Comparative Analysis of Virulence Genes, Genetic Diversity, and Phylogeny of Commensal and Enterotoxigenic *Escherichia coli* Isolates from Weaned Pigs†‡

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Received 26 April 2006/Accepted 9 October 2006

If the acquisition of virulence genes (VGs) for pathogenicity were not solely acquired through horizontal gene transfers of pathogenicity islands, transposons, and phages, then clonal clusters of enterotoxigenic *Escherichia coli* (ETEC) would contain few or even none of the VGs found in strains responsible for extraintestinal infections. To evaluate this possibility, 47 postweaning diarrhea (PWD) ETEC strains from different geographical origins and 158 commensal *E. coli* isolates from the gastrointestinal tracts of eight group-housed healthy pigs were screened for 36 extraintestinal and 18 enteric VGs using multiplex PCR assays. Of 36 extraintestinal VGs, only 8 were detected (*fimH*, *traT*, *fya*, *hlyA*, *kpsMII*, *k5*, *iha*, and *ompT*) in the ETEC collection. Among these, *hlyA* (α-hemolysin) and *iha* (nonhemagglutinating adhesin) occurred significantly more frequently among the ETEC isolates than in the commensal isolates.Clustering analysis based on the VG profiles separated commensal and ETEC isolates and even differentiated serogroup O141 from O149. On the other hand, pulsed-field gel electrophoresis (PFGE) successfully clustered ETEC isolates according to both serotype and geographical origin. In contrast, the commensal isolates were heterogeneous with respect to both serotype and DNA fingerprint. This study has validated the use of VG profiling to examine pathogenic relationships between porcine ETEC isolates. The clonal relationships of these isolates can be further clarified by PFGE fingerprinting. The presence of extraintestinal VGs in porcine ETEC confirmed the hypothesis that individual virulence gene acquisitions can occur concurrently against a background of horizontal gene transfers of pathogenicity islands. Over time, this could enable specific clonotypes to respond to host selection pressure and to evolve into new strains with increased virulence.

Enterotoxigenic *Escherichia coli* (ETEC) is the major cause of postweaning diarrhea (PWD) in weaned pigs. ETEC strains colonize the small intestine with the aid of adhesion factors (e.g., *F*4 or *F*18 fimbriae). Once attached, the release of either heat-labile or heat-stable (STa or STb) enterotoxins induces diarrhea by affecting the electrolyte balance of the small intestine. The presence of virulence genes (VGs) encoding these determinants is a key requirement for pathogenicity in ETEC and is generally used to distinguish these pathogens from the nonpathogenic or commensal *E. coli* normally carried in the intestine (37).

Characterization of isolates from outbreaks of PWD has shown that ETEC strains lacking recognized fimbriae, such as *F*4 and *F*18, are becoming more common (19, 26, 44). This may be related to the widespread use of vaccines incorporating fimbriated strains, providing selection pressure for the acquisition and carriage of novel, unrecognized VGs. For example, Noamani et al. (37) found that most of the PWD isolates recovered in the period 1998 to 2001 in Ontario, Canada, were associated with a new type of O149 ETEC, which possessed VGs for STa and an enteroaggregative heat-stable enterotoxin (EAST1) not found previously in O149 isolates from the same region. Over time, porcine ETEC acquisition of new VGs associated with PWD may therefore change their VG profile and, potentially, their pathogenicity.

Numerous genes encoding virulence factors such as adhesins, host cell surface-modifying factors, toxins, and secretion systems are involved in mechanisms of pathogenicity in *E. coli*. Strains of the same pathotype and serotype normally carry a defined set of VGs that are critical for infection. For instance, enteropathogenic *E. coli* (EPEC) is associated with virulence factors encoded by the locus of enteroagglutinin, including *tir*, *eaeA*, and *esp* genes, while extraintestinal pathogenic *E. coli* (ExPEC) that causes urinary tract infections (UTI) or septicemia carries *pap*, *afa/draBC*, *sfa/focDE*, *kpsMTI*, and *iuA* genes (29).

However, using recent technological advances such as microarrays and multiplex PCR to explore the global virulence pattern of strains, combinations of VGs predictive of differ-
ent pathotypes have been observed in unusual pathogenic and commensal E. coli strain backgrounds (2, 31). For example, Bekal et al. (2) found a human enterohemorrhagic (EHEC) strain harboring a number of VGs typically involved in extraintestinal infections. Additionally, a bovine ETEC strain was found to contain ExPEC-associated genes (traT, ompT, and fimH) and an EHEC-associated gene (etiD) as well as genes for F5 fimbrae and STa. A human ETEC strain (H10407) was found to contain two invasion genes (tia and tib) that direct the bacterium to invade human intestinal cell lines (17).

Population analysis of the autochthonous E. coli bacteria inhabiting the gastrointestinal tract (GIT) of pigs has shown them to be very diverse, with greater diversity being demonstrated between the different regions of the gut than between different animals (12). In terms of microbial fitness, a potential advantage that porcine ETECs have over commensal E. coli strains is greater amplification and dispersion in pigs with diarrheaa compared to healthy pigs with normal feces and, thus, greater opportunity for acquisition of VGs from other strains (37). Characterization and comparison of VG profiles between ETEC and gut commensal E. coli isolates can provide important insights into the evolution and spread of VGs in potentially pathogenic lineage. The aims of this work were to determine whether PWD ETECs also carry VGs that are representative of other pathotypic E. coli strains and to assess whether combinations of VGs are associated with ETEC strains belonging to different serotypes. It was also our intention to examine if VG combinations could distinguish clinical isolates from different geographic origins and to explore the presence of VGs in commensal isolates. Additionally, molecular variation between the PWD ETEC and commensal isolates has been characterized by pulsed-field gel electrophoresis (PFGE) and genomic fingerprinting and compared with VG profiling as a predictor of genotype.

MATERIALS AND METHODS

E. coli strains used in this study. A total of 205 porcine E. coli strains were analyzed in this study. The collection consisted of 47 isolates recovered from pigs with PWD and 158 E. coli isolates obtained from different parts of the GIT of healthy pigs. These isolates have been described in a previous publication (12) and are referred to as nonidentical commensal isolates or strains based on DNA fingerprinting. The PWD isolates were obtained from diagnostic submissions (fecal swabs or intestinal contents) from animals of different geographic origins. Diagnostic criteria for designation of an isolate as a PWD strain have been given previously (13). Seventeen isolates were from southern New South Wales (NSW), Australia, 23 from Queensland (Qld), Australia, and 7 from Vietnam. The isolates were represented by two major serogroups, O149 (n = 39) and O141 (n = 8). The 158 commensal E. coli strains were isolated from eight different 13-week-old male pigs (hybrids of Large White and Landrace), weighing 35 to 45 kg each, from eight different litters at Elizabeth Macarthur Agricultural Institute, Australia. On average, four to five isolates were taken from three different gut regions (duodenum, ileum, and colon) and fecal samples for each individual pig. The isolates were confirmed as E. coli by indole test (positive), minimal lactose agar growth (positive), and Simmons citrate agar growth (negative), as described by Dixit et al. (12). Strains used as reference sources of VGs for PCR analysis are listed in Table S1 in the supplemental material. All strains were grown on Luria-Bertani (LB) media and routinely stored at -80°C in 15% (vol/vol) glycerol. PCR template DNA was prepared by boiling 1 ml of overnight LB broth culture for 10 min, and 2-μl aliquots of the supernatant were subjected to PCR.

Serotyping porcine E. coli isolates. E. coli isolates were serotyped by the Microbiological Diagnostic Unit, University of Melbourne, Australia, using slide agglutination tests as described previously (5, 6). Strains which failed to achieve motility on semisolid medium were considered nonmotile and designated H-.

Detection of virulence genes and rapid phylogenetic analysis by PCR. ExPEC-associated virulence factors were detected by multiplex PCR testing of 36 different VGs associated with extraintestinal diseases. In addition to the 30 extraintestinal VGs that have been published already (28), 6 additional genes (univcnf, iha, irone, ompT, iss, and ireA) were added as shown in Table 1 (J. R. Johnson, University of Minneapolis, personal communication). Primers were sorted into six pools based on primer compatibility and amplicon size. The multiplex PCR assay was modified from Johnson et al. (29). Briefly, PCR was

![Image](http://aem.asm.org/)

**TABLE 1. Virulence genes and PCR sizes for 36 ExPEC genes**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description/function</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAI</td>
<td>PAI marker malX from strain CFT073</td>
<td>930</td>
</tr>
<tr>
<td>papAH</td>
<td>Major structural subunit of pilus associated with pyelonephritis (P fimbrae); defines F antigen</td>
<td>720</td>
</tr>
<tr>
<td>fimH</td>
<td>β-Mannose-specific adhesin, type 1 fimbrae</td>
<td>508</td>
</tr>
<tr>
<td>kpsMTIII</td>
<td>Group III capsular polysaccharide synthesis (e.g., K3, K10, and K54)</td>
<td>392</td>
</tr>
<tr>
<td>papEF</td>
<td>Minor tip pilins; connect PapG to shaft (PapA)</td>
<td>336</td>
</tr>
<tr>
<td>iheA</td>
<td>Invasion of brain endothelium</td>
<td>170</td>
</tr>
<tr>
<td>hlyA</td>
<td>Yersinia siderophore receptor (ferric versiniabactin uptake)</td>
<td>880</td>
</tr>
<tr>
<td>bmaE</td>
<td>M-agglutinin subunit</td>
<td>507</td>
</tr>
<tr>
<td>sfa/focDE</td>
<td>Central region of sfa (S fimbrae) and foc (FIC fimbrae) operons</td>
<td>410</td>
</tr>
<tr>
<td>iutA</td>
<td>Ferric aerobactin receptor (iron uptake; transport)</td>
<td>300</td>
</tr>
<tr>
<td>papG allele III</td>
<td>Cysteine-associated (prs or pap-2) papG variant</td>
<td>258</td>
</tr>
<tr>
<td>kpsMT K1</td>
<td>Specific for K1 (group II) kpsMT</td>
<td>153</td>
</tr>
<tr>
<td>hlyA</td>
<td>α-Hemolysin</td>
<td>1,177</td>
</tr>
<tr>
<td>rfc</td>
<td>O4 lipopolysaccharide synthesis</td>
<td>788</td>
</tr>
<tr>
<td>rfaE</td>
<td>Nonfimbrial adhesin I assembly and transport</td>
<td>559</td>
</tr>
<tr>
<td>papG I (internal)</td>
<td>J96-associated papG variant</td>
<td>461</td>
</tr>
<tr>
<td>kpsMT II</td>
<td>Group II capsular polysaccharide synthesis (e.g., K1, K5, and K12)</td>
<td>272</td>
</tr>
<tr>
<td>papC</td>
<td>Pilius assembly; central region of pap operon</td>
<td>200</td>
</tr>
<tr>
<td>gafD</td>
<td>N-Acetyl-D-glucosamine-specific (G) fimbrial adhesin</td>
<td>952</td>
</tr>
<tr>
<td>evcC</td>
<td>Colicin V; conjugative plasmids</td>
<td>680</td>
</tr>
<tr>
<td>cdtB</td>
<td>Cytotoxid distending toxin</td>
<td>430</td>
</tr>
<tr>
<td>focG</td>
<td>Pilius tip molecule, FIC fimbrae (sialic acid specific)</td>
<td>360</td>
</tr>
<tr>
<td>traT</td>
<td>Surface exclusion, serum survival</td>
<td>290</td>
</tr>
<tr>
<td>papG II</td>
<td>Pylonephritis-associated papG variant</td>
<td>190</td>
</tr>
<tr>
<td>papG I</td>
<td>J96-associated papG variant</td>
<td>1,190</td>
</tr>
<tr>
<td>papG II and III</td>
<td>J96-associated papG variant</td>
<td>1,070</td>
</tr>
<tr>
<td>afaA/draABC</td>
<td>Central region of Dr antigen-specific fimbrial and afimbrial adhesin operons</td>
<td>559</td>
</tr>
<tr>
<td>cnf1</td>
<td>Cytotoxic necrotizing factor 1</td>
<td>498</td>
</tr>
<tr>
<td>sfaA</td>
<td>Pilius tip adhesin, S fimbrae (sialic acid specific)</td>
<td>240</td>
</tr>
<tr>
<td>kpsMT K5</td>
<td>Specific for non-K1 and non-K2 group II kpsMT</td>
<td>159</td>
</tr>
<tr>
<td>univcnf</td>
<td>Universal primer for cytotoxic necrotizing factor 1</td>
<td>1,105</td>
</tr>
<tr>
<td>iha</td>
<td>Novel non-hemagglutinin adhesin (from O157:H7 and CFT073)</td>
<td>827</td>
</tr>
<tr>
<td>iroNEx-coli</td>
<td>Novel catecholate siderophore</td>
<td>665</td>
</tr>
<tr>
<td>ompT</td>
<td>Outer membrane protein T (protease)</td>
<td>559</td>
</tr>
<tr>
<td>iss</td>
<td>Serum survival gene</td>
<td>323</td>
</tr>
<tr>
<td>ireA</td>
<td>Iron-regulated element, a siderophore receptor</td>
<td>254</td>
</tr>
</tbody>
</table>

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performed with 0.2-ml PCR tubes (INTERPATH, Australia) on a PC-960 thermal
cycler (Corbett Research, Australia) with a reaction volume of 25 μl. The
DNA template (2 μl) was added to a mixture containing 0.6 mM of each dATP,
dGTP, dCTP, and dDTP (Astral Scientific, Australia), 1/1000× buffer solution
(QIAGEN, Australia), 0.06 mM of each primer, and 1.5 U of HotStar
Taq polymerase (QIAGEN). Each PCR program was preceded by a step
of 15 min at 95°C to activate the HotStar Taq polymerase, followed by 25 cycles
of 94°C for 30 s, 63°C for 30 s, and 68°C for 3 min, with an extension step at 72°C
for 10 min. MilliQ H₂O was included in each PCR run as a negative control.
DNA from each strain was also subjected to PCR for 18 different VGs (Table 2),
which represent various E. coli pathotypes causing enteric disease, including
ETEC, EPEC, enteropathogenic E. coli (EaggEC), enteroinvasive E. coli
(EIEC), and EHEC. The designations of all the primers in both Table 1 and 2 are
described in Table S2 in the supplemental material.

The phylogenetic grouping for each isolate was determined based on the PCR
method described by Clermont et al. (10). A two-step triplex PCR was performed
using three gene primers (chuA, yjaA, and DNA fragment TspE4C2) under the
following conditions: denaturation for 4 min at 94°C, followed by 30 cycles of 5 s
at 94°C and 10 s at 59°C, and a final extension step of 5 min at 72°C. The
following criteria were used for assignment of strains to phylogenetic groups:
group B2, chuA+, yjaA+; group D, chuA+ yjaA−; group B1, chuA− mutant, TspE4C2+; and group A, chuA mutant, TspE4C2 mutant (15).

PCR products were separated by agarose gel electrophoresis. Five microliters
of amplified product was mixed with 3 μl of loading dye containing 0.25%
bromophenol blue in 15% Ficoll solution and loaded onto 2.5% agarose gels for
electrophoresis (Ultrapure Agarose; Life Technology, Australia) with ethidium
bromide (1 μg/ml), using 0.5× Tris-borate-EDTA (TBE) as running buffer.
DNA in the gel was visualized by exposing the gel to UV light and was photo-
graphed with a digital capture system (Gel Doc; Bio-Rad). PCR product lengths
were verified by comparison with a 100-bp DNA ladder (Promega, Australia).

Pulsed-field gel electrophoresis (PFGE). Genomic DNA for PFGE was pre-
pared using the technique of Barrett et al. (1), with modifications. In brief,
bacterial colonies from overnight LB agar were embedded in 2% of low-melting-
point agarose (Bio-Rad). Genomic DNA was extracted in situ by treatment with
lysozyme (0.5 mg/ml; Sigma, Australia) and protease K (0.5 mg/ml; Sigma) lysis
buffers. The agarose-packaged DNA was then digested with the endonuclease
NotI or XbaI (New England Biolabs, Australia). The resulting fragments were
separated using 1.2% PFGE-grade agarose gel (Bio-Rad) in a GeneNavigator
system (Amersham Pharmacia Biotech), with a 5- to 35-s pulse time and 200 V
for 25 h in 0.5× TBE buffer at 12°C. Gel images were saved in TIFF format and
processed using GelCompar software (version 4.2; Applied Maths, Kortrijk,
Belgium) for computer analysis. PFGE chromosomal fingerprints were com-
pared by use of the criteria of Tenover et al. (46). Similarity between fingerprints
was further determined on the basis of the Dice coefficient. A band position
tolerance of 1.0% and a maximal optimization shift of 0.5% were set, and
dendograms were generated by the unweighted pair group method with arith-
metic mean analysis.

DNA sequencing. PCR fragments were partially sequenced for gene verifica-
tion. The DNA band of the target PCR product was excised from the agarose gel
and purified using a QIAAGEN Gel Purification kit. A total of 70 ng of purified
DNA was subjected to AmpliTaq cycle-sequencing reactions with the Prism
ready reaction dye dideoxy terminator cycle-sequencing kit (Applied Biosystems)
according to the manufacturer’s instructions. Electrophoresis of amplified prod-
ucts was performed on 7% polyacrylamide gels with an automated sequencer
(model 373A; Applied Biosystems). The nucleotide sequences were analyzed
with the ANALYSIS program (Applied Biosystems) and the ANGUS program
(Australian National Genomic Information Service, University of Sydney).

Statistical analysis. The chi-squared test of deviance was used to analyze the
correlation between the presence of virulence genes and the distribution of
virulence genes within different serogroups.

### Table 2. Virulence genes and PCR sizes for 18 enteric pathogenic E. coli virulence genes and 3 Clermont PCR genes

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Description</th>
<th>Amplicon size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterotoxigenic E. coli (ETEC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>faeG</td>
<td>F4 fimbrial adhesin (K88)</td>
<td>86</td>
<td>22</td>
</tr>
<tr>
<td>fedA</td>
<td>F18 fimbrial adhesin</td>
<td>128</td>
<td>22</td>
</tr>
<tr>
<td>fapC</td>
<td>F5 fimbrial adhesin (K91)</td>
<td>450</td>
<td>13</td>
</tr>
<tr>
<td>fhaC</td>
<td>F6 fimbrial adhesin</td>
<td>333</td>
<td>13</td>
</tr>
<tr>
<td>F41</td>
<td>Fimbrial adhesin</td>
<td>431</td>
<td>13</td>
</tr>
<tr>
<td>estL</td>
<td>Heat-stable enterotoxin (StA)</td>
<td>166</td>
<td>13</td>
</tr>
<tr>
<td>estLII</td>
<td>Heat-stable enterotoxin (StB)</td>
<td>172</td>
<td>13</td>
</tr>
<tr>
<td>elA</td>
<td>Heat-labile toxin</td>
<td>696</td>
<td>13</td>
</tr>
<tr>
<td>Enteropathogenic E. coli (EPREC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>bfpA</td>
<td>Type IV bundle-forming pili</td>
<td>326</td>
<td>23</td>
</tr>
<tr>
<td>Enteroinvasive E. coli (EIEC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ipaH</td>
<td>Invasion plasmid antigen</td>
<td>600</td>
<td>41</td>
</tr>
<tr>
<td>Shiga toxin E. coli (STEC)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>stx1</td>
<td>Shiga toxin I</td>
<td>180</td>
<td>39</td>
</tr>
<tr>
<td>stx2</td>
<td>Shiga toxin II</td>
<td>255</td>
<td>39</td>
</tr>
<tr>
<td>stx2a</td>
<td>Shiga toxin 2e</td>
<td>139</td>
<td>22</td>
</tr>
<tr>
<td>estE</td>
<td>Intimin</td>
<td>384</td>
<td>39</td>
</tr>
<tr>
<td>estH</td>
<td>Enterohemolysin</td>
<td>534</td>
<td>39</td>
</tr>
<tr>
<td>Cytotoxic distending toxin-producing E. coli</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cdt</td>
<td>Cytotoxic distending toxin</td>
<td>108</td>
<td>11</td>
</tr>
<tr>
<td>Clermont phylogeny PCR markers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>chuA</td>
<td>A heme transport gene found in O157:H7</td>
<td>279</td>
<td>10</td>
</tr>
<tr>
<td>yjaA</td>
<td>An unknown-function gene in K-12</td>
<td>211</td>
<td>10</td>
</tr>
<tr>
<td>TspE4C2</td>
<td>An anonymous DNA fragment associated with neonatal meningitis E. coli</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
RESULTS

Prevalence of virulence genes in commensal and clinical porcine *E. coli* isolates. Using multiplex PCR screening of 36 ExPEC-associated VGs, 8 genes were identified in the porcine isolates: *fimH*, *fyuA*, *hlyA*, *kpsMII*, *k5*, *iba*, *traT*, and *ompT*. The prevalence of these VGs in PWD isolates and the commensal isolates is shown in Table 3. Two genes, *hlyA* and *iba*, occur significantly more frequently among the PWD isolates, respectively, which was rarely found in PWD isolates. Interestingly, none of the O149 isolates contained any of the ExPEC-associated VGs, 8 genes were identified in the porcine *E. coli* commensal isolates: *faeG* and *ompT*.

Nine of 18 enteric pathogenic VGs were also detected in the porcine *E. coli* isolates. Using multiplex PCR screening of 36 ExPEC-associated VGs, 8 genes were identified in the porcine isolates: *fimH*, *fyuA*, *hlyA*, *kpsMII*, *k5*, *iba*, *traT*, and *ompT*. The prevalence of these VGs in PWD isolates and the commensal isolates is shown in Table 3. Two genes, *hlyA* and *iba*, occur significantly more frequently among the PWD isolates (P < 0.01). *fimH* and *traT* genes were present in most of the isolates, but *fimH* was not found in any PWD isolates belonging to serogroup O149. A number of commensal isolates possessed *fyuA* or *ompT* (23 and 13 out of 158 isolates, respectively), which was rarely found in PWD isolates.

Nine of 18 enteric pathogenic VGs were also detected in the isolates (*eltA*, *estI*, *estII*, *stx*$_{2e}$, *faeG*, *fedA*, *east-1*, *eae*, and *stx*$_2$). Most PWD isolates contained a typical porcine ETEC fimbrial gene, either *faeG* or *fedA*, and at least one enterotoxin gene, such as *eltA*, *estI*, or *estII*, with the exception of one O141 serogroup O149 isolate, which was *faeG*$^+$ and mutant for *eltA*, *estI*, and *estII*. The *F4* fimbrial gene (*faeG*) was exclusively associated with serogroup O149 isolates, and all of the O141 isolates harbored the *F18* fimbrial gene (*fedA*), except for one isolate in which only the type 1 fimbriate gene (*fimH*) was found. The *east-1* gene, which encodes the heat-stable toxin EAST1 that is unrelated to STa and STb, was found to be particularly associated with isolates of serogroup O149 (Table 3). Notably, *east-1* was only present in one O141 strain and 8 out of 158 commensal isolates. Moreover, none of the O149 isolates possessed any Shiga toxin *E. coli* (STE) genes (*stx*$_1$, *stx*$_{2e}$, *eae*, or *exhA*), whereas *stx*$_2$ was of reasonably high prevalence in serotype O141. Comparably, the isolates designated commensals were found on the whole to lack enteric VGs. For example, *faeG* and *fedA* were not detected, only three strains contained *stx*$_{2e}$, and *estI* and *east-1* were only detected in 9 and 8 out of 158 isolates, respectively, confirming that isolates from healthy pigs generally do not carry VG combinations to enable pathogenicity.

It is important to note that in the present study, different primer pairs were used for the detection of *stx*$_2$ and *stx*$_{2e}$ allelic variations within the *stx*$_2$ gene. The majority of strains which possessed *stx*$_2$ were found to have the *stx*$_{2e}$ allele (six out of eight O141 and three out of three commensal isolates). This result corresponds to the finding by Fratamico et al. that confirmed that *stx*$_{2e}$ was the most frequent *stx*$_2$ variant found in *E. coli* isolated from pig feces (20). In addition, three *cdt* gene primer pairs were used (a1/s1, a2/s2, and 3A/3B) for the PCR detection of three different *cdt* gene alleles: *cdtIB*, *cdtIB*, and *cdtIBB*, respectively. None of these three alleles were present in the ETEC or commensal isolates.

Phylogenetic group analysis of porcine PWD and commensal isolates. Phylogenetic analysis using the triplex PCR technique developed by Clermont et al. (10) revealed that group A *E. coli* isolates were predominant in the collection of isolates, with a prevalence of 100% in serotype O149, 92.9% in O141, and 70.5% in commensal isolates. Group B1 was only found in commensals (29.5%). Only one O141 isolate was identified as group B2, and one commensal isolate was found in group D. Interestingly, none of the O149 isolates contained any of the three Clermont PCR gene markers, and most of the O141 and commensal isolates harbored *yjaA* or *TspE4C2*.

Clustering analysis of porcine *E. coli* based on virulence gene attributes. Two major VG combinations were identified...
among the clinical isolates in this study: O149 (hlyA, iha, traT, east-I, est-II, eltA, and faeG) and O141 (fimH, hlyA, iha, stx2, est-I, est-II, fedA, and yjaA). Thirty VG combinations were present in the 158 commensal isolates, of which 13 were represented only by single isolates. The VG combinations of high prevalence were the following: 20/158 (12.7%; fimH and yjaA), 19/158 (12.0%; fimH, traT, and TspE4C2), 18/158 (11.4%; fimH, fyuA, and TspE4C2), 17/158 (10.8%; fimH, traT, and yjaA), 13/158 (8.2%; fimH), and 10/158 (6.3%; yjaA). The VG profiles identified in the ETEC isolates were significantly different from the VGs possessed by commensal isolates ($P < 0.01$). Clustering analysis of the isolates based on VG profiles differentiated the commensals from ETEC isolates by segregation into two differentiated clusters (cluster 1 for all commensal isolates and cluster 2 for all ETEC isolates). The ETEC isolates were further separated into two subclusters in accordance with serogroups (O141 and O149), but the ETEC isolates from different geographic origin could not be differentiated based on their VG profiles. As presented in Fig. 1, commensal isolates were grouped separately into cluster 1, whereas isolates in O141 and O149 were separated into clusters 2a and 2b, respectively. There was one exception: a serogroup O141 strain was found to be more closely related to the commensal E. coli group, because it was the only ETEC strain lacking hlyA, iha, faeG, and fedA genes, although it did possess fimH, fyuA, traT, east-I, and est-II genes.

**PFGE analysis of PWD isolates.** Forty-five PWD isolates were further analyzed by PFGE fingerprinting after restriction enzyme digestion with NotI and XbaI. These isolates included 7 serogroup O141 isolates (NSW), 9 serogroup O149 isolates (NSW), 7 serogroup O149 isolates (Vietnam), and 22 serogroup O149 isolates (Qld). Relatedness of each DNA fingerprint was clustered using the GelCompar software, as presented in Fig. 2. Based on the PFGE pattern, 45 isolates were separated into two main clusters (I and II) in accordance with serogroups (O141 and O149). There are three subclusters in serogroup O149, Ia, Ib, and Ic, primarily based on geographical origin. Queensland isolates fell into two distinct groups, with one group (Qld A in Ia) more closely related to Vietnamese isolates than the second Queensland group (Qld B in Ia).
in IIc). These groups were both distinct from the New South Wales isolates, which also fell into a single subcluster (IIb).

**Analysis of porcine commensal isolates by PFGE and serotyping.** PFGE profiles and serotype were obtained for 158 pig commensal isolates. A total of 45 different serotypes were identified, comprising 29 O groups and 19 H types, as well as 58 nonmotile (H+/H11002) strains. Seventeen serotypes were identified as nontypeable O antigens (Ont). The predominant commensal serotype was Ont 63 (39.9%), followed by this breakdown: O40, 19 (12.0%); O5, 9 (5.7%); O8, 5 (3.2%); O82, 5 (3.2%); O25, 4 (2.5%); O51, 4 (2.5%); O105, 3 (1.9%); O2, 3 (1.9%) and O71, 3 (1.9%). The typical PWD ETEC serogroups, such as O141, O149, and O139, were not identified in any of the commensals.

Isolates within the same serotype normally possessed identical PFGE patterns. For example, 16 out of 18 isolates within serotype O40:H25 displayed PFGE pattern 20# regardless of sampling animal or location. There were some exceptions: five isolates with serotype O114:H+/H11002 had four distinct PFGE patterns, and 23 isolates belonging to Ont:H+/H11002 had 15 PFGE patterns.

**DISCUSSION**

Porcine ETEC isolates associated with PWD belong to a limited number of serogroups, with O8, O138, O139, O141, O147, O149, and O157 being the most commonly reported worldwide (22, 36). Serotyping of porcine ETEC for epidemiological purposes utilizes surface-associated determinants of the bacterium, such as lipopolysaccharide, capsular antigens, and fimbrial antigens (3). Nonetheless, different serogroups still cause similar pathology and clinical disease and should therefore share many features that contribute to their virulence. An important and yet unclear issue addressed in this study is whether these clonal ETEC isolates have in common virulence genes typical of enteric *E. coli* or whether they also...
possess additional virulence genes or virulence gene combinations that can be found in other pathotypes, such as EHEC, EPEC, EIEC, EaggEC, and ExPEC.

It is likely that the acquisition of certain VGs by transferable genetic elements (plasmids and transposons) endows ETECs with the potential ability to broaden their host range or evolve into new serogroups over time (19, 33). The deployment of a multivirulence gene panel assembled from different E. coli pathotypes may provide some insight into the acquisition of VGs in different serogroups of porcine ETECs and whether such diversity may endow better survival attributes because of selection pressure. In this study, both PWD and nonidentical E. coli strains from healthy pigs (commensals) were screened by PCR for a panel of 54 VGs assembled from seven major human pathotypes (ExPEC, ETEC, EPEC, EHEC, STEC, EIEC, and EaggEC). The PWD strains were confirmed to be ETEC due to the possession of at least one of the typical porcine ETEC virulence genes encoding fimbriae and/or enterotoxins. VGs such as 

\[
\text{east-1} \quad \text{and} \quad \text{stx}_{2e}
\]

were also identified in this PWD isolate collection, mirroring previous findings (9, 22, 38). \(\text{stx}_{2e}\) is normally possessed by porcine STEC strains that cause edema disease, but some porcine E. coli strains were found to produce \(\text{stx}_{2e}\) together with enterotoxins capable of causing diarrhea (42). The \text{east-1} gene was initially found in human pathogenic E. coli and encodes the heat-stable toxin termed EAST1, associated with persistent, watery diarrhea in young children (48, 52). Recent surveys have shown that \text{east-1} is widely distributed among porcine diarrhea-related strains, although the role of EAST1 in diarrhea caused by porcine ETEC has still not been determined (37, 49). The results of this study showed a significantly higher incidence of \text{east-1} in O149 ETEC isolates, while the majority of porcine commensal E. coli isolates are \text{east-1} negative, providing incentive for further investigating the pathogenic significance of this gene in porcine PWD.

In 1985, Levine et al. suggested that factors in addition to enterotoxin production may be important in the pathogenesis of ETEC infection in animals (34). Later efforts have focused on searching for additional factors shown to be important for virulence in other intestinal pathotypes, but the extraintestinal virulence factors have been largely ignored. In the present study, eight ExPEC-associated VGs were found in the porcine E. coli isolate collection. Among these, \text{fimH}, which encodes type 1 fimbriae, was frequently detected in both PWD and commensal isolates, with the exception of serogroup O149 isolates. \text{fimH} was present in the majority of commensal porcine isolates, which may indicate a potential role for type 1 fimbriae in the adherence of resident E. coli to pig GIT via a mannose-resistant mechanism. Interestingly, contrary to the other PWD and neonatal diarrhea serogroups (data not shown) in which \text{fimH} prevalences were significantly high, none of the isolates in serogroup O149 contained \text{fimH}, which concurs with the results of a previous study (47).

In this investigation, O149 differed from O141, O8, and commensal strains because of the absence of \text{fimH}, \text{fyuA}, \text{ompT}, \text{chuA}, \text{yjaA}, and TspE4C2. However, all these serogroups did have in common \text{faeG}, \text{hlyA}, \text{iha}, and \text{east-1} virulence genes. O149 is reported to be the most prevalent serogroup isolated from pigs with PWD in Europe and North America (22). The reason for this dominance is still unclear. According to Hampson (24), somatic O antigen or associated factors confers the ability to establish and proliferate in the pig intestine, thus, strains expressing O149 antigen possess certain advantages over other types. O149 dominance could also be related to virulence factor composition if these factors enhance competitiveness in the swine gastrointestinal tract. Larsen (32) has shown that the PWD O149 strains differed from other serotypes in carbohydrate fermentation, urease activity, and colicinogenicity tests. In the present study, PFGE analysis demonstrated that the DNA fingerprint of O149 isolates differed markedly from other ETEC and non-ETEC isolates, indicating that possession of the unique O149 VG profile is linked with genotype. Interestingly, the PFGE analysis showed that isolates from different geographical origins were highly clonal, and the O149 strains from Vietnam were more related to a group of Qld isolates than NSW isolates, confirming that there has been relatively little movement of E. coli populations in pigs between Qld and NSW (13). This also suggests that the Vietnamese strains probably originated from stock imported from Queensland, most probably during agricultural development programs sponsored by the Australian government (D. Trott, University of Queensland, Australia, personal communication). It is also in agreement with the previous report from Hampson et al. (25), which demonstrated that isolates of serogroup O149 from suckling piglets in Scandinavia, Australia, and Indonesia belonged to a single clonal grouping based on multilocus enzyme electrophoresis analysis and shared a close genetic relationship with O149 isolates associated with PWD. The clonal nature of O149 suggests that the deployment of commensal E. coli strains may provide an effective measure to control PWD via competitive exclusion mechanisms. However, the possibility that the data and conclusions may only represent a restricted geographical distribution of genotypes cannot be excluded. To support this hypothesis, a broad range of PWD E. coli strains from a wide variety of sources and serotypes will need to be analyzed.

Two virulence genes, \text{hlyA} and \text{iha}, were present in significantly higher proportions in PWD isolates than in the commensals. \text{hlyA} encodes \(\alpha\)-hemolysin, which is a gram-negative bacterial membrane pore-forming cytolytic toxin of the RTX family with glycine-rich repeats (50). \(\alpha\)-Hemolysin is predominantly detected in ExPEC strains. It is also a major marker of most porcine ETEC and STEC strains, causing PWD and edema disease in pigs. Porcine ETEC strains associated with PWD are almost universally hemolytic, and \(\alpha\)-hemolysin in these isolates is considered to enhance virulence and colonization (43). The cytotoxic necrotizing factor (cnf-1) was reported to be strongly associated with \(\alpha\)-hemolysin and virulence in both human ExPEC and pig diarrheal isolates. The \text{cnf1} gene is closely linked with \text{hlyA} on a pathogenicity island (7, 16). This cooccurrence, however, was not shown in our porcine isolates. It has been demonstrated that porcine cnf-1-producing E. coli also expresses P. S, and F1C fimbriae in strains causing septicaemia and/or diarrhea (14). Our results indicate that cnf-1-producing strains are more likely to be ExPEC strains which possess a repertoire of VGs that differ from the enteric PWD strains.

To our knowledge, this is the first report of the presence of the \text{iha} gene in porcine PWD isolates with significantly higher carriage rates than gut commensal isolates. \text{iha} encodes a novel...
nonhemagglutinating adhesin found in E. coli O157:H7, which facilitates the adherence of O157:H7 strains to epithelial cells in environment such as the GIT of animals (45). iha also facilitates adherence to HeLa cells when transformed into non-adherent E. coli K-12. Prior to this study, iha has only been found in STEC and ExPEC pathotypes. In addition to O157: H7, other STEC strains also contain iha, including strains of serotype O111:H- and O91:H+ (21). Additionally, iha occurs significantly more frequently in UTI or bacteremia isolates than human commensal E. coli isolates (29). The role of iha in colonization of both humans and animals is still being elucidated. Porcine ETEC strains harboring additional fimbrial adhesins, such as iha and hlyA, could possibly exploit several alternative pathways for colonization of the host. The association of iha and hlyA with porcine PWD suggests that these VGs could be used as additional markers of pathogenicity in PWD isolates. More importantly, due to the fact that the use of vaccines against fimbriated strains may allow the emergence of other ETEC strains which lack recognized fimbriae such as F4 and F18, iha might be a potential target for anti-ETEC intervention. Future work will be focused on investigating the role of iha in pig gut colonization by ETEC strains, the mechanisms underlying iha expression, and the presence of this gene in other PWD serogroups.

It is widely believed that a positive correlation exists between environmental stability, community stability, and high microbrial diversity (40). A highly diversified microflora may reflect a stable community with greater “colonization resistance” to enteric pathogens. In the suckling piglet, coliforms isolated from normal feces differ from diarrheal coliforms, because the latter is dominated by pathogenic clones with reduced diversity, and these express only limited combinations of virulence-associated factors (30). The present study investigated serotype, VG profile, and DNA fingerprint patterns for 158 E. coli isolates designated commensals on the basis of their isolation from healthy pigs. The study confirmed that none of the healthy pigs examined were colonized with typical ETEC strains. Very few isolates were of a serotype that is associated with porcine-pathogenic E. coli strains, and fewer still contained virulence genes such as estI, stx2+, and east-1, which may be associated with enteric disease in swine. The majority of commensals only possessed “fitness or adaptive” genes such as fimH, traT, and fyuA, which are involved in colonization, serum resistance, and iron utilization, respectively. This result corresponds to a previous report by Hinton et al. (27), in which a complex E. coli microbiota was demonstrated in the GIT of healthy weaned pigs in the absence of ETEC serotypes such as O149. The present study also established a remarkable clonal diversity among the commensal isolates between pigs. This was an interesting finding, considering that pigs all shared the same environment and feed and water sources. A high level of diversity among commensal E. coli (12) has also been observed in cattle and human feces based on serotype identities (4, 6). By comparing E. coli biochemical fingerprints from five healthy piglets from 7 to 63 days of age, Melin et al. (35) suggested that a disturbed (i.e., decreased diversity) fecal coliform microbiota close to weaning may reflect a situation contributing to an increased susceptibility to various enteric diseases. This information, together with previous work on the diversity of E. coli isolates from different intestinal compartments of the gastrointestinal tract of pigs (12), supports the contention that a highly diverse spectrum of commensal E. coli strains that have colonized the gut epithelial surface can provide a competitive barrier against incoming pathogenic E. coli. The deployment of a large panel of virulence genes from a wide range of E. coli pathotypes has revealed that porcine ETECs have, in the course of their evolution, also acquired extraintestinal virulence genes. Consequently, virulence gene combinations selected from both the intestinal and extraintestinal pathotypic panel can be used to characterize particular ETEC serogroups. The absence of virulence gene combinations can also be used as an epidemiological tool to characterize clonal clusters of commensal E. coli. The advantage of such an approach over serotyping is its potential to evaluate the population structure of entire E. coli communities without the need for multiple analysis of many individual and nonrepresentative clones (8).

ACKNOWLEDGMENTS

Xi-Yang Wu is a recipient of a CRC Beef Quality Postgraduate Research Scholarship, funded through International Animal Health, Pty. Ltd., Australia. We thank Idris Barchia (special biometrician, EMAI, Australia) for support of the statistical analyses and Alexander Kuzevski for help with the serotyping.

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