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Clonal Diversity in *Haemophilus pleuropneumoniae*

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Genetic diversity among 135 isolates of nine serotypes of *Haemophilus pleuropneumoniae* recovered from pigs with pleuropneumonia or other invasive diseases in 14 countries was estimated by multilocus enzyme electrophoresis, which detects allelic variation in structural genes. Thirty-two multilocus genotypes (electrophoretic types [ETs]) were distinguished on the basis of allele profiles at 15 enzyme loci, and 36 distinctive combinations of ET and serotype were identified. The recovery of isolates with identical properties in widely separated geographic regions and over a 20-year period indicated that the population structure of *H. pleuropneumoniae* is clonal. Isolates of the same ET generally shared the same serotype and electrophoretic pattern of the outer membrane proteins, but some ETs were represented by isolates of several different serotypes, outer membrane protein patterns, or both. On average, the genetic diversity among ETs of the same serotype was 56% of the total genetic diversity in the species. Isolates of serotype 1, which are unusually pathogenic, belong to a distinctive group of clones that are closely related to clones marked by serotype 9.

The gram-negative bacterium *Haemophilus pleuropneumoniae* is the major cause of pleuropneumonia (lobar pneumonia with fibrinous pleuritis) in pigs (29). It is transmitted primarily by direct contact between animals, and the resulting infection produces a clinical course varying from peracute to chronic.

Most epidemiological and immunoprophylaxis research on *H. pleuropneumoniae* has relied on serologic typing for strain classification (5, 6, 12–15, 19, 25, 26). Nine types have been distinguished on the basis of serologic assays of antigens that are poorly characterized but presumed to be capsular in origin (5, 19). Recently, methods were developed for classifying isolates by the electrophoretic mobility pattern of their major outer membrane proteins (OMPs). In a survey of 95 isolates from the midwestern United States, Rapp et al. (24) identified three dominant associations of serotype with OMP profile and suggested that these character combinations mark clones. But because serotyping and OMP typing do not estimate the extent of variation in the genome as a whole, the genetic relationships of isolates classified by serotype or OMP type to those of other serotypes and OMP types and to serologically nontypable strains remain unresolved. It is, for example, not known whether strains of serotypes 1, 5, 7, and 9, which together are responsible for more than 95% of cases of pleuropneumonia in pigs in the midwestern United States (25), are genetically similar or a heterogeneous assemblage of genotypes.

Here we report the results of an analysis of genetic variation and relatedness in a collection of isolates of *H. pleuropneumoniae* from the continental United States, Canada, and Europe, based on multilocus enzyme electrophoresis, serologic typing, and OMP analysis. From comparisons of the electrophoretic mobilities of enzymes encoded by 15 structural genes, we determined that natural populations of *H. pleuropneumoniae* are highly variable in multilocus genotypes and have a clonal structure. Although isolates of the same serotype are, in general, genetically quite diverse, those of serotype 1, which are unusually pathogenic (11, 27), form a distinct cluster of closely related clones.

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MATERIALS AND METHODS

Bacterial isolates. We examined 135 isolates of *H. pleuropneumoniae*, including 45 isolates from Iowa, Illinois, and California; 60 isolates from Quebec, Ontario, and Saskatchewan; 8 isolates from Switzerland; 8 isolates from Denmark; 3 isolates from the Netherlands; 2 isolates from the Federal Republic of Germany; 2 isolates from Yugoslavia; and 1 isolate each from Argentina, Ireland, Sweden, Finland, Italy, Japan, and Australia (Table 1). Almost all isolates were recovered from pigs with pleuropneumonia or other invasive disease, and some isolates have been described previously (8, 24, 25).

Bacterial identification, serotyping, and OMP analysis. Isolates were classified as *H. pleuropneumoniae* according to morphologic and biochemical criteria (25). Clinical isolates were serotyped by rapid slide agglutination and an indirect fluorescent antibody test (25). The serotypes of other isolates were those of the source laboratory; however, some isolates were tested by the rapid slide agglutination method to confirm the serotype designations. Electrophoretic OMP profiles of Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.)-enriched preparations from selected strains were determined as described previously (24).

Growth of bacteria and electrophoresis of enzymes. Each isolate was grown in 150 ml of brain heart infusion broth (Difco Laboratories, Detroit, Mich.), supplemented with 10 μg of NAD per ml, overnight at 37°C on an orbital shaker (250 rpm) and harvested by centrifugation at 6,000 × *g* for 10 min at 4°C. Following suspension in 2 ml of 50 mM Tris hydrochloride buffer containing 5 mM EDTA (pH 7.5), the bacteria were sonicated with a Sonifier Cell Disruptor (model 200; Branson Sonic Power Co., Danbury, Conn.) equipped with a microtip for 30 s at 50% pulse, with ice water cooling, and centrifuged at 20,000 × *g* for 20 min at 4°C. The clear supernatant (lysate) was stored at −70°C.

Lysates were electrophoresed on starch gels and selectively stained for 15 metabolic enzymes by methods described elsewhere (30). Polymorphism was studied in the following enymes: leucylalanine peptidase, adenylate

TABLE 1. ET, collection locality, serotype, OMP pattern, and source for 135 isolates of *H. pleuropneumoniae*

ET	Isolate	Locality	Serotype	OMP pattern[a]	Source[b]
1	WF83	Canada (Ontario)	7	6	D
	Rapp 43	United States (Iowa)	7	6	H
	Rapp 53	United States (Iowa)	7	6	H
	Rapp 114	United States (Illinois)	7	6	H
	Rapp 194	United States (Iowa)	7	6	H
2	S1181/70	Switzerland	3	ND	G
3	Rapp 160	United States (Illinois)	7	6	H
	STH85-32	Canada (Quebec)	7	A	A
	STH85-62	Canada (Quebec)	7	6	A
	1648	Switzerland	7	6	B
4	10169	Denmark	8	A	C
	11616	Denmark	8	A	C
5	405	Ireland	8	7	C
6	84-4285	Canada (Quebec)	3	A	A
7	83-5437	Canada (Quebec)	3	A	A
8	ATCC 27090	Switzerland	3	3	I
9	STH85-49	Canada (Quebec)	6	2	A
10	STH85-001	Canada (Quebec)	6	2	A
	STH85-22	Canada (Quebec)	6	2	A
11	Rapp 200	United States (Iowa)	5	5	H
	Rapp 2	United States (Iowa)	5	5a	H
	Rapp 11	United States (Iowa)	5	5a	H
	Rapp 30	United States (Iowa)	5	5	H
	Rapp 41	United States (Illionis)	5	5	H
	Rapp 59	United States (Illinois)	5	5	H
	Rapp 66	United States (Iowa)	5	5	H
	Rapp 73	United States (Iowa)	5	5	H
	Rapp 84	United States (Iowa)	5	5	H
	Rapp 90	United States (Iowa)	5	5	H
	Rapp 100	United States (Illinois)	5	5	H
	Rapp 104	United States (Illinois)	5	5	H
	Rapp 116	United States (Illinois)	5	5	H
	Rapp 191	United States (Iowa)	5	5	H
	Rapp 201	United States (Iowa)	5	5	H
	KISL	United States (Iowa)	3	5	H
	11507	United States (Iowa)	3	5	H
	M-1	United States (Iowa)	5	5	H
	B+B	United States (Iowa)	5	5	H
	81-750	Canada (Quebec)	5	5	A
	85-43A	Canada (Quebec)	5	ND	E
	85-1041	Canada (Quebec)	5	5	A
	85-17841B	Canada (Quebec)	5	ND	E
	Q85-3502	Canada (Quebec)	5	5	A
	STH85-30#1	Canada (Quebec)	5	ND	E
	STH85-35	Canada (Quebec)	5	ND	E
	F85-1720P'	Canada (Quebec)	5	ND	E
12	ATCC 33377	United States (California)	5	5a	I
13	Rapp 74	United States (Iowa)	NT[c]	5	H
	Rapp 76	United States (Illinois)	NT	5a	H
14	Rapp 49	United States (Iowa)	5	5	H
	Rapp 125	United States (Illinois)	5	5	H
	Rapp 138	United States (Iowa)	5	5	H
	Rapp 149	United States (Illinois)	5	5	H
	Rapp 178	United States (Iowa)	5	5	H
	Rapp 142	United States (Iowa)	NT	5a	H

Continued on following page

TABLE 1—*Continued*

ET	Isolate	Locality	Serotype	OMP pattern[a]	Source[b]
15	Rapp 172	United States (Illinois)	5	5	J
16	Rapp 50	United States (Iowa)	5	5	H
17	SG141	Switzerland	2	2	B
	S97	Switzerland	2	2	B
	S292	Switzerland	2	2	B
	4226	Denmark	2	2	C
	6795	Denmark	2	2	C
	Bd166	Sweden	2	ND	G
	STH85-37	Canada (Quebec)	2	2	A
18	HK358	Denmark	2	ND	G
	HK360	Denmark	2	ND	G
	S853/70	Switzerland	3	ND	G
	HP8	Japan	2	ND	G
19	ATCC 27089	Switzerland	2	2	I
20	3015	Denmark	6	2	C
21	Femø	Denmark	6	2	D
22	ATCC 27088	Argentina	1	1	I
	Rapp 33	United States (Iowa)	1	1	H
	Rapp 38	United States (Illinois)	9	1	H
23	Aust 3933	Australia	1	1	B
24	VLS288	Canada (Ontario)	1	ND	F
25	CVJ 13261	Netherlands	9	1	C
	94935	Netherlands	9	1	C
	97655	Netherlands	9	1	C
	726	Federal Republic of Germany	9	1	B
	776	Federal Republic of Germany	9	1	B
	X1	Switzerland	9	1	B
26	Rapp 40	United States (Iowa)	1	1	H
	Rapp 48	United States (Illinois)	1	1	H
	Rapp 87	United States (Illinois)	1	1	H
	Rapp 95	United States (Iowa)	1	1	H
	Rapp 123	United States (Illinois)	1	1	H
27	Q85-3758	Canada (Quebec)	1	1	A
	N85-453	Canada (Quebec)	1	ND	E
	STH85-36	Canada (Quebec)	1	ND	E
	85-26-738	Canada (Quebec)	1	ND	E
	85-26-746	Canada (Quebec)	1	ND	E
	85-26-754	Canada (Quebec)	1	ND	E
	85-26-765	Canada (Quebec)	1	ND	E
	JK82	Canada (Ontario)	1	ND	F
	MA69	Canada (Ontario)	1	ND	F
	MA70	Canada (Ontario)	1	ND	F
	MA71	Canada (Ontario)	1	ND	F
	MA73	Canada (Ontario)	1	ND	F
	MA74	Canada (Ontario)	1	ND	F
	MA75	Canada (Ontario)	1	ND	F
	MA76	Canada (Ontario)	1	ND	F
	MA77	Canada (Ontario)	1	ND	F
	RH82	Canada (Ontario)	1	ND	F
	VSB91	Canada (Ontario)	1	ND	F
	VSB92	Canada (Ontario)	1	ND	F
	VSB98	Canada (Ontario)	1	ND	F
	VSB99	Canada (Ontario)	1	ND	F
	VSB102	Canada (Ontario)	1	ND	F

Continued on following page

TABLE 1—*Continued*

ET	Isolate	Locality	Serotype	OMP pattern[a]	Source[b]
	VLS286	Canada (Ontario)	1	ND	F
	VLS287	Canada (Ontario)	1	ND	F
	VLS289	Canada (Ontario)	1	ND	F
	VLS324	Canada (Ontario)	1	ND	F
	VLS325	Canada (Ontario)	1	ND	F
	VLS336	Canada (Ontario)	1	ND	F
	VLS340	Canada (Ontario)	1	ND	F
	WLN344	Canada (Ontario)	1	ND	F
	S352	Canada (Saskatchewan)	1	ND	F
	S354	Canada (Saskatchewan)	1	ND	F
	S355	Canada (Saskatchewan)	1	ND	F
	S356	Canada (Saskatchewan)	1	ND	F
	S357	Canada (Saskatchewan)	1	ND	F
	S358	Canada (Saskatchewan)	1	ND	F
	S359	Canada (Saskatchewan)	1	ND	F
28	85-4219	Canada (Quebec)	1	1	A
	Q85-1525N	Canada (Quebec)	1	ND	E
	STH85-43	Canada (Quebec)	1	ND	E
	STH85-45	Canada (Quebec)	1	ND	E
	85-26-188	Canada (Quebec)	1	ND	E
29	Rapp 136	United States (Illinois)	NT	A	H
30	ATCC 33378	United States (California)	4	4	I
31	Jugo 36	Yugoslavia	4	2	C
	Jugo 37	Yugoslavia	4	2	C
	Ital 687	Italy	4	2	C
32	Finland	Finland	5	ND	G

[a] See Table 1 of reference 24 for a description of OMP patterns 1 through 7; A, atypical pattern not corresponding to any of the reference strains; ND, not determined; 5a, pattern similar but not identical to predominant serotype 5 profile.

[b] A, K. R. Mittal to V. J. Rapp; B, J. Nicolet to V. J. Rapp; C, R. Nielsen to V. J. Rapp; D, S. Rosendal to V. J. Rapp; E, K. R. Mittal to J. M. Musser: F, S. Rosendal to J. M. Musser; G, M. Kilian to J. M. Musser; H, clinical isolate; I, American Type Culture Collection (Rockville, Md.); J, nasal isolate.

[c] NT, Nontypable.

kinase, glutamate dehydrogenase, mannose phosphate isomerase, indophenol oxidase, 6-phosphogluconate dehydrogenase, malic enzyme, malate dehydrogenase, esterase, leucine aminopeptidase, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, phosphoglucomutase, glutamic oxaloacetic transaminase, and phosphoglucose isomerase.

Glutamate dehydrogenase, glutamate oxaloacetic transaminase, and indophenol oxidase were electrophoresed in a Poulik buffer system; leucylalanine peptidase and leucine aminopeptidase were electrophoresed in a Tris-malate (pH 7.4) buffer system; adenylate kinase, glyceraldehyde-3-phosphate dehydrogenase, and glucose 6-phosphate dehydrogenase were electrophoresed in a phosphate (pH 7.0) buffer; and for all other enzymes except esterase, we used a Tris-citrate (pH 8.0) buffer system. Esterase was electrophoresed in a lithium hydroxide buffer system (gel buffer, pH 8.3; tray buffer, pH 8.1) (30), and activity was demonstrated with α-napthyl propionate as a substrate.

For each enzyme, distinctive mobility variants were designated as electromorphs and numbered in order of decreasing rate of anodal migration. Electromorphs of an enzyme were equated with alleles at the corresponding structural gene locus, and an absence of enzyme activity was attributed to a null allele, designated as 0. Because most isolates showed activity for all 15 enzymes, we presume that the corresponding structural gene loci are located on the chromosome rather than on plasmids.

Each isolate was characterized by its combination of alleles at the 15 enyzme loci, and distinctive profiles of electromorphs (Table 2), corresponding to unique multilocus genotypes, were designated as electrophoretic types (ETs) (30).

Statistical analyses. Genetic diversity at an enzyme locus (h) among either ETs or isolates was calculated from the allele frequencies as $h = (1 - \Sigma x_i^2)(n/n - 1)$, where x_i is the frequency of the ith allele and n is the number of ETs or isolates (30). Mean genetic diversity per locus (H) is the arithmetic average of h values for all loci.

Genetic distance between pairs of ETs was expressed as the proportion of enzyme loci at which different alleles were represented (mismatches), and clustering of ETs was performed from a matrix of pairwise genetic distances by the average-linkage method (30).

RESULTS

Overall genetic diversity. In our collection of 135 isolates of *H. pleuropneumoniae*, 13 of the 15 enzyme loci were polymorphic for from two to six alleles encoding electrophoretically distinguishable variant proteins, and two loci were monomorphic (Table 2). The average number of alleles per locus was 3.3.

A total of 32 ETs were identified (Table 2), among which the mean genetic diversity per locus (H) was 0.428 (Table 3). There was considerably less diversity among the isolates (H = 0.370), because 15 of the ETs were represented by 2 or more isolates (mean, 7.9 isolates; range, 2 to 37) and ET-11

TABLE 2. Allele profiles for 15 enzyme loci in 32 ETs of *H. pleuropneumoniae*

ET	Reference isolate	No. of isolates	Allele at the indicated enzyme locus[a]:														
			PE1	ADK	GLD	MPI	IPO	6PG	MAE	MDH	EST	LAP	G3P	G6P	PGM	GOT	PGI
1	WF83	5	1	2	1	2	3	1	1	4	0	2	2	2	2	3	3
2	S1181/70	1	1	2	1	2	3	1	1	4	1	2	2	2	2	3	3
3	Rapp 160	4	1	2	1	2	3	1	1	4	0	2	2	2	1	3	3
4	10169	2	1	2	1	2	3	1	1	4	0	2	1	2	2	4	2
5	405	1	1	2	1	2	3	1	1	4	2	3	1	2	2	1	3
6	84-4285	1	1	2	1	2	3	1	1	4	2	2	2	1	2	1	3
7	83-5437	1	1	2	1	2	3	1	1	4	2	1	2	1	2	1	3
8	ATCC 27090	1	1	2	1	2	3	1	0	4	2	4	2	2	2	2	3
9	STH85-49	1	1	1	1	2	3	1	0	4	2	3	2	1	2	2	3
10	STH85-001	2	1	1	1	2	3	1	0	4	2	3	2	2	2	2	3
11	Rapp 200	27	1	2	1	2	3	1	2	1	2	2	2	2	4	2	3
12	ATCC 33377	1	1	2	1	2	3	1	2	1	2	2	2	2	4	2	4
13	Rapp 74	2	1	2	1	2	3	1	2	1	2	2	3	2	4	2	3
14	Rapp 49	6	1	2	1	2	1	1	4	2	2	2	2	2	4	2	3
15	Rapp 172	1	1	2	1	2	1	1	4	2	2	2	2	2	4	2	4
16	Rapp 50	1	1	2	1	2	1	1	4	2	2	1	2	2	4	2	3
17	SG141	7	1	2	1	2	3	1	1	4	2	3	3	2	4	2	3
18	HK358	4	1	2	1	2	3	1	1	3	2	3	3	2	4	2	3
19	ATCC 27089	1	1	2	1	2	3	2	1	3	2	3	3	2	4	2	3
20	3015	1	1	2	1	1	3	1	1	4	2	2	2	2	3	5	3
21	Femø	1	1	2	1	1	3	1	0	2	2	2	1	1	3	5	3
22	ATCC 27089	3	1	2	1	2	2	3	0	4	2	2	3	1	3	2	2
23	Aust 3933	1	1	2	1	2	2	3	1	4	2	2	3	1	3	2	2
24	VLS288	1	1	2	1	2	2	3	0	4	2	3	3	1	3	2	2
25	CVJ13261	6	1	2	1	2	2	3	0	4	0	2	3	1	3	2	2
26	Rapp 40	5	1	2	1	2	2	3	0	4	2	2	3	2	3	2	2
27	Q85-3758	37	1	2	1	2	2	3	0	4	2	2	3	1	2	2	2
28	85-4219	5	1	2	1	2	2	3	0	4	2	2	3	1	2	2	5
29	Rapp 136	1	1	2	1	2	2	1	3	4	2	1	3	2	4	2	2
30	ATCC 33378	1	1	2	1	2	3	1	1	4	3	3	3	2	3	3	1
31	Jugo 36	3	1	1	1	2	3	3	0	4	1	2	1	2	3	3	4
32	Finland	1	1	2	1	2	3	2	0	4	0	2	3	2	3	0	4

[a] PE1, leucylalanine peptidase; ADK, adenylate kinase; GLD, glutamate dehydrogenase; MPI, mannose phosphate isomerase; IPO, indophenol oxidase; 6PG, 6-phosphogluconate dehydrogenase; MAE, malic enzyme; MDH, malate dehydrogenase; EST, esterase; LAP, leucine aminopeptidase; G3P, glyceraldehyde 3-phosphate dehydrogenase; G6P, glucose 6-phosphate dehydrogenase; PGM, phosphoglucomutase; GOT, glutamic oxaloacetic transaminase; PGI, phosphoglucose isomerase.

TABLE 3. Allele frequencies and genetic diversity at 15 enzyme loci in 32 ETs of *H. pleuropneumoniae*

Enzyme locus[a]	Frequency of the indicated allele:						h^b
	1	2	3	4	5	0	
PE1	1.000						0.000
ADK	0.094	0.906					0.175
GLD	1.000						0.000
MPI	0.063	0.938					0.121
IPO	0.094	0.250	0.656				0.514
6PG	0.688	0.063	0.250				0.476
MAE	0.406	0.094	0.031	0.094		0.375	0.698
MDH	0.094	0.125	0.063	0.719			0.470
EST	0.063	0.750	0.031			0.156	0.421
LAP	0.094	0.625	0.250	0.031			0.554
G3P	0.125	0.438	0.438				0.621
G6P	0.313	0.688					0.444
PGM	0.031	0.344	0.313	0.313			0.708
GOT	0.094	0.625	0.156	0.031	0.063	0.031	0.589
PGI	0.031	0.250	0.563	0.125	0.031		0.623

[a] See Table 2, footnote *a*, for definitions of abbreviations.
[b] $H = 0.428$.

and ET-27 accounted for 64 (47%) of the isolates examined (Table 2).

ET-13 and ET-29 were represented exclusively by isolates that failed to react with any of the nine specific antisera used for serotyping, and ET-14 was represented by five isolates of serotype 5 and one nonserotypable isolate. Among the 30 ETs of the serotypable isolates, *H* was 0.430, which is almost identical to the estimate for the entire sample.

Genetic relationships among multilocus genotypes. Estimates of the genetic relationships of the 32 ETs are summarized in the dendrogram in Fig. 1. The smallest observed genetic distance (0.07) between ETs corresponds to a single-locus difference. At a genetic distance of 0.30, there were 10 lineages or clusters of lineages, designated A through J. Clusters A through F each were composed of from two to seven ETs (mean, 4.7), and lineages G through J each consisted of a single ET.

Clusters A through E diverged from clusters F through J at a genetic distance of 0.52, which means that ETs of lineages belonging to the two primary branches of the dendrogram are only distantly related in overall chromosomal genotype, differing, on average, at 7 of the 15 loci assayed. ETs of clusters C and F contained 28 and 43%, respectively, of the total isolates. Except for ET-14, there was no case of sharing

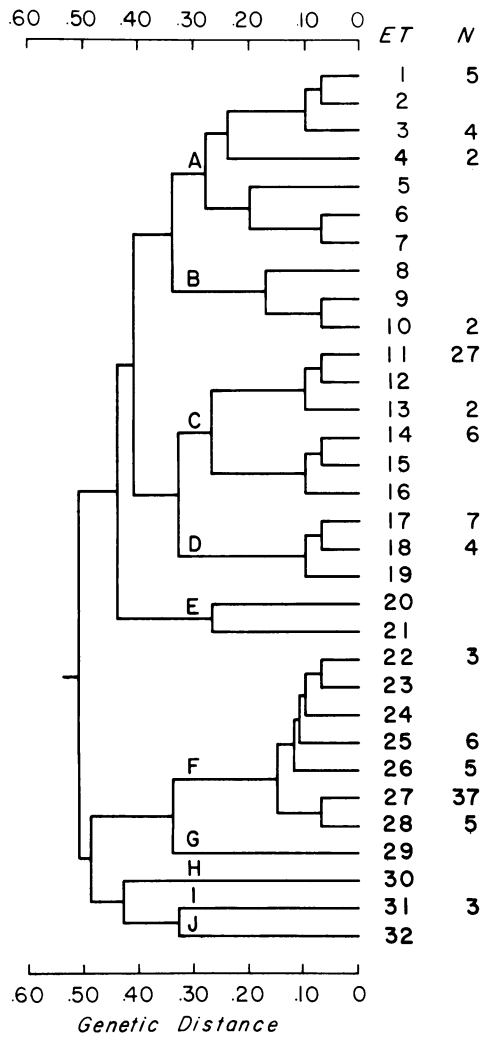

FIG. 1. Genetic relationships of electrophoretic types of *H. pleuropneumoniae* strains. The dendrogram was generated by the average-linkage method of clustering from a matrix of coefficients of pairwise genetic distances, based on 15 enzyme loci. ETs are numbered sequentially from top to bottom in the order of listing in Table 2. *N* is the number of isolates in each ET represented by multiple strains; all other ETs were represented by single isolates. Ten lineages or clusters of lineages, identified at a genetic distance of 0.30, are indicated by the letters A through J.

of multilocus genotype between serotypable and nonserotypable isolates.

Genetic variation in relation to serotype. Several cases of variation in serotype among isolates of the same ET or clusters of closely related ETs were detected. Five of the six clusters of ETs identified at a genetic distance of 0.30 (clusters A through F) were represented by isolates of two or three serotypes (mean, 2.2 serotypes per cluster). For example, the seven ETs of cluster A were represented by isolates of serotypes 3, 7, and 8; and the seven ETs of cluster F included isolates of serotypes 1 and 9.

There were four cases of variation in serotype among isolates of the same ET. As shown in Table 1, ET-11 included 2 isolates of serotype 3 and 25 isolates of serotype 5; ET-18 was represented by 3 isolates of serotype 2 and 1 isolate of serotype 3; ET-22 included 2 isolates of serotype 1 and 1 isolate of serotype 9; and ET-14 was represented by 5 isolates of serotype 5 and 1 nonserotypable isolate. However, 10 of the ETs were each associated with a single serotype, as, for example, ET-17, which was represented by 7 isolates of serotype 2 from four countries, and ET-27, which included 37 isolates of serotype 1 from three provinces in Canada.

Serotypes 4, 7, 8, and 9 each were represented by isolates of two ETs; serotype 2 and the nonserotypable class included isolates of three ETs; serotype 6 was represented by isolates of four ETs; and serotypes 1, 3, and 5 each included isolates of six ETs (Table 1).

Estimates of the extent of genetic variation among ETs of the same serotype are presented in Table 4. The *H* value among ETs of a given serotype was, on average, 0.239, which is 56% of that in the total sample of 32 ETs. But the magnitude of diversity was variable, ranging fom 0.089 for serotype 2, all isolates of which belonged to three closely related ETs of cluster D, to 0.467 for serotype 4, which was represented by the two very dissimilar ETs of lineages 30 and 31. There were, on average, 3.6 ETs per serotype, and all serotypes were represented by isolates of 2 or more ETs (range, 2 to 6).

There were 36 distinctive combinations of ET and serotype, of which ET-27–serotype 1 and ET-11–serotype 5 were the most common, being represented by 37 (27%) and 27 (20%), respectively, of the total isolates examined.

Genetic diversity within geographic regions. Cases of pleuropneumonia in a local region frequently are caused by strains of one or a few serotypes (29). One such area is the midwestern United States, where most of the disease has been attributed to isolates of serotype 5 (25). The 24 isolates of serotype 5 recovered from pigs in Iowa and Illinois in the period from 1980 to 1982 represented four ETs, all of which were in cluster C; 17 isolates were ET-11, five were ET-14, and one each was ET-15 and ET-16 (Table 1). Similarly, 80% of pleuropneumonia in Quebec, Canada, has been attributed to isolates of serotype 1 (11), and our analysis of 12 isolates from that province identified two ETs differing at only one locus; seven of the isolates were ET-27 and five were ET-28 (Table 1).

OMP analysis. The OMP patterns of Sarkosyl-insoluble preparations from the reference strains of serotypes 1 through 9 and of isolates from the midwestern United States have been reported previously (24). The OMP patterns of these isolates, as well as preparations from additional strains from Canada, Europe, Australia, and the United States, are presented in Table 1.

TABLE 4. Mean genetic diversity (*H*) at 15 enzyme loci among ETs of *H. pleuropneumoniae* classified by serotype

Serotype	No. of:		Diversity among ETs	
	Isolates	ETs	*H*	Variance
1	51	6	0.124	0.035
2	11	3	0.089	0.055
3	7	6	0.298	0.097
4	4	2	0.467	0.267
5	34	6	0.311	0.072
6	5	4	0.367	0.100
7	9	2	0.067	0.067
8	3	2	0.267	0.210
9	7	2	0.067	0.067
NT[a]	4	3	0.333	0.192
Total	135	32	0.428	

[a] NT, Nontypable.

As was the case with serotype 1 and 9 isolates from the United States, preparations from all Canadian, European, and Australian serotype 1 and 9 strains examined had the pattern 1 OMP profile, represented by the reference strain ATCC 27088. Similarly, serotype 2 and 6 strains all had the pattern 2 OMP profile, which is characteristic of the reference strains for these serotypes (ATCC 27089 and Femø, respectively).

Four serotype 3 strains were examined in addition to the serotype 3 reference strain ATCC 27090. Two strains, originating from the United States, had profiles indistinguishable from the serotype 5 pattern. The other two strains, orginating from Canada, had a unique pattern which could be distinguished from all other strains examined. Three serotype 4 isolates from Europe were examined; the patterns of OMP-enriched preparations from three isolates were distinguished from that of the reference strain ATCC 33378. The three Canadian serotype 5 strains examined had OMP patterns typical of the serotype 5 isolates from the United States.

All serotype 7 isolates from the United States and one isolate each from Switzerland and Canada exhibited OMP pattern 6, which is characteristic of the reference strain WF83. One Canadian serotype 7 isolate, however, had a unique OMP profile. Two Danish serotype 8 strains were examined; OMP profiles of these strains were different from that of the serotype 8 reference strain 405. Finally, preparations from three of four nontypable isolates from the United States had OMP profiles similar to the serotype 5 pattern; one nontypable isolate had a unique OMP pattern.

DISCUSSION

Clonal nature of *H. pleuropneumoniae*. The clone concept of bacterial population structure was first advanced for *Escherichia coli* to explain certain nonrandom associations of O:K:H serotypes and biotypes among enterotoxigenic strains and the rarity of these serobiotypes among nondisease isolates (22). Subsequently, the clonal structure of natural populations of *E. coli* was clearly demonstrated by studies of multilocus enzyme variation (20) and OMP profiles (1). More recently, electrophoretic enzyme variants have been extensively used as chromosomal markers to measure genetic relatedness among strains and to determine the genetic structure of populations of *Legionella* spp. (32), *Haemophilus influenzae* (16, 17), *Bordetella* spp. (18), *Neisseria meningitidis* (2, 3, 21), and other bacteria (30). Because evolutionary convergence to the same multilocus genotype is highly improbable (31), isolates of identical ET are considered members of the same clone or cell line. Isolates of a given ET with different serotypes are regarded as subclones.

Our findings confirm the hypothesis, originally suggested by observations of nonrandom associations between OMP and serotype (24), that the population structure of *H. pleuropneumoniae* is clonal; they also indicate that only a small fraction of all possible multilocus genotypes are represented in natural populations. Evidence that chromosomal recombination among cell lines is very infrequent is provided by the repeated recovery of isolates of the same ET-serotype-OMP combination from pigs in different parts of the world at different times. For example, ET-3–serotype 7–OMP-6 isolates have been collected in Switzerland, Quebec, and Illinois. Similarly, isolates of ET-17–serotype 2–OMP-2 have been recovered from pigs in Switzerland, Denmark, and Quebec; isolates of ET-25–serotype 9–OMP-1 have been recovered in the Federal Republic of Germany, The Netherlands, and Switzerland; and isolates of ET-22–serotype 1–OMP-1 have been collected in Argentina and the United States at an interval of more than 20 years. The fact that isolates of the same serotype and OMP profile frequently are of the same ET or closely related ETs is also consistent with a basically clonal population structure.

Genetic variation among isolates within serotypes. Most porcine pleuropneumonia in the midwestern United States is caused by strains of serotypes 1, 5, and 7 (25). Data from our ET and OMP analysis of additional strains isolated from a broader geographic region corroborate a previous OMP analysis indicating close relationship of isolates within the serotype 1, 5, and 7 populations (24). Our finding that strains of serotypes 1 and 9 represent a few closely related ETs is consistent with the previously demonstrated serologic cross-reactivity (19) and similarity of OMP profiles (24) of serotype 1 and 9 strains.

We detected five multilocus genotypes among 34 isolates of serotype 5, which is the predominant serotype of isolates recovered from pigs in Iowa and Illinois (25); but two of the five ETs, ET-11 and ET-14, represented 88% of isolates of serotype 5 from that geographic region. The reference strain for serotype 5, ATCC 33377 (K17), which was recovered from a lamb with arthritis in California in the early 1960s (6), was the only isolate of ET-12, a result that is in accord with an earlier observation that K17 has a unique OMP pattern (24).

Serotype 7 strains cause approximately 8% of pleuropneumonia in pigs in the midwestern United States. Rapp et al. (24) found no variation in OMP profile among nine field isolates (from Iowa and Illinois) and reference strain WF83 (from Ontario), but enzyme electrophoresis of eight field isolates and strain WF83 revealed two multilocus genotypes, ET-1 and ET-3. ET-1 was represented by four field isolates and the reference strain; and ET-3 was represented by an isolate from the United States, two isolates from Quebec, and an isolate from Switzerland.

Our analysis also revealed that strains of serotype 2 represent a closely related group of clones. In contrast, considerable heterogeneity was apparent in both OMP profile and multilocus enzyme genotype among strains of each of the three serotypes 3, 4, and 8. Although serotype 6 strains from Canada were indistinguishable from European strains on the basis of serology and OMP profile, the ETs of these two geographic populations were markedly dissimilar.

Genetic variation in relation to disease. Organisms of a wide variety of genotypes cause pleuropneumonia and other invasive diseases. Although we have no information about the clinical condition of the animals from which most of our isolates were obtained, we note that many investigators have concluded that isolates of serotype 1 are more virulent than those of other serotypes (11, 27). All isolates of serotype 1 were of six ETs in cluster F. Inasmuch as these ETs are very closely related to one another and are somewhat divergent from ETs in all other clusters, our findings can be interpreted as evidence either that the serotype 1 antigen itself is responsible for increased virulence or that there are other virulence factors in linkage disequilibrium (nonrandom association) with the multilocus genotypes marked by serotype 1.

Because there is experimental and statistical evidence that the electrophoretically demonstrable enzyme variation observed in bacteria is selectively neutral or nearly so (7), the existence of extensive genetic diversity in natural populations of *H. pleuropneumoniae* suggests that the species has maintained a large effective population size for a long period

of time (9). Hence, although *H. pleuropneumoniae* was first found to be associated with pleuropneumonia in pigs in 1957 (23), our findings effectively rule out the possibility that it evolved within historical times.

How can we interpret data which appear to be somewhat paradoxical—a very recent association with disease throughout the world with extensive genetic diversity? One parsimonious hypothesis is that recent modification of demographic or other features of the host population increased the fitness (and, hence, the abundance) of already existing ET-serotype clones. This hypothesis is fully consistent with and supportive of the idea, developed elsewhere (28, 29), that increases in the incidence of pleuropneumonia can be attributed to the overcrowding and stress of pigs that are associated with industrialization of pork production.

Variation in serotype in relation to population structure. Apart from the study of OMPs by Rapp et al. (24), previous research on *H. pleuropneumoniae* has been conducted within a framework provided by serotyping (5, 6, 12–15, 25, 26). Although heterogeneity of antigenic types has been detected by immunofluorescence, agglutination, and other serologic methods, only nine serotypes have been distinguished, and within a local region, one or a few serotypes predominate (29). Our data show that all serotypes occur in association with a variety of multilocus genotypes and that, in certain cases, isolates of highly divergent ETs have identical serotypes. The situation is similar to that in *E. coli* and *Legionella pneumophila*, in which genetic diversity among isolates of the same serologic class may be almost equivalent to that in the species as a whole and in which otherwise unrelated clones may be serologically identical (4, 30a, 32). Serotyping, as performed on *H. pleuropneumoniae* and most bacterial species, does not provide a basis for estimating overall genetic relatedness among isolates because serotypes reflect variation in a generally unknown but, in all probability, small number of genetic loci encoding enzymes involved in the synthesis of antigenic moieties, the chemical structures of which are, in general, poorly characterized. It is apparent that serotypes are not meaningful units of population structure. The most dramatic example of the failure of serotyping to reflect accurately the overall genetic relationships of strains is provided by serotypes 3 and 6, both of which are associated with ETs that are only distantly related in overall chromosomal genetic makeup (Fig. 1).

From evolutionary, epidemiological, and immunoprophylactic perspectives, it is important to understand the mechanisms responsible for enzyme diversity among isolates sharing the same antigenic determinants (antigenic convergence) and antigenic diversity among isolates of the same ET class (antigenic divergence). Hypotheses accounting for antigenic convergence and divergence in *E. coli* have been discussed by Caugant et al. (4). Evidence has been presented (5), and it is generally presumed, that the type-specific antigens of *H. pleuropneumoniae* are associated with capsular material; but because the exact chemical structure of the antigenic determinants is unknown, it is difficult to present a plausible hypothesis to account for our findings. We note that isolates of *Neisseria meningitidis* of the same ET can produce structurally different polysaccharide capsules (2), and that different antigenic structures of *E. coli* may be recognized by the same antiserum (10).

Multilocus enzyme electrophoresis has several advantages over serotyping as a method for identifying and classifying isolates of *H. pleuropneumoniae*, including greater resolving power and the fact that all isolates can be assigned to ETs. Moreover, enzyme electrophoresis yields estimates of the degree of genetic differentiation between isolates, whereas serologic data indicate only the existence of differences. We do not consider these advantages as arguments for abandoning serologic methods because of the likelihood that serotype antigens are adaptive characters that may be more informative of the clinical properties of strains than is the ET. However, results of this study demonstrate that multilocus enzyme electrophoresis has important contributions to make to the study of *H. pleuropneumoniae*, a pathogen of great veterinary and economic importance.

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