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Clonal Analysis of *Escherichia coli* of Serogroups O9, O20, and O101 Isolated from Australian Pigs with Neonatal Diarrhea

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The genetic diversity of 87 isolates of *Escherichia coli* recovered from Australian pigs with neonatal diarrhea was examined by multilocus enzyme electrophoresis. The isolates were of serogroups O9, O20, and O101, and although most isolates lacked K88(F4), K99(F5), 987P(F6), and F41 fimbriae, they were considered to be involved in the etiology of the diarrhea. The isolates were extremely diverse, considering their origin from a single pathological condition in one country. There were estimated to be 18, 16, and 12 clones of the three respective serogroups in the collection, with serogroup diversities of 0.387, 0.448, and 0.275, respectively. Comparison with the results previously obtained for isolates from piglets with postweaning diarrhea suggested that bacteria from piglets with these two conditions did not come from any particular common genetic background. The overall genetic diversity for the combined collection was the same as that reported by others for representative isolates selected from throughout the species (0.47). The current results indicate that if isolates of these O groups are involved in porcine diarrhea, their pathogenicity is directly linked to their O somatic antigen type and is not simply due to the wide distribution of a small number of virulent clones.

Neonatal diarrhea (ND) is a common and important infectious disease of swine (4). The condition is associated with certain of a relatively small number of serotypes of *Escherichia coli* which proliferate in the small intestines of newborn piglets. Currently, in Australia, the most common serogroups that are isolated from piglets with the condition are O9, O20, and O101 (3, 8), and these are also gaining in frequency of isolation in North America (4) and Sweden (17). In the past, most isolates have been found to possess one or more of the following adhesive fimbriae: K88(F4), K99(F5), 987P(F6), or F41 (11). More recently, following the incorporation of these antigens into vaccines for ND, it appears that strains lacking these fimbriae are becoming more common. For example, in Sweden, only 39 of 170 (23%) isolates of O groups 9, 20, and 101 from piglets with ND possessed these fimbriae (17). The emergence of nonfimbriated strains could be the result of selection for and the wide-scale dissemination of a limited number of clones of *E. coli* following the use of fimbriated strains in vaccines. The purpose of the present study was to examine this possibility by multilocus enzyme electrophoresis to determine the genetic diversity of a collection of mainly nonfimbriated strains from Australian pigs with ND. Results were compared with those obtained previously (5) for isolates recovered from pigs with postweaning diarrhea (PWD).

MATERIALS AND METHODS

Bacteria. The 87 isolates of *E. coli* that were selected for analysis in the present work were each isolated as the predominant serogroup from the feces of piglets suffering from ND. Each isolate was from a different Australian piggery in which that particular serogroup was regularly associated with the condition. Representative isolates were previously demonstrated to have the genetic capacity to produce enterotoxin (ST1) when tested by DNA hybridization techniques (9). The bacteria were originally isolated from rectal swabs submitted to the *E. coli* Reference Laboratory, Regional Veterinary Laboratory, Bendigo, Australia, during the period 1989 to 1991.

Serotyping. Selected colonies of *E. coli* were incubated overnight on 7.5% sheep blood agar and Minca agar. The bacteria were resuspended in physiological saline and were subjected to either slide agglutination or *Staphylococcus aureus* coagglutination by using rabbit antisera prepared against the recognized fimbriae K88(F4), K99(F5), 987P(F6), and F41. The remaining bacterial suspension was autoclaved at 121°C for 2 h and then tested by slide agglutination against a panel of rabbit antisera prepared against the common O serogroups. Isolates that reacted clearly with only one antiserum sample were selected for use in the analysis.

Bacterial growth and enzyme preparation. Isolates were subcultured into nutrient broth and were grown overnight at 37°C with stirring. Approximately 10^{11} bacteria were harvested from 100-ml batches of broth culture by centrifuging at 20,000 × *g* for 10 min at 4°C. The bacterial pellets were resuspended in phosphate-buffered saline (pH 7.2) and were centrifuged again, and the pellet was resuspended in 2 ml of sonication buffer (10 mM Tris, 1 mM EDTA, 0.5 mM NADP [pH 6.8]). The bacteria were lysed by two 1-min cycles of sonication at 4°C by using an MSE Laboratory sonicator set at 100 W. The cell debris was removed by centrifugation at 20,000 × *g* for 10 min, and the supernatant was dispensed into 100-μl aliquots and stored at −70°C until required for electrophoresis.

Electrophoresis. The bacterial lysates were electrophoresed in 11.4% horizontal starch gels by the procedures described by Selander et al. (14). The following 19 enzymes were assayed: 6-phosphogluconate dehydrogenase (6PGD), leucyl glycylglycine peptidase 1 (LGG1), leucyl glycylgly-

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cine 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 (G6PD), malate dehydrogenase (MDH), and NADP-dependent glutamate dehydrogenase (GD2).

FDP, IDH, and PGI were electrophoresed on Tris-citrate (pH 6); 6PGD, G6PD, MDH, LGG1, and LGG2 were electrophoresed on Tris-citrate (pH 8); NSP, LAP, LT1, and LT2 were electrophoresed on Tris-citrate gel buffer and LiOH electrode buffer; ACP and PGM were electrophoresed on Tris-maleate buffer (pH 8.2); MPI and GD2 were electrophoresed on Poulik system (Tris-citrate gel buffer, borate electrode buffer); and potassium phosphate buffer (pH 7) was used for HEX and SOD.

For each enzyme, distinctive mobility variants were numbered in order of decreasing anodal migration and were 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 then grouped together according to these allele profiles and were designated a distinct electrophoretic type (ET).

Analysis. Genetic diversity (h) 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 (12). 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 O9, O20, and O101, the mean (D) and the variance of these pairwise differences were calculated (1). D is a measure of allelic diversity in structural genes among isolates of a given serogroup.

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 clustering strategy (16).

Comparison with postweaning isolates. The results for the current study were compared with those obtained previously (5) with a collection of 79 *E. coli* isolates from cases of PWD, which were examined by the same techniques (5). Genetic diversity was calculated for the whole collection. A phenogram was constructed as described previously (5), but in the present study, we used allelic profiles from only single representative isolates from each of the 13 neonatal and 14 postweaning subclusters, identified at a genetic distance of 0.2 (subclusters a to m for the current neonatal isolates and a to n for the previous postweaning isolates [5]).

RESULTS

Enzyme activities and genetic diversity. Thirteen of the 19 enzyme loci were polymorphic for from two to five alleles. MDH, NSP, LT1, LT2, LAP, and SOD were monomorphic. The mean number of alleles per locus was 2.47. Seventy-three ETs were identified, with a mean genetic diversity of 0.422. Genetic diversity was calculated as 0.395 when the number of isolates in each ET were considered. The phenogram that was produced is shown in Fig. 1, in which 13 subclusters (marked a to m) are outlined at a genetic distance of 0.2.

Serogroups and ETs. The 87 isolates were of three serogroups, O9 (39 isolates), O20 (22 isolates), and O101 (26 isolates). The distribution of isolates into ETs is shown in Table 1. Isolates of the same serogroup that were closely clustered together in Fig. 1 but that differed at three or fewer enzyme loci were considered to represent clonal groupings. On this basis, the three serogroups contained 18, 16, and 12 clones, respectively (Table 1). Ten of the 73 ETs contained more than one isolate (range, two to four isolates), of which

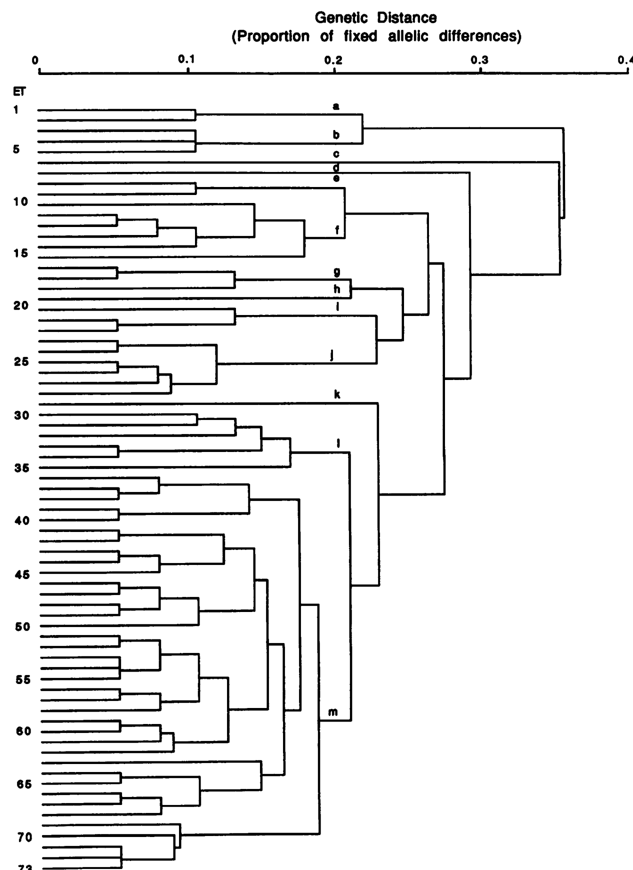

FIG. 1. Phenogram of genetic distance (expressed as percent fixed allelic differences) among ETs of 87 isolates of *E. coli* from piglets with ND clustered by the unweighted pair group method with averages strategy. The phenogram is divided into 13 subclusters (marked a to m) at a genetic distance of 0.2.

TABLE 1. ETs of *E. coli* from Australian pigs with ND

Serogroup	ET[a]	Estimated no. of clones[b]
O9	1; 3, 4, 5; 17, 18; 20; 22; 23, 24; 25, 26, 27, 28; 30, 31; 34; 35; 36; 41; 43, 44, 45; 46; 51, 52, 53, 54; 56, 57, 58; 60, 61; 67	18
O20	2; 6; 7; 8, 9; 10; 11, 12, 13, 14; 15; 21; 29; 38; 40; 42; 54, 55; 57; 59; 63	16
O101	16; 19; 32; 33; 37; 39; 47, 48, 49, 50; 52; 60, 62; 64, 65; 66, 68; 69, 70, 71, 72, 73	12

[a] The ETs are given in Fig. 1.

[b] Bacteria of the same serogroup, clustered together, with different alleles at three or fewer enzyme loci.

TABLE 2. Serogroup diversity among *E. coli* from Australian piglets with ND[a]

Serogroup	No. of isolates	No. of ETs	$\bar{D}$	V	D_{max}	H
O9	39	34	4.513	3.954	10	0.387
O20	22	21	5.076	3.137	9	0.448
O101	26	22	3.376	2.208	8	0.275

[a] $\bar{D}$, average number of electromorph differences between all pairs of isolates within a serogroup; V, variance; D_{max}, largest number of electromorph differences between pairs of isolates; H, genetic diversity for isolates of that serogroup.

3 ETs contained isolates of different serogroups. These were ETs 54 and 57 (O9 and O20) and ET 60 (O9 and O101). Genetic diversities for the three serogroups were all high, being 0.387, 0.448, and 0.275, respectively, with average electromorph differences between pairs of isolates in the three serogroups being 4.513, 5.076, and 3.376, respectively (Table 2).

Fimbriae. Only 6 of the 87 isolates included in the present study possessed fimbriae, as detected by slide agglutination. These were two O20 isolates which produced 987P and one which produced K88 and two O101 isolates which produced K99 and one which produced F41. Fimbriae were not detected on any of the O9 isolates.

Comparison with PWD isolates. The overall mean genetic diversity for the combined neonatal and postweaning isolates was 0.470, and these isolates were divided into 124 ETs. Six of these ETs contained isolates from neonatal and postweaning piglets. A phenogram comparing the relationships of the 13 neonatal subclusters with 14 subclusters of isolates from piglets with PWD, at a genetic distance of 0.2, is presented as Fig. 2. Strains from neonatal piglets and piglets with PWD were located throughout the combined phenogram.

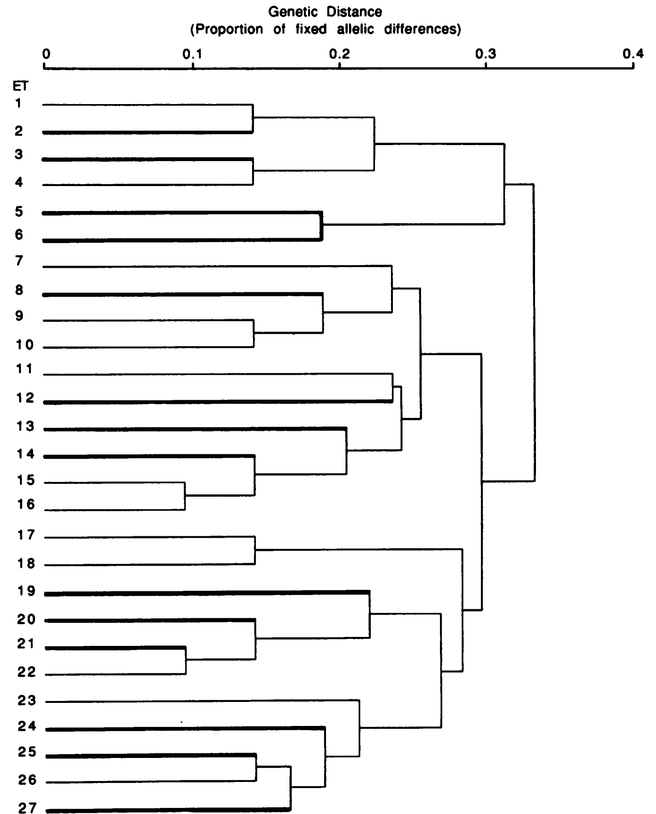

FIG. 2. Phenogram of genetic distance (expressed as percent fixed allelic differences) among single representatives from each of 13 subclusters of isolates of *E. coli* from neonatal piglets (a to m in Fig. 1) and from 14 subclusters of isolates from postweaning piglets (5) clustered by the unweighted pair group method with averages strategy. The 14 ETs of isolates from postweaning piglets are marked in bold.

DISCUSSION

A striking feature of the *E. coli* isolates used in the present study was their general lack of recognized fimbriae. It was possible, however, that certain isolates may have had other adhesive factors, such as F42 (7), for which we did not assay. All the isolates were of serogroups recognized as being associated with neonatal diarrhea (O9, O20, O101), and were smooth or smooth-mucoid and nonhemolytic. In the past, in Australia, isolates from neonatal piglets usually did have recognized fimbriae (10), but since the advent of wide-scale vaccination for neonatal colibacillosis, fimbriae are now rarely detected (2). The isolates were all assumed to be the cause of the diarrhea in the piglets from which they were isolated, although this was not proven. They were likely thought to be pathogens because they were regularly recovered as the predominant organism in the feces of piglets with diarrhea on the farms in question. Furthermore, the inclusion of organisms of these serogroups in vaccines for ND has greatly reduced the problem in many Australian piggeries (2). We cannot, however, exclude the possibility that the isolates were simply efficient colonizers of piglets with ND.

The isolates that were examined were remarkably diverse, given that they were all of only three serogroups and were from Australian piglets with ND. For the purpose of analysis, it was assumed that isolates of the same serogroup that were clustered together on the phenogram and that differed at three or fewer loci were members of the same clonal line. By this definition, the collection contained 18, 16, and 12 clones of the three serogroups, respectively. This diversity was not just due to the fact that the isolates came from different states, since the relative diversity for the serogroups in some states was greater than that seen for the whole country. Thus, in both New South Wales and Victoria, from which 61% of the collection originated, the mean number of isolates per clonal grouping for the three serogroups was 1.26 compared with 1.89 for the whole collection. The existence of such genetic heterogeneity among *E. coli* isolates belonging to the same serogroup has been recorded previously (1, 13). The use of serological grouping is not an accurate marker for analyzing isolates for epidemiological studies.

When the current neonatal strains were compared with those from piglets with PWD, the overall genetic diversity increased to 0.47, a value previously given for representative isolates of the whole species (15). Strains from neonatal and postweaning piglets were intermingled on the combined phenogram, and there was no evidence to suggest that isolates recovered from piglets with ND and PWD were more likely to come from any particular genetic background.

In contrast to this, it has been shown that isolates of serotype O149:K91:K88(F4) from piglets with ND from Scandinavia, Australia, and Indonesia belong to a single clonal grouping (5, 6). The difference between the isolates

from piglets with ND assayed in the present study and those of serotype O149:K91:K88(F4) used in the previous study (5) may have been the availability of a more complete antigenic formula for the O149 isolates. It has been shown that the apparent diversity among isolates of *E. coli* sharing the same serogroup is reduced as additional antigenic determinants are included in the analysis (1). This is unlikely to be the whole explanation for the difference that we observed however, because there is still considerable diversity in strains of serotype O149:K91:K88(F4) from piglets with PWD (5).

Assuming that the isolates used in the current study were the cause of the piglets' neonatal diarrhea, their virulence would appear to be linked to their O somatic antigen type rather than to their overall genetic origin. The lipopolysaccharide could conceivably be involved in facilitating intestinal colonization, although it is more likely to be linked, for example, to the presence of novel fimbriae which have not yet been characterized. Further work should be directed at examining this possibility and looking for other common properties possessed by isolates of these serogroups.

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