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Genetic analysis of *Escherichia coli* from porcine postweaning diarrhoea

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SUMMARY

A total of 79 Australian isolates of beta-haemolytic *Escherichia coli* from cases of porcine postweaning diarrhoea (PWD), and 18 isolates of serotype O 149:K91:K88 (F4) from unweaned pigs from Australia, Indonesia and Denmark, were examined by multilocus enzyme electrophoresis. These were divided into 57 electrophoretic types (ETs), with an overall mean genetic diversity per enzyme locus of 0·466. This value closely resembled that previously recorded for the whole species. Not only was the collection diverse, but there was considerable genetic heterogeneity amongst PWD isolates of the same serogroup. Isolates from serogroups O 8 and O 138 were most varied, whilst many from serogroups O 141 and O 149 were more closely related. In contrast, the isolates from the unweaned pigs all belonged to only one ET.

INTRODUCTION

Pigs frequently develop diarrhoea starting 3–10 days after they are weaned. This condition, called porcine postweaning diarrhoea (PWD), is associated with the proliferation of a restricted range of predominantly enterotoxigenic serogroups of *Escherichia coli* in the anterior small intestine [1–3]. The serogroups most commonly involved at O 8, O 138, O 141, O 149 and O 157 [4, 5]. These bacteria are nearly always beta-haemolytic, although under experimental conditions the haemolysin is not essential as a virulence determinant [6]. It is not clear why such a restricted group of serological types is involved in the condition, although one explanation could be that bacteria with these phenotypic properties are representatives of a small number of widely disseminated virulent clones. The purpose of the present study was to test this hypothesis by examining the genetic structure of a population of Australian PWD strains, using multilocus enzyme electrophoresis (MEE).

MATERIALS AND METHODS

Source of bacteria

The 79 *E. coli* isolates used in the main part of the study were all beta-haemolytic and had been isolated as the predominant serotype from the faeces of pigs with PWD. The isolates were from piggeries in various States of Australia,

Table 1. *Electrophoretic types of* Escherichia coli *from pigs with postweaning diarrhoea*

Serotypes	Number of isolates	Electrophoretic type*
O 8:K87	3	16, 25, 55
O 8:K87:K88 (F4)	12	3, 4, 6, 7, 8(3), 13, 21, 22, 27, 54
O 45:K'E65'	1	30
O 98:K'SNT'	1	57
O 138:K81:K88 (F4)	4	2, 5(2), 9
O 138:K81	5	11, 12, 17, 28, 29
O 139:K82	1	1
O 141:K85ab	27	10, 15, 23, 24, 26, 39(2), 40, 41, 42(3), 43, 44(2), 47(2), 49, 50, 51(6), 52(2)
O 141:K85ac	8	35, 45, 46, 51(4), 53
O 147:K89	1	19
O 149:K91:K88 (F4)	15	14, 18, 20, 33, 34(5), 36(2), 37, 28, 48, 56
O 157:K'V17'	1	31
Total	79	

* Electrophoretic type in Figure 1. Numbers in parentheses are the number of isolates, when more than one was present. All isolates from pigs with preweaning diarrhoea were located in ET 32.

and were isolated and serotyped at the *E. coli* Reference Laboratory, Bendigo, Victoria, during the period 1989–91. An additional 18 haemolytic isolates of serotype O 149:K91:K88 (F4) from unweaned pigs were included for comparative purposes. These were 5 isolates from newborn Australian piglets with neonatal diarrhoea (ND), 12 isolates from Indonesian piglets 5–11 days old, all with diarrhoea (from Dr Supar, Research Institute for Veterinary Science, Bogor, Indonesia), and a Danish reference strain supplied by the National Veterinary Institute, Copenhagen.

All isolates were serotyped [2], utilizing hyperimmune rabbit sera prepared against the major somatic, capsular and fimbrial antigens (K88 [F4], K99 [F5], 987P [F6] and F41). The serotypes of the PWD isolates are indicated in Table 1.

Bacterial growth and preparation of enzymes

Isolates were subcultured into nutrient broth and grown overnight at 37 °C whilst being stirred. Approximately 10^{11} bacterial were harvested from 100 ml batches of broth culture by centrifuging at 20000 g for 10 min, at 4 °C. The bacterial pellets were resuspended in phosphate buffered saline (pH 7·2), centrifuged and resuspended in 2 ml of sonication buffer (10 mM Tris – 1 mM EDTA – 0·5 mM NADP, pH 6·8). The bacteria were then lysed by two cycles of sonication, each of 1 min duration, at 4 °C, using an MSE Laboratory Sonicator set at 100 watts. The cell debris was removed by centrifugation at 20000 g for 10 min, and the clear supernatant containing constitutive enzymes collected, dispersed in 100 μl amounts, and stored at −70 °C until analysed.

Electrophoresis

The bacterial sonicates were subjected to electrophoresis in horizontal starch gels, using the buffers and conditions described by Selander and colleagues [7]. After initial screening, the following 19 metabolic enzymes were selected for use: 6-phosphogluconate (6PG), leucyl glycylglycine peptidase 1 (LGG1), leucyl glycylglycine peptidase 2 (LGG2), mannose-6-phosphate isomerase (MPI), fructose-1,6-diphosphatase (FDP), isocitrate dehydrogenase (IDH), acid phosphatase (ACP), hexokinase 1 (HEX1), hexokinase 2 (HEX2), phosphoglucose isomerase (PGI), nucleoside phosphorylase (NSP), leucyl tyrosine peptidase 1 (LT1), leucyl tyrosine peptidase 2 (LT2), leucine aminopeptidase (LAP), superoxide dismutase (SOD), phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G6P), malate dehydrogenase (MDH), and NADP-dependent glutamate dehydrogenase (GD2).

The buffers used were Tris-citrate (pH 6) for FDP, IDH, and PGI; Tris-citrate (pH 8) for 6PG, G6P MDH, LGG1 and 2; Tris-citrate gel buffer, LiOH electrode buffer for NSP, LAP, LT1 and 2; Tris-maleate buffer (pH 8·2) for ACP and PGM; Poulik system (Tris-citrate gel buffer, borate electrode buffer) for MPI and GD2; and potassium phosphate buffer (pH 7) for HEX and SOD.

For each enzyme, distinctive mobility variants were numbered in order of decreasing anodal migration and interpreted as products of different alleles at the corresponding structural gene locus. Isolates were characterized by the combination of alleles at the enzyme loci, and were grouped together according to these allele profiles and designated a distinct electrophoretic type (ET).

Analysis

Genetic diversity at each locus among ETs or isolates was calculated as $h = (1-\Sigma x_i^2)(n/n-1)$, where x_i is the frequency of the ith allele among ETs and n is the number of ETs or isolates [8]. Total genetic diversity (H) was calculated as the mean of h over all loci.

The number of electromorph differences between pairs of isolates of serogroups O 8, O 138, O 141 and O 149, the mean (D) and the variance of these pairwise differences were calculated [9]. D is a measure of allelic diversity in structural genes among isolates of a given serogroup, and can range from 0 (no diversity) to 19 (no identity) [10].

Genetic distance between pairs of ETs was expressed by the proportion of loci fixed for different alleles. A phenogram of relationships between ETs was constructed from a matrix of distance coefficients by the unweighted pair group method with averages (UPGMA) clustering strategy [10].

RESULTS

Thirteen of the 19 enzyme loci were polymorphic for 2–4 alleles. GD2, NSP, LT1, LT2, LAP and SOD were monomorphic. The mean number of alleles per locus was 2·32. A total of 57 ETs were identified, with a mean genetic diversity per locus of 0·466. When the number of isolates in each ET was included in the calculations, genetic diversity was reduced to 0·423. The phenogram produced was complex,

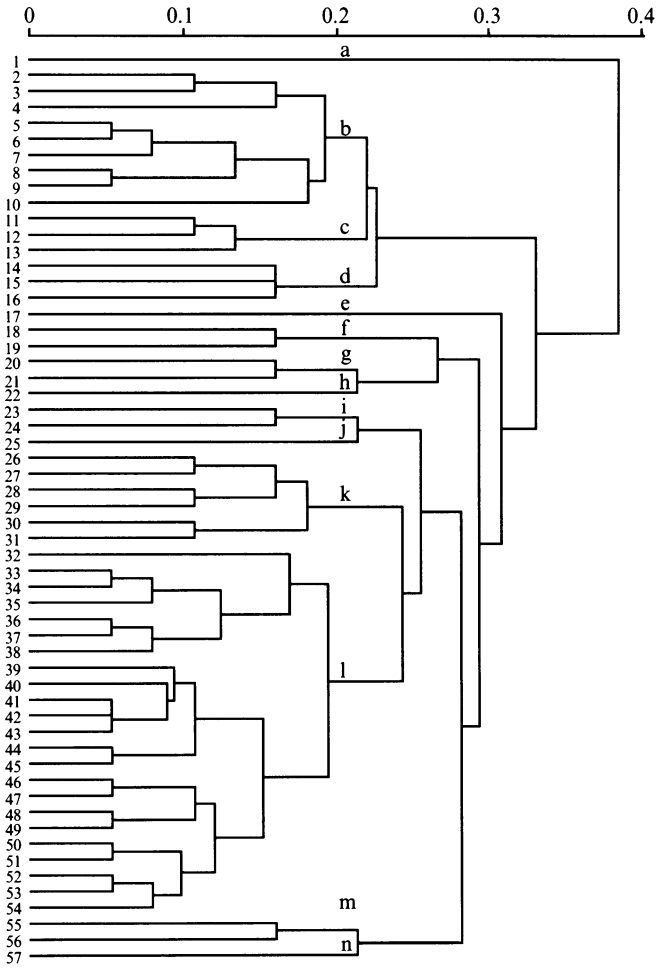

Fig. 1. Phenogram of genetic distances amongst ETs of *Escherichia coli*, clustered by UPGMA strategy. The phenogram is divided into 14 subclusters (marked a–n), at a genetic distance of 0·2.

and 14 subclusters, marked a–n on Figure 1, are outlined at a genetic distance of 0·2.

The ETs in which isolates of specific PWD serotypes were located are indicated in Table 1. All isolates from unweaned pigs were located in ET 32. Only 10 of the 56 ETs of PWD isolates contained more than 1 isolate (range 2–5 isolates), and only 1 ET (51) contained isolates with different serological properties, and these were capsular antigenic variants of serogroup O 141.

The diversity of the four most prevalent serogroups (O 8, O 138, O 141 and O 149) is presented in Table 2. Diversity for individual serotypes within serogroups O 8, O 138 and O 141 was not calculated because of the small numbers of isolates. Genetic diversity was greater for serogroups O 8 and O 138 than for O 141 and O 149. Despite the overall diversity, there was some clustering of isolates within the same serogroup. Seven of the 12 (58%) isolates of serotype O 138:K81:K88 (F4) were located in subcluster b on the phenogram, and 28 of 35 (80%) isolates of serogroup O 141 were located in ETs 39–53, which comprised part of subcluster **l**.

Table 2. *Serogroup diversity amongst* Escherichia coli *from pigs*

Serogroup	Number of isolates	Number of ETs	$\bar{D}$	V	D_{max}	H
O 8	15	13	5·769	3·972	9	0·485
O 138	9	9	4·905	2·943	8	0·488
O 141	35	20	3·647	3·797	10	0·238
O 149	33	11	3·491	1·760	6	0·196

$\bar{D}$ is the average number of electromorph differences between all pairs of isolates within a serogroup. V is the variance. D_{max} is the largest number of electromorph differences between pairs of isolates. H is the genetic diversity.

Ten of 15 (67%) PWD isolates of serotype O 149:K91:K88 (F4) were present in ETs 33–38, in the other part of subcluster 1.

DISCUSSION

This study demonstrated the existence of considerable genetic diversity amongst Australian *E. coli* isolates of a limited range of serotypes, isolated from cases of PWD. The diversity of 0·466 was the same as the figure of 0·47 reported for the whole species [11]. Among the four main serogroups only certain isolates of O 141 and O 149 in subcluster **1**, and some of O 8 and O 138 in subcluster **b**, were relatively closely related. These clusters could be clonal groupings (e.g. O 149 in ETs 33–38; O141 in ETS 39–45 and 46–54), although there was diversity even amongst these. This diversity may have arisen through mutational change or acquisition of new genetic material in the period since the original clones were introduced into Australia. In contrast, all 18 isolates of serotype O 149:K91:K88 (F4) from unweaned pigs with diarrhoea in Australia, Indonesia and Denmark belonged to a single ET. This ET was related to the main group of PWD isolates of this serotype (ETs 33–38), but was distinct. Using a phenotypic typing system, Kuhn and colleagues [12] have demonstrated that virtually all isolates of this O-group from neonatal pigs in Sweden belonged to a single clone which has been present in the country since the mid-1960s. One of the isolates in the current study was from Denmark. Assuming that this belonged to the same clone as present in Sweden, this suggests that this clone also exists as a cause of diarrhoea in unweaned pigs in Australia and Indonesia. Interestingly, this clonal group is distinct from those causing PWD, which are themselves genetically heterogenous.

Previous studies have demonstrated that in the general *E. coli* population, isolates sharing single antigenic determinants (e.g., somatic antigen type) can be as diverse as randomly selected strains [9]. Such diversity was not expected in the present study, because all the isolates were from a limited range of serotypes, associated with a specific syndrome in weaned pigs. Our results suggest that these isolates share virulence determinant(s), linked to the O-types involved, that allow them to proliferate selectively in the small intestines of newly-weaned pigs. Using DNA hybridization techniques, selected isolates from among this collection have previously been shown to have the genetic potential to produce either heat stable toxins (STa and STb), heat labile toxin (LT), shiga-like toxin II variant (SLT-IIV), or combinations of these (I. D. Connaughton, unpublished data). Such

toxins are, however, also produced by other *E. coli* strains that are not involved in PWD. Previous studies have suggested that *E. coli* isolates from PWD may have certain metabolic properties that are distinct from isolates involved in neonatal diarrhoea [13, 14], and it is possible that such shared properties could also make the PWD strains more capable of colonizing the piglet intestine after weaning. Again, however, this was not specifically examined. A common feature of the PWD isolates in this study was that they were all beta-haemolytic. This property is apparently linked to the O-type, and could conceivably assist in bacterial competition, acquisition of nutrient and/or bacterial adhesion in the newly-weaned pig intestine. Non-haemolytic strains do occasionally cause PWD [15, 16], but this may only reflect an absence of haemolytic strains that might otherwise out-grow them in the intestine. The haemolysin is not an essential virulence determinant, since strains cured of the Hly plasmid can still induce diarrhoea under conditions of experimental challenge [6]. This does not exclude the possibility however that, under natural conditions, strains possessing haemolysin have a selective advantage that allows them to proliferate in preference to non-haemolytic strains. Finally, colonization factors that are genetically linked to the O-groups of the PWD strains would be good candidates as virulence determinants. Only certain of the isolates examined possessed the K88 (F4) fimbrial adhesion, but others could have possessed more recently described colonization factors [17–20]. These were not assayed for in this study.

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REFERENCES

1. Richards WPC, Fraser CM. Coliform enteritis of weaned pigs. A description of the disease and its association with hemolytic *Escherichia coli*. Cornell Vet 1961; **51**: 245–57.
2. Sojka WJ. In '*Escherichia coli* in domestic animals and poultry', 1965. Commonwealth Agricultural Bureau, Weybridge, England.
3. Svendsen J. Enteric *Escherichia coli* diseases in weaned pigs. Nord VetMed 1974; **26**: 226–38.
4. Dam A, Knox B. Haemolytic *Escherichia coli* associated with enteritis and enterotoxaemia in pigs in Denmark, with particular reference to the rapid spread of serogroup O 149:K91. Nord VetMed 1974; **26**: 219–25.
5. Bertschinger HU, Jucker H, Pfirter HP. Control of *Escherichia coli* infection in weaned pigs by use of oral immunization combined with a diet low in nutrients. Fortschr VetMed 1979; **29**: 73–81.
6. Smith HW, Linggood MA. Observations on the pathogenic properties of the K88, hly and ent plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. J Med Microbiol 1971; **4**: 467–85.
7. Selander RK, Caugant DA, Ochman H, Musser JM, Gilmour MN, Whittam TS. Methods of multilocus enzyme electrophoresis for bacterial population genetics and systematics. Appl Environ Microbiol 1986; **51**: 873–84.
8. Nei M. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 1978; **89**: 583–90.
9. Caugant DA, Levin BR, Orskov I, Orskov F, Eden CS, Selander RK. Genetic diversity in relation to serotype in *Escherichia coli*. Infect Immun 1985; **49**: 407–13.
10. Sneath PHA, Sakal RR. Numerical taxonomy. San Francisco; CA: WH Freeman 1973: 573.

11. Selander RK, Levin BR. Genetic diversity and structure in *Escherichia coli* populations. Science 1980; **210**: 545–7.
12. Kuhn I, Franklin A, Soderlind O, Mollby R. Phenotypic variations among enterotoxigenic *Escherichia coli* from Swedish piglets with diarrhoea. Med Microbiol Immunol 1985; **174**: 119–30.
13. Vasenius H. The influence of dietary carbohydrate on the multiplication and colonization of inoculated *Escherichia coli* strains in pigs intestines. II. Investigations in pigs at weaning age. Nord VetMed 1969; **21**: 535–44.
14. Larsen JL. Differences between enteropathogenic *Escherichia coli* strains isolates from neonatal *E. coli* diarrhoea (NCD) and postweaning diarrhoea (PWD) in pigs. Nord VetMed 1976; **28**: 417–29.
15. Hoblet KH, Kohler EM, Saif LH, Theil KW, Ingalls WL. Study of porcine post-weaning diarrhoea involving K88 (–) hemolytic *Escherichia coli*. Am J Vet Res 1986; **47**: 1910–2.
16. Soderlind O, Thafvelin B, Mollby R. Virulence factors in *Escherichia coli* strains isolated from Swedish piglets with diarrhea. J Clin Microbiol 1988; **26**: 879–84.
17. Kennan RM, Monckton RP. Adhesive fimbriae associated with porcine enterotoxigenic *Escherichia coli* of the O 141 serotype. J Clin Microbiol 1990; **28**: 2006–11.
18. Bertschinger HU, Bachman M, Mettler C, et al. Adhesive fimbriae produced *in vivo* by *Escherichia coli* O 139:K12 (B):H1 associated with entertoxaemia in pigs. Vet Microbiol 1990; **25**: 267–81.
19. Nagy B, Casey TA, Whipp SC, Moon HW. Susceptibility of porcine intestine to pilus-mediated adhesion by some isolates of piliated enterotoxigenic *Escherichia coli* increases with age. Infect Immun 1992; **60**: 1285–94.
20. Salajka E, Salajkova Z, Alexa P, Hornich, M. Colonization factor different from K88, K99, F41 and 987P in enterotoxigenic *Escherichia coli* strains isolated from postweaning diarrhoea in pigs. Vet Microbiol 1992; **32**: 163–75.