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Use of an rRNA Probe and Restriction Endonuclease Analysis To Fingerprint *Pasteurella multocida* Isolated from Turkeys and Wildlife

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Twenty-five isolates of the bacterium *Pasteurella multocida* were characterized (fingerprinted) phenotypically and genotypically in order to compare the abilities of various techniques to differentiate strains for epidemiologic studies of fowl cholera. Isolates were obtained over a 16-month period from turkeys dying from fowl cholera (six outbreak flocks) and from wildlife captured on premises with a history of the disease. The characteristics compared included (i) serotype, (ii) subspecies, (iii) antibiogram, (iv) presence of plasmid DNA, (v) restriction endonuclease analysis patterns of whole-cell DNA, and (vi) ribotype. Ribotyping, a method of highlighting DNA restriction site heterogeneity by using an rRNA probe, worked well for differentiating the strains of *P. multocida* when the majority of the other techniques could not. Ribotyping results correlated directly with and confirmed results obtained from restriction endonuclease analysis. Ribotyping demonstrated the presence of up to three strains of *P. multocida* in one outbreak flock, recurrence of a single strain in five of the flocks over an 11-month period, and the presence of common strains in turkeys and wildlife on the premises.

Fowl cholera, a major bacterial disease of domestic and wild fowl, has been recognized and studied since the late 1700s (16). Many unanswered questions remain regarding the epidemiology of fowl cholera, making disease control difficult. The California Turkey Project (CTP) has been studying the epidemiology of fowl cholera in meat and breeder turkeys in California since 1985. A major goal of this research has been to elucidate transmission patterns and the reservoir(s) of the causative gram-negative bacterium *Pasteurella multocida*. In order to investigate the dynamics of the microorganism within and between turkey flocks, isolates of *P. multocida* from turkeys dying from fowl cholera, live turkeys, and wildlife captured on turkey premises have been collected by the CTP during the past 3 years for characterization and comparison in the laboratory. The correlation of laboratory strain fingerprint data with epidemiologic data collected from the flocks and premises from which isolates were obtained has provided the opportunity for more in-depth studies of the transmission and reservoirs than have been previously possible.

At present, the most commonly used method for the differentiation of avian strains of *P. multocida* is somatic antigen serotyping by the method of Heddleston et al. (6). For the past 3 years in California, the majority of isolates recovered from turkeys dying from fowl cholera have been of serotype 3,4 (unpublished data). However, within a given serotype designation, e.g., 3,4, it appears that substantial additional phenotypic variation may occur in avian isolates. For example, the CU and M9 attenuated live vaccine strains are serotype 3,4, but they were developed to be avirulent for turkeys. Thus, a major difference exists in an important phenotypic trait (virulence) in these strains of the same somatic serotype, implying that all strains with similar serotype designations are not necessarily identical. Capsular serotyping has also been used to group *P. multocida* (4); however, nearly all isolates collected by the CTP from turkeys have been the same capsular type (type A; unpublished data).

In epidemiologic studies involving *Pasteurella* species and other bacteria, the fingerprinting techniques (other than serotyping) used for the differentiation of strains of the same species have included bacteriophage typing, determination of antibiogram, biotyping, plasmid DNA analysis, and whole-cell protein analysis (1, 5, 8, 10, 12, 21, 22, 25). With the exception of phage typing, the CTP has attempted to fingerprint turkey isolates of *P. multocida* by all of these methods. In the overall population of strains, a large degree of variation in biotype has not been detected, and the majority of strains do not contain plasmids (unpublished data). Antibiograms have been very similar between isolates, with resistance to any of eight antimicrobial agents tested being uncommon. To date, whole-cell protein analysis has proved difficult to interpret, with complex yet similar protein separation profiles observed on polyacrylamide gels.

All of the techniques listed above have limitations because typing or grouping relies on phenotypic traits which are inconsistently expressed, and as witnessed by many of our results obtained with *P. multocida*, the sensitivity level is inadequate to differentiate strains accurately. An additional fingerprinting technique that has proved its value for epidemiologic studies is restriction endonuclease analysis (REA) of whole-cell (primarily chromosomal) DNA. This technique has been shown to be highly sensitive and reproducible in a variety of bacteria (2, 15, 24). A major advantage of REA is the fact that one is examining the genotype of the organism, which is more stable than the phenotype. A disadvantage of REA is that, depending on the restriction enzyme used, it may be difficult to interpret the results observed following electrophoresis, when a multitude of chromosomal bands (or fragments) are present and subtle differences may be missed.

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To circumvent this problem, investigators have used nucleic acid probes, both DNA and RNA, to highlight DNA restriction site heterogeneity (7, 20, 23). Stull et al. (20) and Irino et al. (7) have demonstrated the usefulness of rRNA probes as a widely applicable system for the differentiation of strains of different gram-negative bacteria. Homologous rRNA probes, as well as an *Escherichia coli* rRNA probe, have been successfully used to type strains of *E. coli*, *Pseudomonas cepacia*, and *Haemophilus influenzae* (20). This procedure, which compares highly conserved rRNA genes and their adjacent sequences, has been denoted ribotyping by some researchers.

In this study we used REA and ribotyping (using an *E. coli* rRNA probe) to help differentiate 25 isolates of *Pasteurella multocida*, all of which were recovered from the same meat turkey premises in central California between August 1985 and December 1986. Our goal was to explore the use of these genomic fingerprinting techniques for studying the epidemiology of pasteurellosis, in this case on a single turkey farm with a history of fowl cholera, and to compare them with several other phenotypic characteristics that were studied.

MATERIALS AND METHODS

Premises and flock management description. The premises discussed in this study, designated by code 0727, were a commercial meat turkey-raising facility located at an elevation of 500 ft (ca. 152 m) in the foothills adjacent to the Central Valley of California. During this study period, the CTP was monitoring (for fowl cholera) approximately 95% of all meat turkey premises and their respective flocks in the state, nearly all of which were located in the Central Valley region.

Premises 0727 consisted of four turkey poult brooder houses (in which poults were kept after arriving from the hatchery until approximately 7 weeks of age) and 13 adult turkey grow-out houses (to which turkeys were transferred after the brooder house). Grow-out houses contained, on average, 6,350 turkeys per house at any given time. All grow-out buildings were of the confinement type of configuration, in which an attempt was made to limit turkey access to the outside environment while they matured to market age.

A flock was defined as a group made up of turkeys that were placed in brooder houses on the premises within 3 to 4 days of each other and then marketed as a company unit 4 to 5 months later. All flocks placed on the premises were mixed-sex flocks (males and females). Female turkeys were sent to market at approximately 16 weeks of age, and males were sent to market at about 20 weeks of age. At any given time, two flocks were present on the premises, one in brooder houses and one in grow-out houses. The average flock size was 82,500 birds.

Seven flocks were placed and raised on the premises during the study period, six of which suffered outbreaks of fowl cholera; all of these outbreaks occurred in the grow-out houses. An outbreak was identified by three criteria: (i) above-normal weekly mortality rates for a flock (the normal rate is 0.25 to 0.35%), (ii) clinical signs indicative of fowl cholera (as determined by company flock managers), and (iii) isolation of *P. multocida* from dead turkeys submitted to the California Veterinary Diagnostic Laboratory System.

Trapping of wild animals for subsequent bacterial culturing. Wild mammals and birds were captured on premises 0727 to determine their role as reservoirs for *P. multocida*, as described previously (18). Traps and nets were set adjacent to grow-out houses that contained turkeys dying from fowl cholera. A total of 30 mammals and 30 birds were captured for sampling on two separate occasions, once in November 1985 and again in December 1986.

Bacterial isolates. Fourteen isolates of *P. multocida* from turkeys dying from fowl cholera were isolated from carcasses submitted to the California Veterinary Diagnostic Laboratory System. Eleven isolates from wildlife were obtained by culturing the oropharynx of trapped wild mammals and birds as described previously (17). Isolate designations and dates of their isolation are indicated in Table 1.

Determination of serotype. The system of Heddleston et al. (6) for determining the somatic serotype was used.

Determination of subspecies. Broths (Purple Base; Difco Laboratories, Detroit, Mich.) containing trehalose, maltose, xylose, arabinose, mannitol, sorbitol, and dulcitol were inoculated with each isolate and incubated at 37°C to determine fermentation results and, hence, the subspecies (14).

Determination of antimicrobial susceptibility. The MICs of eight antimicrobial agents were measured by an agar dilution method (26). The antimicrobial agents tested were as follows (ranges of doubling dilutions in micrograms per milliliter are given in parentheses): (i) chloramphenicol (0.25 to 8.0), (ii) gentamicin (0.25 to 32), (iii) kanamycin (1.0 to 128), (iv) penicillin G (0.25 to 2.0), (v) streptomycin (1.0 to 128), (vi) tetracycline (0.25 to 128), (vii) sulfonamides (1.0 to 256), and (viii) trimethoprim-sulfonamide (ratio of 1:20; 0.25 to 1.0, trimethoprim fraction).

Plasmid DNA analysis. A colony lysis technique was used to screen isolates for plasmid DNA by agarose gel electrophoresis as described previously (9, 13).

Preparation of whole-cell DNA. Isolates were grown in 100 ml of brain heart infusion broth overnight at 37°C with shaking. Cultures were harvested by centrifugation (15,000 × *g*, 10 min, 4°C), washed once in 50 mM Tris–5 mM EDTA–50 mM NaCl (TES), and suspended in 1.6 ml of 25% sucrose in 50 mM Tris–1 mM EDTA (pH 8.0). The suspension was transferred to ultracentrifuge tubes, lysozyme was added (0.4 ml of approximately 5 mg/ml), and the tubes were placed on ice for 15 min. Ten microliters of proteinase K (20 mg/ml) was then added, and the tubes were again placed on ice for 15 min. After treatment with the proteinase K, 0.4 ml of 0.5 M EDTA was combined with 0.2 ml of 10% Sarkosyl (CIBA-GEIGY Corp., Summit, N.J.) and was added to the tubes. The tubes were then covered and incubated at 65°C overnight. A total of 35 ml of cesium chloride solution (75.65 g of CsCl in 60 ml of TES buffer containing 50 μg of phenylmethylsulfonyl fluoride per ml) was added, and the lysate was centrifuged for 42 h at 31,000 rpm in a rotor (Ti 50.2; Beckman Instruments, Inc., Fullerton, Calif.). The DNA fraction was collected from the side of the tube with a 16-gauge needle and dialyzed against 50 mM Tris–5 mM EDTA buffer for 48 h. The final DNA concentration was determined spectrophotometrically.

Restriction endonuclease digestion of DNA. Purified bacterial DNA (2.0 to 2.5 μg) was mixed with 1.0 μl of restriction enzyme in a 20-μl reaction mixture containing the appropriate buffer, as recommended by the manufacturer. The restriction enzymes used were *Sma*I and *Sal*I (double digest), *Eco*RI, and *Pst*I. Enzymes were purchased from Bethesda Research Laboratories, Inc. (Gaithersburg, Md.). Following incubation for 1 h at the appropriate temperature for digestion, 5 μl of endonuclease stop mix tracking dye (50 mM EDTA, 0.07% bromphenol blue, 7% sodium dodecyl sulfate, and 33% glycerol in distilled water) was added, and the samples were electrophoresed at 35 V for 16 h in a horizontal

TABLE 1. *P. multocida* isolates recovered from turkeys, wild mammals, and wild birds on premises 0727 from August 1985 to December 1986

Outbreak no.	Isolate[a]	Date isolated (mo/day/yr)	Outbreak flock no.	Serotype	REA type	Ribotype	Plasmid (size [MDa[b]])	*P. multocida* subsp.	MIC (μg/ml)			Source
									Streptomycin	Tetracycline	Sulfonamide	
1	FC 1	08/06/85	3	3,4	1	a	+ (2)	*multocida*	8	<0.25	<1	Turkey
1	FC 3	08/12/85	3	3,4	1	a	+ (2)	*multocida*	8	<0.25	8	Turkey
2	FC 26	11/06/85	24	3	1	a	+ (2)	*multocida*	8	<0.25	4	Turkey
2	FC 25	11/06/85	24	3,4	2	b	−	*multocida*	8	1	16	Turkey
3	FC 41	01/15/86	34	10	2	b	−	*multocida*	8	1	16	Turkey
3	FC 64	02/27/86	34	10	2	b	−	*multocida*	8	1	16	Turkey
4	FC 103	07/29/86	53	3,4	2	b	−	*multocida*	4	0.50	16	Turkey
5	FC 125	09/24/86	62	3,4	3	c	−	*multocida*	16	<0.25	16	Turkey
5	FC 124	09/29/86	62	3,4	2	b	−	*multocida*	8	1	8	Turkey
5	FC 129	10/03/86	62	3,4	2	b	−	*multocida*	4	0.50	8	Turkey
5	FC 127A	10/15/86	62	3,4	3	c	−	*septica*	16	<0.25	8	Turkey
5	FC 127B	10/15/86	62	1,4	4	d	−	*multocida*	8	<0.25	8	Turkey
6	FC 139A	10/23/86	73	3,4	2	b	−	*multocida*	8	0.50	4	Turkey
6	FC 139B	10/23/86	73	3,4	2	b	−	*multocida*	8	0.50	16	Turkey
	1–36	11/24/85		3,4	5	e	−	*multocida*	8	<0.25	4	Opossum (*Didelphia marsupialis*)
	38–5	12/15/86		3,4	3	c	−	*septica*	8	<0.25	32	Feral cat (*Felis catus*)
	38–6	12/15/86		3,4	3	c	−	*septica*	16	<0.25	16	Deer mouse (*Peromyscus maniculatus*)
	38–7	12/15/86		3,4	3	c	−	*septica*	16	<0.25	16	House mouse (*Mus musculis*)
	38–8	12/15/86		3,4	2	b	−	*multocida*	8	0.50	16	Western meadowlark (*Sturnella neglecta*)
	38–10	12/15/86		3,4	2	b	−	*multocida*	8	1	16	House finch (*Carpodacus mexicanus*)
	38–11	12/15/86		3,4	2	b	−	*multocida*	8	1	16	White-crowned sparrow (*Zonotrichia leucophrys*)
	38 –15	12/15/86		3,4	3	c	−	*multocida*	16	<0.25	16	Western meadowlark (*Sturnella neglecta*)
	38–22	12/16/86		3	6	f	−	*multocida*	8	<0.25	16	Feral cat (*Felis catus*)
	38–30	12/16/86		3,4	7	g	−	*multocida*	8	<0.25	16	Killdeer (*Charadrius vociferus*)
	38–51	12/18/86		3,4	2	b	−	*multocida*	8	<0.25	32	Deer mouse (*Peromyscus maniculatus*)

[a] FC, Fowl cholera.
[b] MDa, Megadaltons.

slab gel electrophoresis unit (15 by 20 cm; model HE-99; Hoefer Scientific Instruments, San Francisco, Calif.). The gel consisted of 0.7% agarose (Sigma Chemical Co., St. Louis, Mo.) in Tris borate buffer (pH 8.0; 89 mM Tris, 2.5 mM disodium EDTA, and 8.9 mM boric acid). Following electrophoresis, the gels were stained for 15 to 20 min with ethidium bromide (1.0 μg/ml), DNA was visualized with shortwave UV light, and the gels were photographed.

Preparation of Southern blots. The *P. multocida* DNA used in the Southern blot experiments was digested with *Pst*I and *Eco*RI. Digested and electrophoresed DNA restriction fragments were transferred to a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) by the method of Southern (19). On completion of the DNA transfer, blots were dried at 80°C in a vacuum oven and stored at room temperature until use.

Ribotyping. The ribotyping method used was that of Stull et al. (20). *E. coli* rRNA was purchased from Sigma and was suspended in sterile distilled water to a concentration of 0.25 μg/μl. The rRNA was dephosphorylated with calf intestinal alkaline phosphatase and labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (11). The specific activity of the probe was approximately 10^7 cpm/μg of RNA. A molecular weight standard probe was prepared by digesting lambda phage DNA with *Hae*III, dephosphorylating the DNA by using calf intestinal alkaline phosphatase, and labeling the DNA with [γ-^{32}P]ATP as described above for the rRNA probe.

Blots of restriction endonuclease-digested *Pasteurella* DNA were prehybridized, and then hybridization with ^{32}P-labeled RNA (7.0×10^5 cpm/ml of hybridization solution) and lambda phage DNA was conducted by the method of Stull et al. (20). Briefly, the prehybridization solution consisted of 5× SSPE (0.01 M phosphate buffer containing 0.15 M NaCl and 0.001 M EDTA), 0.1% sodium dodecyl sulfate, 5× Denhardt solution (1% Ficoll [Pharmacia Fine Chemicals, Piscataway, N.J.], 1% polyvinylpyrrolidone, 1% bovine serum albumin in distilled H_2O), and 1.0 mg of salmon sperm DNA per 10 ml of solution. Prehybridization was done for 1 h at 60°C, the solution was discarded, and then the hybridization solution (prehybridization solution plus rRNA and lambda DNA probes) was added to the blot. Hybridization was allowed to proceed overnight from 60 to 40°C. After hybridization, blots were washed once in 0.1× SSPE–0.1% sodium dodecyl sulfate at room temperature and three times at 55°C for 20 min each time. The extent of hybridization of the probes with blotted DNA was analyzed by autoradiography, by using a single intensifying screen, X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.), and incubation at −70°C for 2 days.

RESULTS

Serotypes. Serotypes for all 25 isolates are listed in Table 1. A total of 10 of 14 turkey isolates and 10 of 11 wild animal isolates were serotype 3,4. Different serotypes were isolated during the same outbreak (in certain cases, on the same day) on three occasions, i.e., outbreak 2 (isolates with serotypes 3 and 3,4), outbreak 5 (five serotype 3,4 isolates and one serotype 1,4 isolate), and outbreak 6 (isolates with serotypes 3,4 and 3,4,7).

Subspecies. The different subspecies designations of the isolates are listed in Table 1. A total of 13 of 14 turkey isolates and 8 of 11 wildlife isolates were *P. multocida* subsp. *multocida*. The remainder were *P. multocida* subsp. *septica*.

Antibiograms. Little or no variation was observed in isolate susceptibility to chloramphenicol, gentamicin, kanamycin, penicillin G, or trimethoprim-sulfonamide. All isolates were susceptible to chloramphenicol at levels of ≤0.5 μg/ml (24 of 25 were susceptible to <0.25 μg/ml), susceptible to 1 to 2 μg of gentamicin per ml, susceptible to 4 to 8 μg of kanamycin per ml (one strain, 38-05, was susceptible to 16 μg/ml), susceptible to <0.25 μg of penicillin G per ml, and susceptible to trimethoprim-sulfonamide at <0.25 μg of the trimethoprim fraction per ml. Susceptibilities to the remaining three antimicrobial agents were more variable and are listed in Table 1.

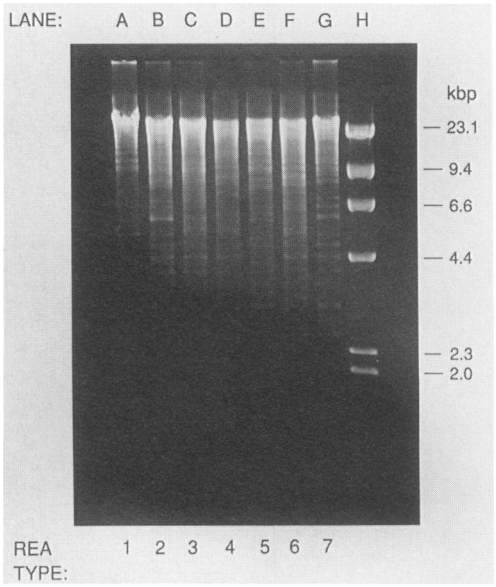

FIG. 1. Agarose gel electrophoresis of whole-cell DNA obtained from *P. multocida* isolated from turkeys with fowl cholera (FC) and from wild mammals and birds on premises 0727 after digestion with *Sma*I and *Sal*I. Lanes contain DNAs from isolates FC 1 (lane A), FC 139B (lane B), FC 125 (lane C), FC 127B (lane D), 1-36 (lane E), 38-22 (lane F), and 38-30 (lane G). Lane H contains DNA isolated from lambda phage and digested with *Hin*dIII, with numbers on the right representing the number of kilobase pairs (kbp) of the respective fragments.

Plasmid analysis. Three isolates were found to contain plasmid DNA, all of which were isolated from dead turkeys (Table 1). The plasmids in these isolates were of the same size (approximately 2 megadaltons) and were cryptic.

REA. Examples of results of *Sma*I-*Sal*I double digests of turkey and wildlife isolate whole-cell DNA are shown in Fig. 1. A total of seven different REA patterns were observed among the 25 isolates studied by this method, and each of these patterns is presented in Fig. 1. Complete REA results for all 25 isolates are indicated in Table 1. Four different patterns, or REA types, numbered 1 to 4, were observed with the turkey isolates. REA type 2 was the most common in turkeys (8 of 14 isolates). Wildlife harbored five different types (types 2, 3, 5, 6, and 7), two of which (types 2 and 3) were also recovered from turkeys. Types 2 and 3 were the most common in wildlife (four isolates of each type).

REA patterns following digestion with *Eco*RI and electrophoresis of isolate DNA were more difficult to interpret than they were with *Sma*I-*Sal*I because of the increased number of DNA fragments, but it was possible to discern similarities and differences (data not shown). These results conform to the same groupings of the isolates as observed with digestion with *Sma*I-*Sal*I. Results with *Pst*I were similar to those with *Eco*RI (i.e., a large number of DNA fragments were pro-

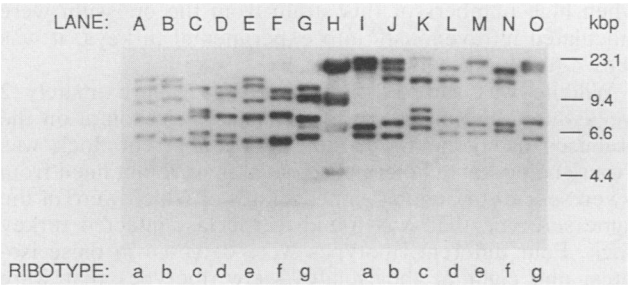

FIG. 2. Autoradiograph of Southern blot of *Eco*RI- and *Pst*I-digested whole-cell DNA obtained from *P. multocida* isolated from turkeys with fowl cholera (FC) and from wild mammals and birds on premises 0727 after hybridization with an *E. coli* rRNA probe. Lanes A to G contain DNA digested with *Eco*RI and hybridized with the probe, and lanes I to O contain DNA digested with *Pst*I. Lanes contain DNAs from isolates FC 1 (lane A and I), FC 139B (lanes B and J), FC 125 (lanes C and K), FC 127B (lanes D and L), 1-36 (lanes E and M), 38-22 (lanes F and N), and 38-30 (lanes G and O). Lane H contains DNA isolated from lambda phage, digested with *Hin*dIII and hybridized with *Hae*III-digested lambda phage probe. Numbers on the right represent the number of kilobase pairs (kbp) of the respective lambda phage fragments.

duced), correlating with the *Eco*RI and *Sma*I-*Sal*I results (data not shown).

Ribotyping. Representative results of ribotyping experiments involving turkey and wildlife isolate DNA digested with *Eco*RI are shown in Fig. 2. Autoradiographs reveal hybridization of the *E. coli* rRNA probe with five to six restriction fragments. Seven different ribotypes were observed in the 25 isolates (types a through g), with ribotypes b and c being the most common in turkey and wildlife isolates. The seven ribotypes are represented in Fig. 2. Overall results conform to the groupings of isolates following digestion with restriction enzymes *Sma*I-*Sal*I. Results of hybridization of the probe with *Pst*I-digested DNA from the turkey and wildlife isolates were similar to those observed with *Eco*RI (Fig. 2). Ribotyping experiments with DNA digested with *Pst*I were included to confirm the results obtained with *Eco*RI.

DISCUSSION

The purpose of this study was to evaluate the use of REA and ribotyping for differentiation (fingerprinting) of *P. multocida* strains for epidemiologic studies. By using isolates obtained from turkeys and wildlife from premises with a history of fowl cholera, the aim was to compare the results obtained by these two genotypic fingerprinting techniques with those obtained by other commonly used typing techniques, in particular, somatic antigen serotyping. Results obtained by the two molecular techniques indicated that these methods possess the potential for revealing information about the epidemiology of fowl cholera that other, more traditional methods do not.

When examining the serotypes of isolates obtained from dead turkeys, it was observed that the majority of isolates were of serotype 3,4 (10 of 14). This percentage (71%) was representative of the overall (statewide) population of strains isolated during this period of time, and again points out the need for more specific methods of strain identification. A total of 10 of 11 (91%) wildlife isolates were also serotype 3,4. To date, common serotypes in wildlife and turkeys have been found on only a limited number of premises in our studies (17).

Plasmid analysis of the 25 isolates also produced results reflective of the overall statewide strain population, i.e., only 3 of the 25 isolates (12%) possessed plasmids. Plasmid analysis was therefore of little use in studying the epidemiology of fowl cholera on the premises. Subspecies determination and MIC profiles were also of limited use, with the majority of isolates producing similar results (as do the majority of strains isolated statewide). Of 25 isolates, 21 were *P. multocida* subsp. *multocida*, and MIC profiles only varied slightly with streptomycin, tetracycline, and the sulfonamides. The inability of these techniques to fingerprint the isolates adequately necessitated the development of molecular techniques to examine the genotypes of the isolates.

REA revealed seven different REA types within the 25 isolates. The use of restriction enzymes such as *Pst*I and *Eco*RI is more sensitive to sequence variation than is the use of the *Sma*I-*Sal*I combination because of the increased number of cut sites. However, interpretation of the large number of fragments becomes more difficult. The use of an *E. coli* rRNA probe to simplify and highlight restriction site heterogeneity following REA proved to work well with restriction endonuclease-digested *Pasteurella* DNA. Additionally, ribotyping has the advantage over REA of comparing highly conserved rRNA genes (and associated sequences). Ribotyping results did correlate directly with REA results, producing an equivalent number of ribotypes ($n = 7$) as REA types. Digesting the DNA with *Eco*RI produced the best results in the ribotyping experiments, in terms of the number and distribution of hybridization bands observed.

Some questions were raised regarding the relationship between serotype and ribotype, because there were isolates with the same ribotypes but different serotypes, as well as isolates with different ribotypes that had the same serotype. The latter case was anticipated and actually was a reason for conducting this study. It would be possible for different genotypes (strains) to contain portions of their genomes that encode the production of certain similar antigens (e.g., serotype) but that have significant other portions of the genome that are different (e.g., highly conserved rRNA genes). Additionally, phenotypic characteristics could be under the control of environmental influences, many of which are unknown (3). A good example of phenotypic variation under the influence of the external environment is the iron-regulated outer membrane proteins of *P. multocida* (18).

The situation in which isolates had the same ribotype but different serotypes can be explained by the fact that serotyping was based on a boiled extract containing a variety of antigens (primarily lipopolysaccharide), and these antigens may have been encoded by genes in DNA fragments that bound the probe, or they may have been encoded by genes in nonhybridizing fragments. In addition, the base sequences in the fragments of a given size that hybridized with the probe were not necessarily completely identical; certain portion(s) may have had sufficient similarities to bind the probe but contained portions of significant size that were different. Given the above explanations and phenotypic typing results, we focused on the most stable epidemiologic marker studied, rRNA genomic fingerprints.

Four different *P. multocida* ribotypes, designated ribotypes a through d, were found in turkeys from the six different outbreaks over the 16-month study period. One ribotype, b, was recovered from turkeys in five of the six outbreaks.

On given premises, because of the overlap in the placement of one flock and marketing of the previously placed flock, the opportunity for interflock transmission of *P. multocida* exists. This may have occurred on several occasions on the premises in our study. Similar ribotypes were recovered from flocks in the first and second outbreaks, the second and third outbreaks, the fourth and fifth outbreaks, and the fifth and sixth outbreaks. All these pairs of flocks overlapped in placement, and interflock transmission could have occurred by contaminated farm workers or service crews and their equipment when they traveled between turkey houses without proper disinfection. Small wild birds, rodents, or other animals presumably could also transmit the organism, either mechanically or as actual biological hosts.

In two of the outbreaks, multiple ribotypes were isolated from the flocks. Two ribotypes were recovered during the second outbreak, and three ribotypes were recovered during the fifth outbreak. In two cases, once from each outbreak, different ribotypes were obtained from turkeys submitted to the diagnostic laboratory on the same day. These facts support the hypothesis that multiple strains may be involved in a given fowl cholera outbreak.

As stated above, ribotype b was isolated from five of six flocks during the study. Ribotype b appeared during the second outbreak and was isolated from all subsequent flocks placed on the premises except during the fourth outbreak. The fourth flock placed on the premises during the study period did not contract fowl cholera. The ribotype b strain isolated from the fifth flock placed on the premises either was reintroduced from an infected flock from other premises or persisted in the environment of the study premises following marketing of the third flock. Persistence could have occurred either in wildlife (e.g., mice) on the premises or in contaminated house litter, water, or turkey carcasses in burial pits. We are currently studying these as potential inanimate reservoirs of *P. multocida*. The final flock to experience an outbreak of fowl cholera was the only flock to receive a live attenuated fowl cholera vaccine, but the vaccine strain was shown to be of a different ribotype than any of those found in isolates from turkeys during the outbreaks (data not shown).

Based on ribotype differences, it appears that four different strains may have been involved in fowl cholera outbreaks on the premises during the study period. The work of Stull et al. (20) and Irino et al. (7) has demonstrated the ability of various restriction enzymes and *E. coli* rRNA hybridization probes to detect chromosomal DNA sequence differences associated with rRNA genes in heterologous species of bacteria (7, 20). On the basis of our ribotyping results, we believe that new strains of *P. multocida* were introduced into the turkey flocks, either by contaminated personnel or wildlife, as opposed to the evolution of existing strains. Different ribotypes would indicate variation in these highly conserved rRNA genes, genes that are not subject to frequent mutation.

The ribotypes of *P. multocida* isolated from wild mammals and birds captured on the premises were compared with those isolated from turkeys. In particular, wildlife isolates were compared with those of two flocks experiencing fowl cholera at or near the time of wildlife sampling. One serotype 3,4 isolate was obtained from an opossum 2.5 weeks after the second flock (flock 24) placed on the premises was first reported to be experiencing increased mortality as a result of serotype 3,4 *P. multocida* infection. However, the ribotypes were shown to differ, and the opossum isolate was observed to be different from all other isolates collected. In addition, when high numbers of this strain from the opossum were inoculated intravenously into experimental turkeys, it was shown to be avirulent (17).

Wildlife was sampled a second time, approximately 2 weeks after the final flock to contract fowl cholera on the premises had gone to market. No concurrent flock was experiencing fowl cholera. Ten isolates were obtained from seven species of animals, nine isolates of which were of the same serotype that was found in the last infected turkey flock. Four different ribotypes were detected in these isolates, and eight of the isolates were ribotypes that were previously found in infected turkeys (ribotypes b and c). Thus, it appears that, at least on the premises in our study, wild animals may be infected with isolates of *P. multocida* that are pathogenic for turkeys. Transmission of *P. multocida* between wildlife and turkeys could occur, particularly involving small birds (e.g., sparrows) and rodents. The animals sampled in this study were captured just outside the turkey houses. Even the most modern confinement buildings have ample locations where a sparrow or mouse can gain access to the interior. It is not uncommon for such animals to be observed in the turkey houses, where they presumably are attracted to the turkey feed and water. Such vehicles would provide a convenient transport for *P. multocida* to susceptible turkeys.

In conclusion, it appears that ribotyping, by using an *E. coli* rRNA probe, and REA are tools that can be used for the further differentiation of strains of *P. multocida* of the same (or different) serotype. In this study, ribotyping was able to provide information regarding the relatedness of isolates from different flocks and wildlife that could not be differentiated by phenotypic methods (e.g., serotyping). At this point, however, genomic fingerprinting probably should not be considered exclusively, but it should be used in conjunction with the established technique of serotyping to answer questions regarding the epidemiology of fowl cholera. More strains need to be analyzed and compared by these techniques to clarify the relationship (if any) between serotype and ribotype, and an overall ribotyping system must be developed to enable the comparison of large numbers of isolates from different premises over time. The use of genomic fingerprinting techniques in the future may help to clarify modes of transmission and reservoirs of *P. multocida* for turkeys.

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