Analysis of Southern Ontario *Actinobacillus (Haemophilus) pleuropneumoniae* Isolates by Restriction Endonuclease Fingerprinting

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ABSTRACT

Isolates of *Actinobacillus (Haemophilus) pleuropneumoniae* were studied by restriction endonuclease fingerprinting (REF) analysis using the enzymes *Bam*HI and *Hind*III. Restriction fragments were resolved by polyacrylamide gel electrophoresis and visualized by silver staining. Except for serotypes 1 and 9, reference strains of *A. pleuropneumoniae* serotypes 1 to 10 had clearly distinguishable REF profiles. Analysis of REF profiles of southern Ontario field isolates revealed limited heterogeneity amongst isolates of serotype 1 or serotype 5. The REF profiles of the serotype 7 isolates studied showed greater variation. Heterogeneity could not be correlated with the presence of plasmids nor with antibiotic resistance. Limited heterogeneity could also be detected amongst REF profiles of *A. pleuropneumoniae* isolates recovered from a closed herd suggesting that there is a small amount of genetic variation within clonal populations.

RÉSUMÉ

Des isolats d'*Actinobacillus (Haemophilus) pleuropneumoniae* furent étudiés par analyse des empreintes générées par les endonucléases de restriction (EER) *BamHI* et *HindIII*. Les fragments obtenus furent séparés par électrophorèse sur gel de polyacrylamide et visualisés grâce à une coloration à l'argent. A l'exception des sérotypes 1 et 9, les souches de référence des sérotypes 1 à 10 de *A. pleuropneumoniae* avaient des profils de EER facilement distinguables. L'analyse des profils de EER d'isolats cliniques en provenance du sud de l'Ontario laissait voir une hétérogénéité limitée au sein des isolats du sérotype 1 ou du sérotype 5. Les profils de EER des isolats du sérotype 7 montraient une plus grande variation. L'hétérogénéité ne put être reliée à la présence de plasmides ou à la résistance aux antibiotiques. Une hétérogénéité limitée fut également détectée parmi les profils de EER d'isolats de *A. pleuropneumoniae* provenant d'un troupeau fermé suggérant ainsi qu'il existe un certain degré de variation génétique à l'intérieur de populations clonales.

INTRODUCTION

The gram-negative bacterium *Actinobacillus (Haemophilus) pleuropneumoniae* is an important pathogen of swine throughout the world (1,2). It causes acute fibrinous pneumonia and pleuritis which is often fatal. Pigs that survive *A. pleuropneumoniae* infection generally suffer from chronic lesions and become subclinical carriers of the pathogen (1).

To date, serotyping has been used extensively to monitor the spread of *A. pleuropneumoniae* infection. Various serotyping techniques including slide agglutination, indirect fluorescent antibody, indirect hemagglutination and gel immunodiffusion have been employed (3-8). Since only twelve serotypes have been described and of those, only a few are present in a given geographical area, serotyping cannot provide detailed epidemiological information (1,3,6). In addition, most isolates of serotypes 3, 6 and 8 and isolates of serotypes 1 and 9 cross-react and may only be differentiated after elaborate antigenic analysis (4-6,9).

Restriction endonuclease fingerprinting (REF) analysis can be used for comparison of closely related microorganisms (10-17). In this study, we have employed REF analysis to compare 53 independent *A. pleuropneumoniae* isolates from southern Ontario and seven isolates from an outbreak of acute pleuropneumonia in a closed herd in Illinois.

MATERIALS AND METHODS

BACTERIAL STRAINS AND GROWTH CONDITIONS

The sources and origins of serotype 1 to 8 reference strains of *Actinobacillus pleuropneumoniae* have been described previously (18). Strains CVJ 13261 (serotype 9) and B2209 (serotype 10), provided by Dr. R. Nielsen, Copenhagen, Denmark, were also included in this study (6). The bacteria were grown on chocolate agar supplemented with 0.01% (wt/vol) nicotinamide adenine dinucleotide (NAD) or in TYE + NAD medium (18).

Field isolates listed in Tables I, II and III were cultured from material provided by Dr. J.L. Lynch, Veterinary Laboratory Services, Guelph,

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TABLE I. Restriction endonuclease fingerprinting (REF), plasmid and antibiotic resistance profiles of Ontario field isolates of *Actinobacillus pleuropneumoniae* serotype 1

Isolate	Date	Source	REF profile *BamHI* #	REF profile *HindIII* #	Plasmid profile[a]	Antibiotic resistance[b]
G242	1/87	Brunner	1	1	-	-
G585	1/87	Dresden	1	1	-	-
G605	2/87	Fergus	1	1	-	-
G1267	2/87	St. Isadore	1	1	-	-
G917	2/87	Shedden	1	1	-	-
G1487	3/87	Wallenstein	1	1	-	-
G2891	5/84	Wallenstein	1	1	-	Ery
G4565	7/84	Drayton	1	1	-	-
G2826	4/84	Petrolia	1	1a	-	Ery
G2874	4/84	Ridgetown	1	1a	2.8	Ery, Tet
G4500	7/84	Milgrove	1	1b	-	-
G1012	2/87	Orville	1	1b	-	-
HP476	2/87	Komoko	1	1c	-	-
G2809	4/84	Cold Spring	1	1d	-	-
G1377	3/87	Listowel	1	2	-	-
G1177	2/87	Blenheim	1a	1b	-	-
HP586	2/87	Zurich	1a	1c	-	Neo, Tms
G922	2/87	St. Clements	1a	1a	-	-
G1454	3/87	Shedden	1b	1b	-	-
G1540	3/87	Kent Bridge	1c	1b	-	-
G137	1/87	Waterloo	1d	1b	-	-
G717	1/87	Thamesville	1e	1c	-	-
G1662	3/87	Alma	2	1	2.8, 2.2	Ery
G36	1/87	Kennilworth	3	1b	-	-

[a]Size in megadaltons

[b]Abbreviations: Amp, ampicillin; Car, carbenicillin; Ery, erythromycin; Gen, gentamicin, Kan, kanamycin; Neo, neomycin; Pen, penicillin; Tet, tetracycline, trimethoprim-sulpha

Ontario and Dr. S.E. Sanford, Veterinary Laboratory Services, Huron Park, Ontario. The isolates submitted to Veterinary Laboratory Services, Guelph, were collected from south central and southeastern Ontario; those submitted to Veterinary Laboratory Services, Huron Park, were collected from southwestern Ontario. The isolates submitted to the Guelph laboratory have an identification number prefixed by "G"; those submitted to the Huron Park Laboratory have an identification number prefixed with "HP". The isolates studied were obtained from different farms or in a few cases, from the same farm at different times. Thus we consider the isolates to be independent. From the records available, it was not possible to determine whether the isolates were obtained from chronically or acutely infected animals.

TABLE II. Restriction endonuclease fingerprinting (REF), plasmid and antibiotic resistance profiles of Ontario field isolates of *Actinobacillus pleuropneumoniae* serotype 5

Isolate	Date	Source	REF profile *BamHI* #	REF profile *HindIII* #	Plasmid profile	Antibiotic resistance[a]
G586	1/87	Shedden	1	1	-	Tet
HP337	1/87	Seaforth	1	1	-	Tet
G757	2/87	Stratford	1	1	-	-
G1419	2/87	Old Castle	1	1	-	-
G1703	3/87	Hillsburg	1	1	-	Tet
G1412	3/87	Stratford	1	1	-	Tet
G1510	3/87	Wingham	1	1	-	-
G1565	3/87	Strathroy	1	1	-	Ery, Tet
G1092	2/87	Thedford	1	1a	-	-
G2806	4/84	Rodney	1	1a	-	Tet
G1934	3/87	Old Castle	1	1b	-	-
G1728	3/87	Smithville	1	1b	-	Tet, Kan
G1696	3/87	New Dundee	1	1c	-	Tet
G1090	2/87	West Montrose	1	1d	-	Tet
G1988	4/87	St. Isadore	1	3	-	-
HP2766	10/86	Goderich	1a	1a	-	-

[a]Abbreviations: Amp, ampicillin; Car, carbenicillin; Ery, erythromycin; Gen, gentamicin, Kan, kanamycin; Neo, neomycin; Pen, penicillin; Tet, tetracycline, trimethoprim-sulpha

Seven serotype 1 isolates were received from Dr. A. Davis, Flanagan, Illinois. These isolates were recovered from Farm LVF, a 400 sow farrow to finish total confinement herd. Pleuropneumonia was first diagnosed in this herd in November 1984. For the five years prior to the outbreak, all additions to the herd had been made by embryo transfer, artificial insemination or by introduction of cesarean derived specific pathogen free animals. The source of the *A. pleuropneumoniae* infection was unknown.

Strain VCF7, isolated from the initial outbreak, was used to prepare an autologous bacterin. Animals were routinely vaccinated at six weeks of age and the herd was free of clinical cases of pleuropneumonia until the fall of 1986. Strains VCF1-6 were isolated from acutely infected animals (January 5, 1987 (VCF1); January 16, 1987 (VCF2); February 4, 1987 (VCF3); March 29, 1987 (VCF4) and March 30, 1987 (VCF5 and VCF6).

PREPARATION OF DNA

Deoxyribonucleic acid was prepared using a modification of the method of Marmur as described by Johnson (16,19). For the rapid preparation of small quantities of DNA, cells were washed from one 10 cm plate, suspended thoroughly in 1 mL of 0.15 M NaCl, and lysed by the addition of 50 μL of 10 mg/mL proteinase K and 200 μL of 10% (wt/vol) sodium dodecyl sulfate (SDS). Lysates were phenol extracted and precipitated as described (19) but the RNase step and further extractions were omitted. By the use of this technique, DNA could be conveniently prepared in one day. The DNA concentration was determined spectrophotometrically (19).

RESTRICTION DIGESTS

Deoxyribonucleic acid was incubated for 2 to 4 h in a two-tenfold excess of enzyme using medium or high salt buffer (20). Ten μg of RNase A were added to each 30 μg digest.

TABLE III. Restriction endonuclease fingerprinting (REF), plasmid and antibiotic resistance profiles of Ontario field isolates of *Actinobacillus pleuropneumoniae* serotype 7

Isolate	Date	Source	REF profile *BamHI* #	REF profile *HindIII* #	Plasmid profile[a]	Antibiotic resistance[b]
G1935	3/87	Muirkirk	1	1	3.6, 2.8	Amp, Car, Pen, Tet
G2023	3/87	Muirkirk	1	1	3.6, 2.8	-
G522	1/87	Tottenham	1	1a	3.6, 2.8	Amp, Car, Pen, Tet
G1421	3/87	Hastings	1	1b	3.6	Amp, Car, Pen, Tet, Ery
G1291	2/87	Orono	1	2	-	-
HP413	2/87	Brussels	1	3	3.6	Amp, Pen
G1410	3/87	Stratford	1a	4	3.6, 1.7	Amp, Car, Pen, Tet
HP733	3/87	Shakespeare	1b	5	-	-
G844	2/87	Stayner	2	6	2.2	Ery, Kan, Neo, Gen
G1104	2/87	Drayton	3	7	-	Ery, Tet
HP1584	6/86	Stratford	4	8	-	Amp, Car, Pen, Tet
G1735	3/87	Princeton	5	9	-	-
HP596	2/87	Mitchell	6	10	5.5	-

[a]Size in megadaltons

[b]Abbreviations: Amp, ampicillin; Car, carbenicillin; Ery, erythromycin; Gen, gentamicin, Kan, kanamycin; Neo, neomycin; Pen, penicillin; Tet, tetracycline, trimethoprim-sulpha

The restriction digests were checked for completeness by running 1 μg of digested DNA on an agarose gel using cl 857 Sam 7 Lambda DNA (Boehringer Mannheim Canada Ltd., Dorval Quebec) digested with *Eco*RI and *Hin*dIII as a marker. Restriction enzymes were purchased from Pharmacia (Canada) Ltd, Baie d'Urfe, Québec.

ANALYSIS OF RESTRICTION ENDONUCLEASE FRAGMENTS BY SDS POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The procedure used was based on a modification of the method of McClenaghan *et al* (15). Approximately 7.5 μg of digested DNA were loaded per lane and the fragments were resolved by electrophoresis at 70 V for 17 h in a 7.5% SDS-polyacrylamide gel. Deoxyribonucleic acid fragments were visualized using the procedure of Sammons *et al* (21). The details of these procedures have been described previously (16). Since careful examination of the REF patterns after different electrophoresis times or voltage gradients showed that the relative position of some bands changed, comparisons of REF profiles were always made within, rather than between, gels (22).

PLASMID PROFILE ANALYSIS

Plasmid DNA was isolated by the method of Portnoy and White as described by Crosa and Falkow (23). Plasmid DNA was run on 0.7% agarose gels and visualized by staining with ethidium bromide. The sizes of the plasmids were determined by comparison with plasmids from *Escherichia coli* strain, V517 (24). In isolates where no plasmid DNA could be detected, at least three independent extractions were performed. Plasmid DNA for REF analysis was purified by CsCl gradient centrifugation (20).

ANTIBIOTIC RESISTANCE DETERMINATIONS

Isolates were tested for antibiotic resistance by the disk diffusion method. Broth cultures were adjusted to a 0.5 MacFarland turbidity standard and swabbed on 150 mm plates of Mueller-Hinton agar supplemented with 5% (vol/vol) laked horse blood and 0.1 mL of 2.0% (wt/vol) NAD. Disks containing 10 μg ampicillin, 100 μg carbenicillin, 30 μg cephalothin, 15 μg erythromycin, 10 μg gentamicin, 30 μg kanamycin, 30 μg neomycin, 10 U penicillin G, 300 U polymyxin, 30 μg tetracycline and 1.25/23.75 μg trimethoprim/sulphamethoxazole were used. Isolates were determined to be sensitive or resistant based on the guidelines of Barry and Thornsberry (25).

RESULTS

ANALYSIS OF *A. PLEUROPNEUMONIAE* REFERENCE STRAINS

Deoxyribonucleic acid from representative strains of *A. pleuropneumoniae* was digested with a panel of restriction enzymes to determine which enzymes would give a convenient number of fragments for analysis. For most comparisons, we found that *Bam*HI digests, which produced approximately 25 fragments in the 400 to 1600 basepair (bp) range were most convenient for grouping related strains. *Hin*dIII, which generated approximately 40 fragments in the 400 to 1600 bp range, facilitated the comparison of closely related strains.

Deoxyribonucleic acid from the reference strains of *A. pleuropneumoniae* serotypes 1 to 10 were digested with *Bam*HI (Fig. 1A) and with *Hin*dIII (Fig. 1B). The REF patterns of the DNAs of most reference strains of *A. pleuropneumoniae*, including the antigenically related serotype 3, 6 and 8 strains (lanes 3, 6 and 8), were clearly different. The *Bam*HI profiles of serotypes 1 and 9 (Fig. 1A, lanes 1 and 9) and to a lesser extent, the *Hin*dIII patterns, shared many comigrating fragments (Fig. 1B, lanes 1 and 9).

ANALYSIS OF *A. PLEUROPNEUMONIAE* FIELD ISOLATES

The REF patterns of field isolates of a given serotype were all similar to one another and to their respective reference strain (Figs. 1-3). Twenty-four serotype 1, sixteen serotype 5, and thirteen serotype 7 isolates of *A. pleuropneumoniae* were first digested with *Bam*HI, grouped, and then further analyzed with *Hin*dIII. The source, date of isolation, and the *Bam*HI and *Hin*dIII profile number of each isolate are listed in Tables I, II and III. Isolates with identical REF profiles were arbitrarily assigned the same number. Using the criterion of Kristiansen (11), those which varied by no more than four fragments were designated variants and denoted by a lower case letter. For example, DNAs of serotype 7 isolates G1935 and G2023 designated *Hin*dIII #1, were identical (Fig. 2, lanes 1 and 2). The REF pattern of isolate G1421 (lane 3) had one additional fragment (*Hin*dIII #1b) and one fragment was absent from the pattern of isolate G522 (*Hin*dIII #1a). Although there were numerous common fragments, the REF patterns of isolates G1291, G1410, G844 and G1104 differed at

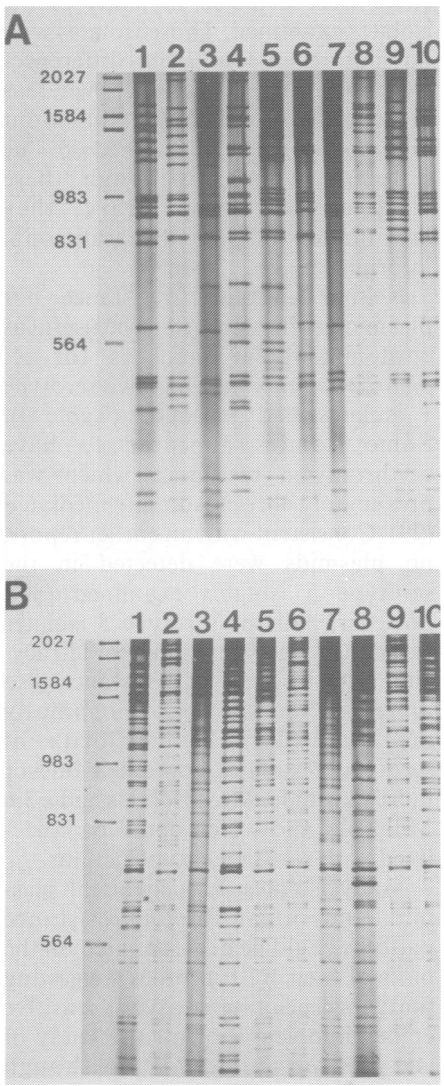

Fig. 1. Restriction endonuclease fingerprinting profiles of *A. pleuropneumoniae* serotypes 1 to 10 DNAs digested with *Bam*HI (Panel A) or *Hind*III (Panel B): Shope 4074 (lane 1), S1536 (lane 2), S1421 (lane 3), M62 (lane 4), K17 (lane 5), Femq/ (lane 6), WF83 (lane 7), 405 (lane 8), CVJ 13261 (lane 9), and D13039 (lane 10). The sources of strains and the details of the procedures are given in the Materials and Methods. The sizes of the *Hind*III/*Eco*RI digested Lambda fragments are indicated in basepairs.

four or more sites and were designated as a different REF profile (*Hind*III # 2, 4, 6, 7). If DNAs from isolates with identical *Bam*HI and *Hind*III profiles were digested with *Eco*RI, no further heterogeneity could be detected.

No association between a particular REF profile and a given geographical region (Tables I, II and III) was found. Isolates from the same town but from different producers, e.g. serotype 1 isolates G917, G1454; serotype 5 isolates G1419, G1934; and serotype 7 isolates G1410 and HP1584 were all different. Similarly, the same variant could sometimes be isolated in widely separated geographic locations. Isolates G585 and G1267 were isolated from Dresden and St. Isadore, Ontario, communities more than 720 km apart.

Strains of *A. pleuropneumoniae*, as defined by their *Bam*HI and *Hind*III REF profiles, were able to persist for several years (Tables I and II). For example, serotype 1 isolate G1487 isolated in 1987 had the same profiles as isolates G2891 and G4565 which were isolated in 1984. Similarly, a 1984 serotype 5 isolate, G2806 and a 1987 isolate, G1092 had identical profiles.

CONTRIBUTION OF PLASMIDS TO THE REF PROFILES

In order to determine if the differences detected were due to the presence of extrachromosomal DNA, all isolates were screened for plasmid DNA (Tables I, II and III). None of the sixteen serotype 5 isolates examined harbored plasmids. Plasmid DNA could be detected in only 2 of 24 serotype 1 isolates but 8 of 13 serotype 7 isolates contained plasmid DNA. When plasmid DNAs were digested with *Bam*HI or *Hind*III and run next to the corresponding digested total DNA preparations, no bands could be detected in the 400 to 1600 bp region. Isolates with different plasmid profiles had, with only one exception, small differences in the chromosomal DNA pattern which were detected in the 400 to 1600 bp range. Only serotype 1 isolates, G2826 and G2874 had identical REF patterns but different plasmid profiles.

PLASMIDS AND ANTIBIOTIC RESISTANCE AS EPIDEMIOLOGICAL MARKERS

The presence of plasmids and/or antibiotic resistance has been used to differentiate different strains of the same organism (11,13). Only five of 24 serotype 1 isolates demonstrated resistance to the antibiotics tested (Table I). Four isolates were resistant to erythromycin but only two of these isolates carried plasmids. Nine of 16 serotype 5 isolates demonstrated antibiotic resistance (Table II). All

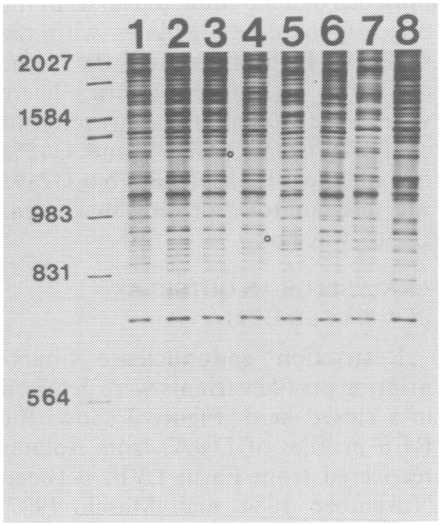

Fig. 2. Restriction endonuclease fingerprinting profiles of DNAs of *A. pleuropneumoniae* serotype 7 field strains digested with *Hind*III: G2023 (lane 1), G1935 (lane 2), G1421 (lane 3), G522 (lane 4), G1291 (lane 5), G1410 (lane 6), G844 (lane 7) and G1104 (lane 8).

were resistant to tetracycline. Eight of 13 serotype 7 isolates were resistant to antibiotics (Table III). Most isolates were resistant to ampicillin, carbenicillin and penicillin. In most cases, this resistance could be correlated with the

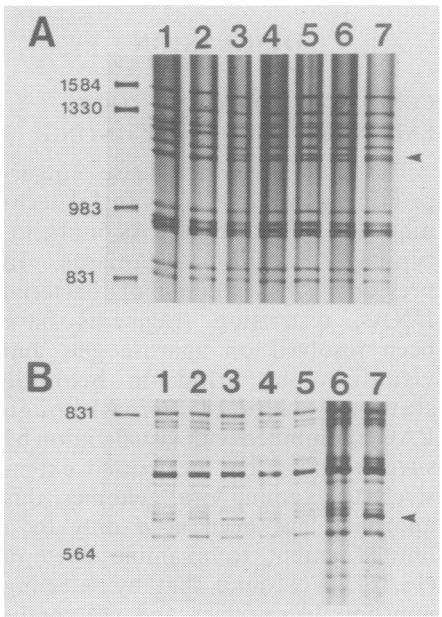

Fig. 3. Restriction endonuclease fingerprinting profiles of DNAs of isolates from Farm LVF digested with *Bam*HI (A) or with *Hind*III (B): VCF1 (lane 1), VCF2 (lane 2), VCF3 (lane 3), VCF4 (lane 4), VCF5 (lane 5), VCF6 (lane 6) and VCF7 (lane 7).

presence of a 3.6 Mdal plasmid. In the majority of cases, isolates with the same REF profiles had the same antibiotic resistance pattern. There were some exceptions, however. For example, isolates G2891 and G4565 had identical REF profiles but G2891 was resistant to erythromycin whereas G4565 was not.

ANALYSIS OF AN OUTBREAK IN A CLOSED HERD

Restriction endonuclease fingerprinting profile variants were detected in a closed herd. Figure 3 shows the REF profiles of DNAs from isolates recovered from Farm LVF, between November 1984 and March 1987. *Actinobacillus pleuropneumoniae* was first detected in the herd in 1984. Strain VCF7 was isolated at that time and was used to prepare an autologous bacterin. Despite routine vaccination, the herd experienced a severe outbreak of pleuropneumonia in the fall of 1986. Isolates VCF1-6 were recovered from animals in the nursery and grower areas during the period of January to March 1987. The REF profiles of all the DNAs were very similar but differences in the *Bam*HI profile were detected in the 1100 bp region and in the *Hin*dIII profile in the 600 bp region (Fig. 3).

DISCUSSION

CONDITIONS FOR RESTRICTION ENDONUCLEASE FINGERPRINTING

Restriction endonuclease fingerprinting analysis is a powerful technique for comparing DNAs of phenotypically similar organisms. In previous REF studies of bacterial DNAs, restriction fragments have been resolved on agarose gels and visualized by ethidium bromide staining (11,12,14,17). Although PAGE combined with visualization by silver staining has been used extensively to examine viral genomes, this approach has been used only to a limited extent to examine bacteria (13,16). We found that by selecting appropriate enzymes and gel conditions, PAGE could be used to analyze *A. pleuropneumoniae* DNAs.

Previous workers have shown that six basepair cutters produce a reasonable number of fragments for REF analysis (10,15). When *A. pleuropneumoniae* DNA, which has a G + C content of 42%, was digested with a restriction enzyme such as *Bam*HI that has a GC-rich recognition site, a simple pattern of approximately 25 fragments in the 400 to 1600 bp range was produced. For comparison of different serotypes or related species, the relatively small number of fragments facilitated analysis (Fig. 1A). For a more detailed examination of variants within a serotype, digestion with *Hin*dIII was used to provide further information (Fig. 1B, Fig. 2).

ANALYSIS OF REFERENCE STRAINS

Various serotyping techniques are currently used to analyze *A. pleuropneumoniae* isolates (3-7). The value of serotyping has been limited, however, because typically there is only one (or a few) serotype(s) in a given area and because some serotypes cross-react (3-6, 9). The reference strains of most serotypes had clearly distinguishable REF profiles (Fig. 1A and 1B). The DNAs of serotypes 1 and 9 had very similar *Bam*HI and *Hin*dIII patterns. Serologically, these two serotypes have been shown to have identical outer membrane protein profiles (26) and closely related electropherotypes (27). The DNA-DNA homology between the reference strains of serotype 1 and 9 is 90%, consistent with the close relationships but not excluding different serotypic affiliation (28). It may be possible to use REF analysis to discriminate between serotype 1 and 9 strains but a large number of serotype 9 isolates would have to be compared and analysis with additional enzymes might be required.

HETEROGENEITY OF FIELD ISOLATES

Based on outer membrane protein analysis of 95 isolates, Rapp *et al* concluded that populations of serotypes 1, 5 and 7 were clonal (26). Using multilocus enzyme electrophoresis, Musser *et al* (27) reported that natural populations of *A. pleuropneumoniae* are genetically diverse but those of serotype 1, form a distinct cluster of closely related clones. Our data are consistent with the notion that *A. pleuropneumoniae* isolates of the same serotype are genetically very similar (Tables I, II and III). Using REF analysis, however, subtle differences were detected in many of the isolates examined. Those isolates with no more than four band differences were considered variants whereas those isolates with more than four differences were considered to be different strains. Although these designations are arbitrary, they are made to provide additional information.

Neither antibiotic resistance nor plasmid profiles proved to be useful as epidemiological markers for *A. pleuropneumoniae*. Only two serotype 1 isolates had plasmids (Table I). Some isolates appeared to have erythromycin resistance which was presumably chromosomally mediated (29). Consistent with an earlier report, no plasmids were detected in the serotype 5 isolates examined (30), however, half the serotype 5 isolates examined were resistant to tetracycline (Table II). This resistance also appeared to be chromosomally mediated (29). The majority of serotype 7 isolates contained one or more plasmids. Most isolates had a 3.6 Mdal plasmid which could be correlated with resistance to penicillins.

Many isolates with identical plasmid and/or antibiotic resistance patterns could be differentiated on the basis of their REF profiles suggesting that fingerprinting is a very sensitive epidemiological tool for the study of *A. pleuropneumoniae*. Although restriction endonuclease fingerprinting was generally more sensitive than other phenotypic methods, the examination of plasmid and antibiotic resistance data suggested that REF analysis does have some limitations. For example, serotype 1 isolates G2874 and G2826 had the same REF profile but only G2874 contained a 2.8 Mdal plasmid. Similarly, only four of eight serotype 5 isolates with *Bam*HI pattern #1 and *Hin*dIII pattern #1 were tetracycline resistant. Three isolates with these patterns were sensitive to antibiotics and one was resistant to both erythromycin and tetracycline.

ANALYSIS OF REF PATTERNS OF ISOLATES FROM A CLOSED HERD

The REF patterns of seven independent isolates of *A. pleuropneumoniae* from a closed herd were similar but not identical (Figs. 3A and 3B).

We cannot rule out the possibility that several variants of *A. pleuropneumoniae* were introduced when the herd first became infected or that there were multiple introductions but these possibilities are unlikely. Most probably, variants of *A. pleuropneumoniae* arose with time and persisted. From a practical point of view, the REF analysis suggested that the outbreak of pleuropneumonia was not likely due to the introduction of several new isolates of *A. pleuropneumoniae* or evolution of six different, more virulent mutants of the original strain, against which the bacterin could not afford protection. If six new strains were introduced then the REF patterns would likely show more variation. If a more virulent mutant appeared then, most likely, all the new isolates would have an identical pattern. Rather, these data suggested that there was a problem with the vaccination protocol or other management factors. Analysis of veterinary records revealed that just prior to the outbreak, the farm had begun to vaccinate piglets at four rather than at six weeks of age. The outbreak probably resulted because piglets of chronically infected sows were vaccinated before the level of maternal antibodies in the piglets had dropped. No new outbreaks of pleuropneumonia have occurred since the vaccination protocol has been changed although it is not possible to rule out a change in the status of herd immunity as the result of the effects of the active infection.

Restriction endonuclease fingerprinting analysis is a powerful technique for studying the genetic diversity of bacterial populations. It is especially useful when looking at species such as *A. pleuropneumoniae* where serological techniques can provide only limited information. Although fingerprinting is more complex and time-consuming than many serological techniques, recent advances in automation of DNA extraction and electrophoresis might make it possible to employ restriction enzyme fingerprinting as a routine epidemiological tool. The nature of the REF data also makes the method readily amenable to automated data entry and analysis.

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ERRATUM

Caudal Epidural Analgesia in Cattle Using Xylazine

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The footnotes on this article should have read as follows: Department of Large Animal Clinical Sciences, Michigan State University, East Lansing, Michigan 48824.

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We apologize for this error and trust it has not caused any inconvenience.