a dynamic evolutionary relationship between aEPEC and STEC.

Diarrheagenic *Escherichia coli* represents a variety of different pathotypes with diverse virulence factors which often are conveniently used to subdivide *E. coli* associated with diarrheal disease (18, 22). For example, the primary virulence determinant of the enteropathogenic *E. coli* (EPEC) pathotype is the formation of attaching and effacing (A/E) lesions on cultured epithelial cells, a phenotype mediated by the locus for enterocyte effacement and the outer membrane protein intimin (*eae*) (15, 30). Similarly, the expression of Shiga toxins associated with lambdoid phage embedded within the chromosome defines the Shiga toxin-producing *E. coli* (STEC) pathotype (18, 22). A/E lesion formation has also been detected in certain STEC serogroups, such as O157, O26, O103, O111, and O145, which are commonly associated with more severe human disease (hemolytic-uremic syndrome [HUS] and hemorrhagic colitis [HC]) and are described as enterohemorrhagic *E. coli* (EHEC) (22). In addition to A/E lesion formation, many human EPEC strains possess the EPEC adherence factor (EAF) virulence plasmid containing an operon associated with the expression of bundle-forming pili (*bfp*) (30). Recent studies have suggested that EAF plasmid-negative EPEC may be associated with human diarrheal disease (1, 2, 16, 17, 23, 26, 31) and have also been isolated from ruminants (3, 9, 10, 14, 19, 20, 24). Consequently, these EAF plasmid-negative EPEC strains lacking the *bfp* locus have been described as atypical EPEC (aEPEC) strains to distinguish them from typical EAF plasmid-positive EPEC strains such as the progenitor strain O127:H6 E2348/69 (30). Loss of virulence determinants is not confined to the EPEC pathotype, however. Consecutive stool sampling of patients with HUS or HC has also revealed that certain EHEC serogroups (O157, O26, and O145) may lose *stx* genes during infection (4, 21). To distinguish these particular *stx*-negative, *eae*-positive serogroups associated with STEC/EHEC infection from aEPEC, it has been suggested that they be described as EHEC-LST (EHEC that lost *stx*) (4). It is uncertain whether ruminants are a reservoir of aEPEC which may cause human diarrheal disease or whether human aEPEC strains are distinct from ruminant aEPEC strains. Previous studies have indicated that plasmid-associated virulence factors more commonly associated with STEC, such as EHEC hemolysin (*ehxA*) (27), serine protease (*espP*) (6, 13), a catalase/peroxidise system (*katP*) (5), and a type II secretion pathway (*etpD*) (28), are occasionally associated with human aEPEC strains (1, 3, 16, 17) and that these same virulence factors may also be associated with ruminant aEPEC (8, 10–12). In addition, subtyping of *ehxA* and *espP* PCR products has indicated that subtypes of these virulence factors are shared between ruminant aEPEC and STEC (10, 12). This study, therefore, was done to investigate the virulence profiles of ruminant aEPEC and to compare them to thevirulence profiles of *eae*-positive STEC to provide insights into the relationship between the two pathotypes. A small number ($n = 15$) of STEC/aEPEC strains from humans were also included in the analyses where isolation was associated with symptoms associated with HUS/HC or diarrhea or there were no clinical details provided.

In total, the virulence profiles of 154 *eae*-positive (91 cattle, 48 sheep, and 15 human) strains were examined using PCR amplification alone or in combination with PCR restriction fragment length polymorphism (RFLP). PCR-RFLP analysis of *eae*, *ehxA*, and *espP* amplicons was performed as described previously (10–12), and amplification of stx_1, stx_2, *etpD*, and *katP* (catalase/peroxidase) was performed using primers and amplification as outlined in other studies (5, 8, 9). Only bac-

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terial strains that were *eae* positive were included in this study. Ruminant strains were originally isolated between November 2002 and January 2003 by using rectoanal mucosal swabs and selected on sorbitol MacConkey agar supplemented with cefixime and tellurite (CT-SMAC) or tryptone X-glucuronide agar (9). The human strains were from the Enteric Reference Laboratory, National Centre for Biosecurity & Infectious Diseases, Wallaceville, New Zealand. Serological analysis was carried out at the Enteric Reference Laboratory according to standard World Health Organization methods using antisera (Statens Serum Institute, Copenhagen, Denmark) raised for all known O and H groups.

Hierarchical clustering (complete linkage) was used to group samples based on the data generated from the seven PCR(-RFLP) analyses: stx_1, stx_2, *eae*, *ehxA*, *espP*, *etpD*, and *katP*. The similarity between samples was calculated by using $s(s_i, s_j) = N(\text{matches})/[N(\text{matches}) + w \cdot N(\text{mismatches})]$ (32), where N(matches) is the number of matches between sample i and sample j and w is a weight applied to the number of mismatches. A Dice coefficient weighting factor (w) of 0.5 was used in this study. The distance metric was therefore calculated as $d = 1 - s(s_i, s_j)$. For example, two samples having the same genotype would have a similarity score of 1. If sample 1 had genotyping scores of (−, −, beta, C, −, +, −) and sample 2 had scores of (−, −, beta, −, −, −, −), N(matches) = 5, N(mismatch) = 2, the similarity score would be 0.83 and the distance score 0.17. The cluster analysis was performed using R (25) with the distance matrix computed by a custom R script.

Using the data generated from the seven PCRs and differentiating by source, the *E. coli* strains clustered into 36 groups comprising five main clusters varying in heterogeneity (Fig. 1): 1, STEC O157; 2, *eae* β, *ehxA*, *espP* positive; 3, *eae*, *ehxA*, *espP* positive; 4, *eae*, *ehxA*, *etpD* positive or negative; 5, mainly (six of eight strains) *eae*, *ehxA*, *espP* positive, *etpD* positive. The *espP* and *ehxA* alleles were more common in cattle than in sheep (chi-square test, $P < 0.001$), and the *etpD* allele was more common in sheep than in cattle ($P < 0.001$). The *katP* allele was associated more commonly with STEC isolates than with aEPEC isolates ($P < 0.001$), whether the human isolates were included or not.

Overall, there was significant heterogeneity within the beta *eae*-positive STEC/aEPEC isolates (from humans, cattle, and sheep) with 12 groups and less variation with zeta isolates (from humans, cattle, and sheep) and theta isolates (from cattle and sheep) *eae*-positive STEC/aEPEC (5 and 7 groups, respectively, of each *eae* type). However, there was also heterogeneity within clusters with respect to the β-glucuronidase and non-sorbitol fermentation characteristics. Intimin subtype epsilon-, iota-, and kappa-positive aEPEC isolates were more homogeneous. Eight of 10 epsilon intimin-positive aEPEC isolates (*ehxA* subtype F) from cattle and sheep were clustered together with only single strains of *ehxA* subtypes B and C from cattle clustering separately. Iota-positive aEPEC isolates (n = 16) were associated with cattle exclusively and formed a single cluster. Kappa intimin-positive aEPEC isolates (n = 12) from cattle and sheep formed a single cluster, with only a single strain from cattle displaying a differing fermentation characteristic on CT-SMAC. Interestingly, multilocus sequence typing (MLST) of seven housekeeping genes from human aEPEC strains previously demonstrated the clustering of iota and kappa intimin types into specific clades where the majority of the strains were of the H8 (iota) or H10 (kappa) flagellum subtype, respectively (29). However, zeta intimin-positive and some theta intimin-positive aEPEC isolates could not be readily clustered using the MLST methodology (29). Furthermore, aEPEC serotypes ONT:H8 (intimin iota positive) and O49:H10 (intimin kappa positive) have been previously associated with human diarrheal disease (23, 29).

Six zeta intimin-positive aEPEC isolates, including an O131:H25 strain from cattle and a single O108:H25 isolates from sheep, had virulence profiles similar to those of two ONT:H− zeta intimin-positive STEC isolates, except for the presence of the stx_1 gene in the STEC strains. Similarly, seven zeta intimin-positive aEPEC strains (six from cattle, one from a sheep), including two O150:H− strains and a single O129:H− strain, had virulence profiles similar to those of the 16 zeta intimin-positive O84:H2/H− STEC strains, except for the presence of the stx_1 gene in the STEC strains. Generally, similarities between the virulence profiles of STEC and aEPEC isolates from sheep were less evident than those of isolates from cattle. Only a single O26:H11 aEPEC strain may have been an EHEC-LST strain, as other *stx*-positive O26:H11 strains with the same virulence profile were identified.

aEPEC strains are rarely isolated from human diarrheal disease in New Zealand, and only 2 of the 15 human strains in this study were *stx* negative (Fig. 1). More than 95% of the STEC isolates from human clinical cases in New Zealand are of serotype O157:H7. Non-O157 STEC strains (including *eae*-negative STEC strains) are isolated less frequently, and there are no uniform methods available for provincial health laboratories to screen for and identify aEPEC from stool specimens that are negative for STEC. Therefore, it is likely that non-O157 STEC strains may be somewhat underreported and aEPEC strains significantly overlooked, as they may be associated with nonbloody diarrheal disease or asymptomatic carriage. Despite the limited number of human isolates, the virulence profiles (*ehxA* subtype C and *etpD* positive) of the two human aEPEC strains (O145:H− and ONT:HND) that were intimin gamma positive matched those of two cattle strains (O172:H− and O145:H46) (Fig. 1) and a single sheep strain (O146:H−) (Fig. 1). As expected, the three STEC O157 strains clustered separately with their contrasting virulence profile containing O157:H7-specific *ehxA* and *espP* subtypes (Fig. 1). In addition, this work suggests that transmission of serogroups O26 and O84 between ruminants and humans may occur.

These data indicate that, except for *katP*, ruminant aEPEC may harbor the same plasmid-encoded virulence factors as STEC and this may be host associated. STEC plasmid-associated virulence factors that may be acquired by horizontal gene transfer, such as EHEC hemolysin, serine protease, and a type II secretion system (7), are commonly found in ruminant aEPEC and the prevalence of these virulence factors in ruminant aEPEC is greater (*ehxA*, 57.8%; *espP*, 45.6%; *etpD*, 53.5%) than their reported presence in human aEPEC strains (≤36%) (1, 4, 16, 17). Bacterial isolates that are intimin types beta (human, cattle, and sheep isolates), zeta (human, cattle, and sheep isolates), and theta (cattle and sheep isolates) form heterogeneous groups which contain both STEC and aEPEC, indicating that horizontal gene transfer of *stx*-containing prophage may occur within these groups. To our knowledge,

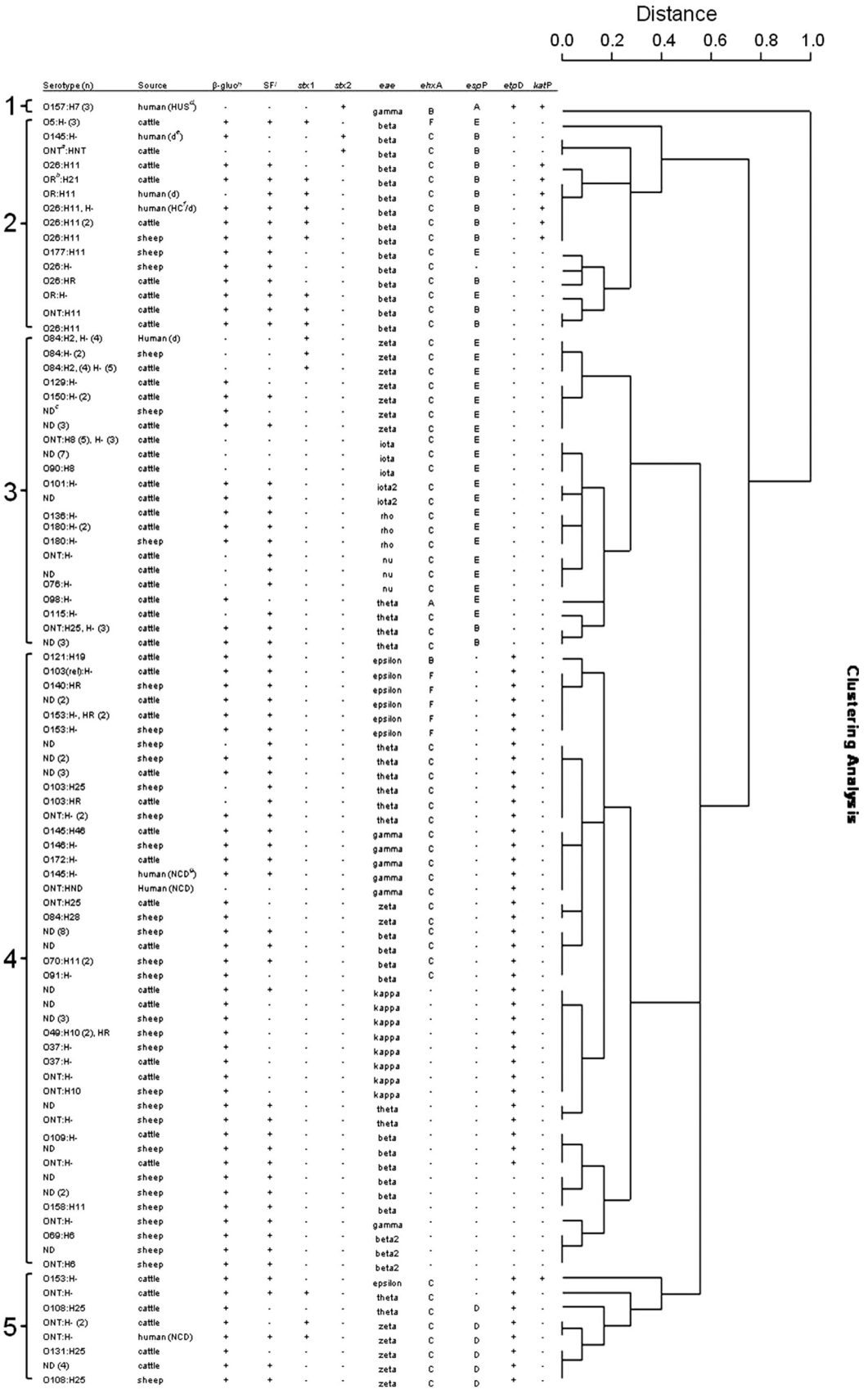

FIG. 1. Hierarchical clustering (complete linkage) analysis of *eae*-positive *E. coli* isolates from cattle, sheep, and humans based on the data generated from seven PCR(-RFLP) analyses: stx_1, stx_2, *eae*, *ehxA*, *espP*, *etpD*, and *katP*. Superscripts: *a*, not typable; *b*, O rough (autoagglutination); *c*, not done; *d*, HUS; *e*, diarrhea; *f*, HC; *g*, no clinical details; *h*, β-glucuronidase activity; *i*, sorbitol fermentation.

intimin types iota and kappa are not readily associated with STEC (10, 19, 20, 24, 33) and appear from this study to be more homogeneous and clonal and could therefore be more refractory to the insertion of *stx*-containing prophage. However, intimin types iota and kappa have been associated with human diarrheal disease (23, 26, 29) and certain serotypes such as O49:H10 and ONT:H8 that have been found in both ruminants and humans may be genuine zoonotic pathogens. We believe that the aEPEC pathotype is a heterogeneous group of bacteria that may encompass several different groups with various host specificities: (i) EHEC-LST, (ii) classical aEPEC strains that have lost the EAF plasmid, (iii) human aEPEC strains associated with diarrheal disease that contain STEC virulence factors, and (iv) commensal aEPEC strains that reside in cattle and sheep and contain STEC virulence factors. Further studies are required to elucidate any zoonotic relationship between humans and ruminant aEPEC and to characterize the integrity or availability of *stx*-containing phage insertion sites within STEC and aEPEC to determine whether a dynamic relationship between aEPEC and STEC occurs within the ruminant and/or human gastrointestinal tract with cycles of horizontal gene transfer and acquisition/loss of *stx*-containing phage by A/E lesion-producing bacteria.

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