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Phylogenetic and molecular insights into the evolution of multidrug-resistant porcine enterotoxigenic *Escherichia coli* in Australia

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Abstract

This study investigated the phylogeny and molecular epidemiology of Australian porcine enterotoxigenic *Escherichia coli* (ETEC) isolates (n = 70) by performing multilocus sequence typing (MLST), random amplified polymorphic DNA (RAPD) analysis, virulence gene analysis, plasmid, bacteriocin, integron and antimicrobial resistance gene typing, and antimicrobial susceptibility phenotyping. Isolates of the most commonly observed O serogroup (O149) were highly clonal with a lower frequency of antimicrobial resistance compared with the less common O141 serogroup isolates, which were more genetically diverse and resistant to a greater array of antimicrobials. The O149 and O141 isolates belonged to sequence types (STs) ST100 and ST1260, respectively. A small number of new STs were identified for the least common serogroups, including O157 (ST4245), O138 (ST4244), O139 (ST4246) and O8 (ST4247). A high frequency of plasmid replicons was observed among all ETEC isolates. However, O149 isolates predominantly carried IncFIB, I1, HI1 and FIC, whereas O141 isolates carried a more varied array, including IncI1, FIB, FIC, HI1, I1, Y and, most significantly, A/C. O141 isolates also possessed a greater diversity of bacteriocins, with almost one-half of the isolates carrying colicin E3 (44.4%; 12/27) and E7 (48.1%; 13/27). This study shows that Australian porcine ETEC are distinct from isolates obtained in other parts of the world with respect to the MLST profile and the absence of resistance to critically important antimicrobials, including third-generation cephalosporins and fluoroquinolones.

Keywords: Enterotoxigenic *Escherichia coli*; Porcine; Antimicrobial resistance; Plasmids; Serogroups; IncA/C

Introduction

Enterotoxigenic *Escherichia coli* (ETEC) is the aetiological agent of both neonatal and post-weaning diarrhoea in pigs [1,2]. Neonatal diarrhoea occurs in piglets of 0–4 days of age and is typically associated with high morbidity and mortality [2,3]. ETEC strains that cause neonatal diarrhoea are usually non-β-haemolytic and belong to serogroups O8, O9, O20, O64, O149 or O101 [3,4]. By

contrast, ETEC strains that cause post-weaning diarrhoea are usually β-haemolytic and belong to serogroups O8, O138, O139, O141, O145, O149 or O157 [1,4]. Both in neonatal and post-weaning diarrhoea, attachment of ETEC strains to the intestinal epithelium is mediated by the fimbrial adhesins F4 (K88), F5 (K99), F6 and F41 (neonatal diarrhoea) or F4 and F18 (post-weaning diarrhoea). Collectively, strains of these two groups of ETEC also release one or more of the heat-labile (LT) and heat-stable (STa and/or STb) enterotoxins, which induce hypersecretory diarrhoea [2,4]. Porcine ETEC virulence factors are typically encoded on F-type plasmids such as IncFIIA, FIB and FIC [5,6].

The occurrence of antimicrobial resistance (AMR) in porcine ETEC has been a longstanding problem in pig production [7,8]. Recently, there has been an increasing tendency for porcine ETEC to express a multidrug-resistant (MDR) phenotype [7,9]. With continuing use of antimicrobials, it is probable that at least some MDR ETEC will eventually develop pan-resistance, a phenotype that includes resistance to all commonly used drugs plus resistance to critical antimicrobials such as fluoroquinolones and third- and fourth-generation cephalosporins [7]. Recent studies have shown that fitness of drug-resistant ETEC in the pig production environment is aided by possession of specific virulence-associated plasmids in combination with resistance-associated plasmids [5]. Virulence genes are typically found on F-type plasmids, whereas resistance genes are encoded on IncFII-like IncFV, IncA/C and IncI1 plasmids [10].

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In addition to encoding for virulence factors and AMR, plasmids also encode for fitness factors such as bacteriocins, which may enhance colonisation capacity [11]. Bacteriocins are antibacterial peptides produced by bacteria to specifically inhibit or kill closely related species [11]. Bacteriocin-producing strains are themselves protected from the toxin owing to co-expression of an immunity factor (antitoxin) [11]. Bacteriocins therefore provide a competitive advantage to the producer strain and play an important role in intraspecific interactions within *E. coli* communities [11]. Colicins and microcins are the two types of bacteriocins produced by *E. coli.* Our recent studies have shown that commensal *E. coli* from the porcine gastrointestinal tract have distinctive bacteriocin profiles [12]. Although the key role of bacteriocins in the adaption of commensal *E. coli* to life in the gut is generally well understood, knowledge of their prevalence and distribution among porcine ETEC is currently lacking [11,12,13].

Due to the geographic isolation of Australia and the decades-long prohibition of the importation of livestock and fresh meat, the Australian porcine ETEC population has evolved in isolation from populations in other parts of the world. Australian porcine ETEC also are subject to less diverse antimicrobial selection pressures because of the restricted range of antimicrobials legally permitted for use in livestock in this country. For instance, Australia is the only country never to have permitted the use of fluoroquinolones and gentamicin in food-producing animals [14]. In addition, tight regulations govern the use of third-generation cephalosporins for mass medication, and fourth-generation cephalosporins are not registered for use [9,15].

Our recent studies have focused on the prevalence of different serogroups, virulence genes and AMR genotypes and phenotypes among porcine ETEC associated with neonatal and post-weaning diarrhoea in Australia [4,9]. The objective of the present study was to examine the phylogeny and genetic relatedness of porcine ETEC strains as well as the prevalence and diversity of plasmid replicons and bacteriocins in clonally related lineages. The results of this study may provide unique insight into the role of plasmids encoding virulence and fitness factors as well as AMR in the evolution of strains from different genetic backgrounds in the Australian context. This may lead to the development of

practical alternatives to antimicrobials for the treatment of ETEC, permitting the intensive management of pigs to remain sustainable.

Materials and methods

Bacterial strains

In total, 70 ETEC strains were collected from clinical cases of neonatal (*n* = 7) and post-weaning (*n* = 63) diarrhoea from pigs in New South Wales (*n* = 33), Victoria (*n* = 14) and Queensland (*n* = 23). Isolates were collected from veterinary diagnostic laboratories between 1999 and 2005 (Supplementary Table S1). These strains have been serogrouped as previously described [4]. The serogroups of the ETEC collection were as follows: O149 (*n* = 31); O141 (*n* = 27); O8 (*n* = 6); O20 (*n* = 2); O109 (*n* = 1); O138 (*n* = 1); O139 (*n* = 1); and O157 (*n* = 1). For ease of discussion and due to low numbers, isolates belonging to serogroups other than O149 and O141 are jointly referred to as isolates of non-prominent serogroups (NPS). All O138, O139, O141 and O149 isolates were from cases of post-weaning diarrhoea, whereas the O20, O109 and three of the O8 isolates were from cases of neonatal diarrhoea.

Maintenance of bacteria and DNA extraction

Strains were grown overnight (ca. 16 h) in Luria–Bertani (LB) broth (Oxoid Australia, Adelaide, SA, Australia) at 37 °C with shaking at 200 rpm in an orbital shaker (Oxoid Australia). Bacterial strains were stored at −80 °C in LB broth containing 20% glycerol (Oxoid Australia). Strains from frozen stocks were recovered onto LB agar plates and single colonies were grown overnight (ca. 16 h) in LB broth for DNA extraction. Extraction of DNA was performed using 6% Chelex® (Bio-Rad, Sydney, NSW, Australia) as previously described [16].

Random amplified polymorphic DNA (RAPD) analysis

RAPD analysis was carried out to assess the clonality of the porcine ETEC isolates using RAPD primers 1254 as previously described [12]. Gel-to-gel variations of the RAPD profiles were

minimised by running the samples on one agarose gel (1.5%) at 120 V for 2 h. BioNumerics 6.1 (Applied Maths, Sint-Martens-Latem, Belgium) was used to normalise the gels using the same DNA ladder (Gene Ruler 100 bp plus; Thermo Scientific, Adelaide, SA, Australia).

Phylogenetic analysis

Phylogenetic grouping was carried out using a multiplex PCR-based assay as described by Clermont et al. [17].

Multilocus sequence typing (MLST)

Phylogenetic relationships of a subset (26/70) of porcine ETEC were determined using the *E. coli* scheme described at http://mlst.warwick.ac.uk/mlst/ [accessed 19 May 2014] [18]. The following seven genes were amplified and sequenced for MLST: *adk*; *fumC*; *gyrB*; *icd*; *mdh*; *purA*; and *recA*.

Plasmid replicon typing

Plasmid replicon typing was carried out to investigate the presence of 18 replicons using three multiplex and one uniplex PCR. These assays were performed using previously described primers [19], multiplex panels and reaction conditions as described previously [20].

Bacteriocin gene assay

The presence of the following 16 bacteriocins was assessed for each strain using multiplex and uniplex reactions: colicins A, B, D, E1, E2, E3, E6, E7, Ia/Ib, K and M; and microcins B17, H47, J25, M and V. The assays were performed using previously described primers and reaction conditions [20].

Enterotoxigenic *E. coli* characterisation

The ETEC pathotyping PCR was used to profile the signature virulence genes associated with porcine ETEC. The assay was carried out in one multiplex PCR reaction targeting the following eight ETEC virulence genes: *stx2e*; F4 (*faeG*); F6 (*fasA*); F18 (*fedA*); LT1 (*eltb*); F5 (*fanA*); STaP (*estA*); and STb (*estB*). The PCR reactions were performed as described by Casey and Bosworth [21].

Phenotypic detection of antimicrobial resistance

Phenotypic detection of AMR was performed by disc diffusion using the Calibration Dichotomous Susceptibility (CDS) test Australia [22]. The following antimicrobials were tested: ampicillin (25 µg); amoxicillin/clavulanic acid (AMC) (60 µg); ticarcillin/clavulanic acid (85 µg); cefalexin (100 µg); cefoxitin (30 µg); cefotaxime (5 µg); cefepime (10 µg); nalidixic acid (30 µg); ciprofloxacin (2.5 µg); imipenem (10 µg); sulfisoxazole (sulfafurazole) (300 µg); trimethoprim (5 µg); tetracycline (10 µg); apramycin (15 µg); neomycin (30 µg); gentamicin (10 µg); azithromycin (15 µg); and chloramphenicol (30 µg). The CDS method is commonly used both in human and veterinary diagnostic microbiology laboratories within Australia and the breakpoints are calibrated by internationally standardised minimum inhibitory concentration (MIC) testing for each antimicrobial [22]. *E. coli* NCTC 10418 was used as the control strain [22]. Isolates resistant to three or more classes of antimicrobials were classified as MDR.

Antimicrobial resistance gene (ARG) profiling

A combination of three multiplex and eight uniplex PCR assays were performed to screen 25 ARGs commonly identified in *E. coli* [23]. The genes tested and the nucleotide sequences are described in Supplementary Table S2.

Detection of integrons

A multiplex PCR assay targeting class 1, 2 and 3 integrons (*int1*, *int2* and *int3*) was performed to investigate the presence of integrons in the ETEC collection. This reaction was performed using previously described primers and conditions [24].

Statistical analysis

BioNumerics 6.1 was used to perform principal coordinate analysis (PCA) using Dice correlation and complete linkage. RAPD profiles were assessed and compared using a band-based dendrogram created using Pearson correlation as the numeric coefficient with 0.5% position tolerance and unweighted pair-group mathematical average (UPGMA) clustering algorithm using BioNumerics 6.1.

Fisher's exact test was used to assess the significance of differences in prevalence of traits (genetic and phenotypic) between the two major serogroups (O141 and O149).

Results

Random amplified polymorphic DNA profiling

RAPD analysis separated the isolates into four major clusters and six independent branches that largely conformed to serogroup identity (Supplementary Fig. S1). The O149 and O141 isolates were divided into two separate clusters with the exception of three (atypical) O141 isolates. The NPS isolates were predominantly grouped into two separate clusters with the exception of four isolates that were clustered independently or intermingled among the O141 and O149 clusters. Interestingly, the three major clusters that separated O141, O149 and one group of NPS isolates shared 90% band similarity, whilst the remaining cluster representing NPS isolates only shared 50% band similarity (Supplementary Fig. S1). The NPS clusters that shared 90% homology with O141 and O149 clusters were all from cases of post-weaning diarrhoea, whereas one-half (5/10) of the isolates in the less related NPS cluster were from neonatal diarrhoea. In addition, O8 isolates from post-weaning diarrhoea were closely related to the O141 and O149 isolates, whereas the O8 isolates from neonatal diarrhoea were less closely related.

Phylogenetic analysis

Clermont phylogenetic grouping revealed that all O141 and O149 isolates belonged to phylogenetic group A. Serogroup O8 isolates belonged to diverse phylogenetic groups including B1, C and E (Supplementary Table S1). NPS isolates belonged to the following phylogenetic groups: O20 (A and E); O109 (B1); O138 (D); O139 (D); and O157 (C).

Multilocus sequence typing

All O149 isolates belonged to sequence type 100 (ST100), whereas all O141 isolates belonged to ST1260. The ST identities for the other serotypes were as follows: O109 (ST29); O8 (ST90, 120,

410); and O20 (ST746). In addition, MLST identified new STs for the following serogroups: O157 (ST4245); O138 (ST4244); O139 (ST4246); and O8 (ST4247).

Comparison with and among serogroups

No statistically meaningful comparisons could be made among the minor serogroups or between the minor subgroups and the O141 and O149 isolates, given the small sample sizes and the diverse genetic background of the minor serotypes. The characteristics of these isolates together with the O141 and O149 isolates are provided in Supplementary Table S1.

Detection of plasmid replicon types

Plasmid replicon typing identified 12 different plasmid types with all isolates carrying at least one or more plasmids (Table 1). IncI1 types were detected most frequently, followed by FIB, HI1 and FIC (Table 1). The IncFrepB group of F plasmids (FI, FII, FIII, FIV and FV) was present in all of the isolates. Of the Inc types detected, there was a significant difference in the carriage of IncFIB and A/C plasmid replicons between O149 and O141 serogroup isolates. IncA/C plasmid replicons that typically contain critical drug resistance genes such as those encoding resistance to third-generation cephalosporins and carbapenems [25] were only identified among O141 (14.8%; 4/27) and O139 (*n* = 1) isolates. IncFIB replicons were more prevalent among O149 (96.8%; 30/31) compared with O141 isolates (59.3%; 16/27), whilst IncY replicons were more prevalent among O141 (18.5%; 5/27) compared with O149 isolates (6.5%; 2/31). In addition, plasmid replicons IncB/O FIA, H12, L/M and P were detected only in NPS isolates. Replicons IncFIIA, K/B, T, X and W were not detected.

Detection of bacteriocin genes

The bacteriocin gene assays targeting 16 colicin and microcin genes revealed that 65.7% of the isolates were positive for at least one bacteriocin gene. A total of 61.4% carried colicin genes whilst 5.7% carried microcin genes. The most commonly detected bacteriocins were all colicins (Ia/Ib, E7, E3, B and M) (Table 1). Bacteriocin profiling revealed significant differences in carriage amongst isolates belonging to different ETEC serogroups (Table 1). The O141 isolates demonstrated greater diversity with nine bacteriocin profiles in comparison with four bacteriocin profiles for the O149

serogroup isolates (Table 1). Approximately one-half of the O141 isolates carried colicins E3 (44.4%) and E7 (48.1%), whereas none of the O149 isolates carried these colicins. Colicins A, D, K and E6 and microcins V, B17 and J25 were not detected in any of the isolates.

Distribution of virulence genes

The STb-encoding *estB* gene was the most frequently detected virulence gene. Genes encoding LT1 (60%), STa (58.6%), F4 (47.1%) and F18 (45.7%) were also widely dispersed among the ETEC isolates (Table 1). O141 isolates carried STa, STb, F18 and *stx2e*, whereas O149 isolates typically carried STa, STb, LT1 and F4.

Phenotypic detection of antimicrobial resistance

Susceptibility testing revealed that the majority of isolates were resistant to one or more antimicrobials (92.8%) and a high proportion showed resistance to three or more classes of antimicrobial (64.3%) and were thus classed as MDR. Overall, 34 multidrug resistance profiles were identified among the ETEC isolates (Supplementary Table S3). None was resistant to AMC, cefotaxime, cefepime, ciprofloxacin or imipenem. Resistance was most frequently detected to sulfisoxazole (72.9%), followed by tetracycline (67.1%), ampicillin (50%) and apramycin (44.3%) (Table 2). In addition, resistance to cefoxitin (14.3%) and cephalexin (7.1%) also was observed. The O141 isolates exhibited resistance to a greater range of drugs in comparison with O149 isolates (Table 2), and a significantly higher proportion of O141 isolates expressed resistance to ampicillin, gentamicin, apramycin, cefoxitin and azithromycin in comparison with O149 isolates (Table 2). However, resistance to sulfisoxazole was more frequently detected among O149 (90.3%) than O141 isolates (51.9%).

Distribution of antimicrobial resistance genes and integrons

ARG typing revealed that a high proportion (97%) of ETEC isolates carried at least one of the targeted ARGs (Table 1). Genes encoding resistance to aminoglycosides [*aac(3)-IV*, *aadA*, *strA* and *strB*] and sulphonamides (*sul1*) were the most frequently detected ARGs in the ETEC collection (Table 1). Of the genes conferring β-lactam resistance, only bla_{TEM} (40%) was

detected in the ETEC isolates. The most prevalent tetracycline resistance gene was *tetA*, being detected in 35.7% of isolates. ARGs bla_{TEM}, *aac(3)-IV*, *strA* and *strB* were more frequently detected among O141 in comparison with O149 (Table 1). In contrast, genes encoding resistance to aminoglycosides (*aadA*), sulphonamides (*sul1*) and tetracycline (*tetA*) were commonly detected mong O149 isolates (Table 1). None of the following genes was detected in the collection: bla_{SHV}; *cat1*; *floR*; bla_{OXA}; bla_{CMY}; bla_{CTX-M}; bla_{MIR-1} or bla_{ACT-1}; $bla_{CTX-M-15}$; *aac(3)-I*; bla_{DHA}; $bla_{FOX1–5}$; and bla_{MOX} or bla_{CMY}.

The prevalence of integron classes 1, 2 and 3 is shown in Table 1. The class 1 integron (*int1*) was the most common integron among the ETEC strains (68.6% of isolates). This integron was present in all but two O149 isolates (93.5%) compared with 40.7% of O141 and 66.7% of NPS isolates. The class 3 integron was absent in the ETEC collection and only one strain harboured a class 2 integron (1.4%).

Variation within serogroups

The O149 isolates were relatively homogeneous with respect to their virulence gene content, plasmid type and AMR profile (data not shown). However, the O141 isolates were heterogeneous and broadly fell into one of three clusters (Fig. 1). The clusters varied with respect to the carriage of large conjugative plasmids (Ia and BM) and small non-conjugative plasmids (colicin E7) as well as their virulence factors (Stx2e and LT) and AMR profile (gentamicin and sulfisoxazole).

Discussion

Neonatal and post-weaning diarrhoea caused by porcine ETEC are amongst the most common infectious diseases identified in young piglets. In this study, we investigated the phylogeny and molecular epidemiology of Australian porcine ETEC. The findings support three main conclusions regarding this pathogen in Australia. First, serogroup O149 isolates, considered to be the most common cause of post-weaning diarrhoea, belong to a single, closely related clonal cluster, whereas the less commonly encountered serogroup O141 isolates are more heterogeneous in their genetic

make-up. Second, the study shows that Australian porcine ETEC are distinct from isolates obtained in other parts of the world owing to their unique multilocus sequence types and the absence of resistance to critically important antimicrobials, including third-generation cephalosporins and fluoroquinolones. Lastly, highly promiscuous, broad-host-range IncA/C plasmids were identified among a significant proportion of O141 isolates.

The O149 serogroup (ST100) is considered to be the most common cause of post-weaning diarrhoea in Australian pigs. In the present study, combined analysis of O149 genetic diversity together with transmissible adaptation traits such as virulence genes, plasmids, bacteriocins and ARGs as assessed by PCA (data not shown) demonstrated that O149 isolates belong to a single, closely related clonal cluster. This indicates that Australian O149 isolates likely arose from the same clonal lineage and have undergone little genetic divergence as they have disseminated throughout the pig industry. Demonstrated O149 clonality, together with a relatively conserved transmissible adaptation trait profile, supports the persistent clone concept similar to that already described for O25b-ST131, a globally disseminated, zooanthroponotic highly virulent and MDR extraintestinal pathogenic *E. coli* (ExPEC) clonal lineage [26,27].

In contrast to the O149 findings, isolates belonging to serogroup O141, recognised as the second most common ETEC serogroup in Australia, possessed a much more diverse array of transmissible adaptation traits despite belonging to the same ST and sharing similar RAPD profiles [4]. This confirms the findings from previous studies which proposed that compared with their O149 counterparts, Australian O141 isolates had developed resistance to a wider range of antimicrobials and possessed a wider spectrum of ExPEC-associated virulence genes (e.g. *bmaE*, *sfa*/*focDE*, *fimH* and *ihA*) [4,9]. The epidemiological significance of these findings is currently unclear, although it is quite likely that O141 strains are more adapted to withstand greater selection pressure arising from host immunity (virulence genes), antimicrobial use (resistance genes) and competition with other *E. coli* in the gut (bacteriocins).

Australian porcine ETEC belonged to different multilocus sequence types compared with porcine ETEC from other countries. For example, a recent phylogenetic analysis of porcine ETEC revealed

that O149 isolates from the USA belong to ST165, and the majority of the US porcine ETEC isolates belong to ST10, ST23 and ST169 [5]. However, Australian O149 (ST100), O141 (ST1260) and O20 (ST746) strains are all members of the phylogroup A ST complex 10, as are O149 (ST165) ETEC isolates from the USA. The O8 isolates (ST410 and ST90) are members of the ST88/23 complex, a group of phylogroup C strains containing many ETEC isolates. Taken together, these results show that Australian ETEC isolates, especially serogroups O149, may have evolved in isolation from other parts of the world.

A major finding of this study was that a subset of O141 isolates (14.8%) were the only porcine ETEC in the collection found to carry IncA/C plasmids. IncA/C plasmids are highly promiscuous with a broad host range and often contain genes that encode resistance to critically important antimicrobials including third-generation cephalosporins ($bla_{\mathrm{CMY-2}}$) and carbapenems ($bla_{\mathrm{NDM-1}}$) [25,28,29]. Recent studies comparing different IncA/C plasmids have shown that these plasmids have a conserved scaffold and a phage integrase–*rhs* region facilitating the acquisition of new ARGs [25]. The IncA/C plasmids identified among the Australian porcine ETEC may therefore be primed to acquire resistance to critical antimicrobials such as third-generation cephalosporins through selection pressure arising from off-label use of ceftiofur owing to the high prevalence of resistance to other registered antimicrobials [9].

The AMR profiling revealed a high frequency of multidrug resistance (67.1%) among the ETEC isolates. Antimicrobial classes to which a large proportion of the ETEC strains were resistant included sulfisoxazole (*sul1*, 57.1%; and *sul2*, 21.4%), trimethoprim (*dhfrV*, 25.7%), aminoglycosides [*aac(3)-IV*, 47.1%; *aadA*, 58.6%; *strA*, 50%; and *strB*, 55.7%], ampicillin (bla_{TEM}, 40%) and tetracycline (*tetA*, 35.7%; *tetB*, 7.1%; and *tetC*, 5.7%) (Tables 1 and Table 2). Overall, O141 demonstrated resistance to a wider range of antimicrobials in comparison with O149 with the exception of sulfisoxazole (Table 2). This is likely to be attributed to the variation in the carriage of specific plasmids by the different serogroups (Table 1). The absence of resistance to critical antimicrobials used in human medicine (fluoroquinolones and third-generation cephalosporins) is noteworthy in comparison with data from other countries where resistance to these critical antimicrobials has already

been identified in porcine ETEC [7,8]. This is likely due to the restriction on the movement of animals and food into the country and comparatively stricter regulation of antimicrobial use in food-producing animals in Australia [14]. However, the presence of the hypervariable promiscuous resistance plasmids IncA/C and I1 among the O141 and NPS isolates indicates that if selection pressure is intensified in the future by increased use of third-generation cephalosporins, strains possessing these plasmids would be most likely to acquire extended-spectrum or AmpC β-lactamases. These resistance-encoding plasmids also have the potential to horizontally transfer to commensal *E. coli* and other Enterobacteriaceae in the pig gut, which routinely enter the environment and the food chain in large numbers [30]. It is of concern that such events could lead to exacerbation of resistance in human pathogens, as evidenced in other countries [30].

Future directions for control of ETEC are informed by several findings from this work. First, the dominance of O149 and O141 strains in the population suggests potential for a vaccine-based approach to control these serogroups. However, the diverse nature of NPS isolates from Australia indicates that development of novel vaccine candidates for the serogroups could be challenging and alternative measures may be required to control NPS infections. It is also essential to understand whether a high rate of carriage of AMR and fitness genes on hypervariable, highly promiscuous plasmids signifies an ability of O141 serogroup porcine ETEC to rapidly evolve and adapt to new environments and control measures.

In summary, this study reveals that Australian porcine ETEC are distinct from isolates obtained in other parts of the world with respect to the MLST profile and the absence of resistance to critically important antimicrobials, including third-generation cephalosporins and fluoroquinolones. Widespread occurrence of virulence and resistance plasmids in the porcine ETEC population is a concern for animal and public health and warrants further attention.

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Table 1. Frequency of Australian porcine enterotoxigenic *Escherichia coli* isolates possessing specific plasmid replicons, bacteriocins, virulence genes, antimicrobial resistance genes and integrons.

Trait	Frequency (%)			
	Overall (n = 70)	Serogroup O149 (n = 31)	Serogroup O141 (n = 27)	P-value (χ^2)[a]
Plasmid replicons				
B/O	4.3	0	0	1
FIC	30	22.6	37	0.26
A/C	7.1	0	14.8	0.041
P	5.7	0	0	1
FIA	4.3	0	0	1
FIB	80	96.8	59.3	<0.001
Y	15.7	6.5	18.5	0.233
I1	85.7	87.1	100	0.116
HI1	30	25.8	29.6	0.776
N	20	16.1	33.3	0.218
H12	5.7	0	0	1
LM	5.7	0	0	1
Bacteriocins				
Colicin E1	1.4	0	0	1
Colicin E2	2.9	0	7.4	0.212
Colicin E3	18.6	0	44.4	<0.001
Colicin E7	20	0	48.1	<0.001
Colicin Ia/Ib	30	32.3	33.3	1
Colicin B	17.1	16.1	11.1	0.712
Colicin M	17.1	16.1	11.1	0.712
Microcin H47	2.9	3.2	3.7	1
Microcin M	2.9	3.2	3.7	1
Virulence genes				
stx2e	21.4	0	51.9	<0.001
F4 (*faeG*)	47.1	96.8	0	<0.001
F6 (*fasA*)	2.9	0	7.4	0.212
F18 (*fedA*)	45.7	0	100	<0.001
LT1 (*eltb*)	60	93.5	29.6	<0.001
F5 (*fanA*)	7.1	3.2	0	1
STaP (*estA*)	58.6	54.8	66.7	0.426
STb (*estB*)	84.3	93.5	85.2	0.402
Antimicrobial resistance genes and integrons				
dhfrV	25.7	22.6	18.5	0.756
aadA	58.6	87.1	40.7	<0.001
bla_{TEM}	40	19.4	48.1	0.026
cmlA	12.9	6.5	25.9	0.068

Trait	Frequency (%)			
	Overall (n = 70)	Serogroup O149 (n = 31)	Serogroup O141 (n = 27)	*P*-value (χ^2)[a]
ereA	7.1	0	0	1
aphA1	27.1	16.1	18.5	1
aac(3)-IV	47.1	25.8	66.7	0.003
tetA	35.7	45.2	14.8	0.022
tetB	7.1	6.5	0	0.494
tetC	5.7	12.9	0	0.116
sul1	57.1	83.9	37	<0.001
sul2	21.4	9.7	29.6	0.091
strA	50	29	63	0.017
strB	55.7	41.9	55.6	0.43
int1	68.6	93.5	40.7	<0.001
int2	1.4	0	0	1

[a] *P*-values assess the significance of differences between serogroups O149 and O141.

Table 2. Frequency of Australian porcine enterotoxigenic *Escherichia coli* isolates possessing single resistance against various antimicrobials.

Antimicrobial	Frequency (%)			
	Overall (*n* = 70)	Serogroup O149 (*n* = 31)	Serogroup O141 (*n* = 27)	*P*-value (χ^2)[a]
AMP	50.0	25.8	66.7	0.003
AZM	25.7	12.9	40.7	0.019
CEX	7.1	3.2	11.1	0.329
NAL	2.9	3.2	0	1
SSX	72.9	90.3	51.9	0.001
TIM	11.4	12.9	11.1	1
TMP	40.0	48.4	29.6	0.184
TET	67.1	74.2	55.6	0.172
APR	44.3	25.8	63.0	0.007
NEO	24.3	22.6	11.1	0.311
CHL	17.1	16.1	25.9	0.518
GEN	34.3	16.1	59.3	<0.001
FOX	14.3	3.2	29.6	0.009

AMP, ampicillin; AZM, azithromycin; CEX, cefalexin; NAL, nalidixic acid; SSX, sulfisoxazole (sulfafurazole); TIM, ticarcillin/clavulanic acid; TMP, trimethoprim; TET, tetracycline; APR, apramycin; NEO, neomycin; CHL, chloramphenicol; GEN, gentamicin; FOX, cefoxitin.

[a]*P*-values assess the significance of differences between serogroups O149 and O141.

Fig. 1. Principal coordinate analysis (PCA) of phenotypic antimicrobial resistance, antimicrobial resistance genes, virulence genes, integrons, plasmid replicons and bacteriocins detected among serogroup O141 isolates. Red, green and blue circles represent the three clusters of O141 isolates. Coordinates of some of the individual traits that distinguish the clusters are also present (plasmid replicons, IncN and IncHI1; colicins, Ia, E7 and BM; virulence factors, Stx2e and LT1; and antimicrobial resistance, gentamicin and sulfisoxazole). For example, the strains indicated by red circles are more likely to harbour IncN plasmids or encode colicin E7 than are strains in the other clusters. (For interpretation of the references to colour in this text, the reader is referred to the web version of the article.)