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DNA Fingerprinting of *Pasteurella multocida* Recovered from Avian Sources

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Repetitive sequence-based PCR (rep-PCR) and amplified fragment length polymorphism (AFLP) were used to characterize a sample of 43 field isolates and 4 attenuated vaccine strains of *Pasteurella multocida* recovered from multiple avian species. Both rep-PCR and AFLP assays were rapid and reproducible, with high indices of discrimination. Concordance analyses of rep-PCR and AFLP with somatic serotyping indicate that, in general, somatic serotyping is a poor indicator of genetic relatedness among isolates of *P. multocida*. In addition, the data provide evidence of host specificity of *P. multocida* clones. Overall, the results of our study indicate that the rep-PCR and AFLP techniques enable rapid fingerprinting of *P. multocida* isolates from multiple avian species and enhance the investigation of fowl cholera outbreaks.

Pasteurella multocida is an aerobic, gram-negative, nonmotile bacterium that causes fowl cholera in domestic poultry and avian pasteurellosis in other avian species. The disease usually occurs in two forms, an acute septicemia with high morbidity and mortality rates and chronic localized infection of joints and sinuses (17). Five capsular serogroups, designated A, B, D, E, and F, and 16 somatic serotypes (designated 1 through 16) of *P. multocida* have been described (17, 18). Although serotyping of both capsular and somatic antigens has proved to be very useful for detection and identification of this bacterium, it is limited in that it provides insufficient information for epidemiological studies to distinguish among different strains of the same serotype.

While a variety of molecular subtyping techniques, including restriction endonuclease analysis (4, 31, 32, 33), plasmid profiling (4, 16), and ribotyping (2, 21), have been used for subtyping of *P. multocida,* these techniques in general have several limitations. Restriction endonuclease analysis and ribotyping are limited in that they are time-consuming and labor intensive. Plasmid profiling is relatively easy and inexpensive but has limited applications in epidemiological investigations because all isolates may not carry plasmids or they might be lost during growth in laboratory media (23).

The objective of this study was to determine the utility of two rapid PCR-based approaches, repetitive sequence-based PCR (rep-PCR) and amplified fragment length polymorphism (AFLP), for differentiation of *P. multocida* isolates from multiple avian species and fowl cholera outbreak investigations to the subspecies level.

Bacterial isolates and DNA isolation. The geographic origin and source of the *P. multocida* isolates are presented in Table 1. Thirty-eight isolates of *P. multocida* were obtained from the National Animal Disease Center, Ames, Iowa, and the University of Minnesota, St. Paul, Minn. An additional nine clinical isolates (96-174, 96-226, 96-235, 96-240, 96-246, 96-248, 96-260, 96-265, and 96-269) were obtained from outbreaks in turkey farms in Minnesota. The methods used to prepare genomic DNA from bacterial isolates have been described previously (1).

rep-PCR. rep-PCR was performed as previously described with some modifications (1, 28, 34). Oligonucleotide primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG) were used to generate DNA fingerprints in this study. To evaluate the reproducibility of the rep-PCR fingerprints, three separate reactions were performed for each isolate.

AFLP. AFLP was performed as described in the AFLP microbial fingerprinting kit protocol (Perkin Elmer-Applied Biosystems Division), Foster City, Calif.).

Selective amplification was performed with the *Eco*RI selective primer containing a fluorescent label on the 5′ end with additional base A (carboxyfluorescein dye [FAM]-*Eco*RI-A: FAM-5′-GACTGCGTACCAATTCA) and the *Mse*I selective primer with no additional base (*Mse*I-O: 5′-GATGAGTCCT GAGTAA). The AFLP fingerprint profiles were automatically analyzed with GeneScan (Perkin-Elmer) with GeneScan-500 Rhodamine X (ROX) size standard as an internal size control. Sizes of amplified products were then tabulated and exported for cluster analysis with Molecular Analyst software (Bio-Rad, Hercules, Calif.). To analyze AFLP fingerprints, DNA fragments from 100 to 500 bp in size were included for cluster analysis.

Computer-assisted analysis of rep-PCR and AFLP fingerprints. Molecular Analyst software (Bio-Rad) was used to compare the rep-PCR and AFLP fingerprint profiles among *P. multocida* isolates. The program automatically computed the similarity for each pair of fingerprints on the basis of band positions with the Dice coefficient (S_D). In this investigation, the cutoff value of S_D was 0.90. This cutoff value is assigned

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TABLE 1. Properties of 47 *P. multocida* isolates

Strain[a]	Host	Source[b]	Somatic type[c]	rep-PCR type	AFLP type
96-265*	Turkey	Minnesota	NA	1	1
96-248*	Turkey	Minnesota	3	1	1
96-246*	Turkey	Minnesota	3	1	1
96-174**	Turkey, lung	Minnesota	3	1	1
2220	Turkey	Iowa	3	1	2
4038	Turkey	Oregon	3	1	2
CU	Turkey	UMN	3,4	1	3
CU	Turkey	UMN	3,4	1	3
4020	Turkey	Texas	3	1	3
4021	Turkey	Texas	3	1	3
96-235***	Turkey	Minnesota	3	2	3
96-226***	Turkey, lung	Minnesota	3	2	3
96-260****	Turkey	Minnesota	NA	3	4
3704	Turkey	South Dakota	3,4,12	3	4
3707	Turkey	South Dakota	3,4,12	3	4
3758	Turkey	Kansas	3,4,12	3	4
4031	Turkey	Iowa	3	3	4
4032	Turkey	Iowa	3	3	4
5314	Turkey	Iowa	3,4	3	4
CU	Turkey	UMN	3,4	3	5
5167	Turkey	Germany	3,4	4	2
1996	Bald eagle	California	1	5	6
2148	Duck	Utah	1	5	6
2410	Duck	Utah	1	5	6
3200	NA	UMN	3	6	16
4601	Quail	Virginia	1	7	7
4602	Quail	Virginia	1	7	7
2846	Chicken	Singapore	1	7	10
2847	Duck	Singapore	1	7	10
2855	Chicken	Singapore	1	7	10
4247	Chicken	Michigan	1	7	10
4251	Duck	Michigan	1	7	10
5439	Chicken	Egypt	1	7	10
5440	Turkey	Egypt	1	7	10
4910	Chicken	Bangladesh	1	8	8
2852	Chicken	Singapore	1	8	10
2848	Duck	Singapore	1	8	11
V2283	NA	UMN	3,4	9	19
2853	Duck	Singapore	1	10	15
5288	Turkey	California	10	11	20
96-240*****	Turkey	Minnesota	3	12	17
96-269******	Turkey	Minnesota	NA	13	18
5162	Turkey	Germany	3	14	12
1896	Turkey	Canada	4	15	13
4052	Chicken	Arkansas	10	16	9
2879	Duck	Maryland	3,12,15	17	14
2887	Duck	Maryland	3,4,12	17	14

[a] *, isolates recovered from farm A; **, isolates recovered from farm B; ***, isolates recovered from farm C; ****, isolates recovered from farm D; *****, isolates recovered from farm E; ******, isolates recovered from farm F; CU, Clemson University.
[b] UMN, University of Minnesota.
[c] NA, not available.

based on experience with the gel analysis software to ensure that the same bacterial strain run on different PCRs and different gel electrophoreses will be identified as the same type. Pairs of isolates with a similarity coefficient (S_D) of 0.90 were considered similar and not distinguishable. Cluster analysis among isolates was performed by the unweighted pair group average (UPGMA) linkage method (20).

Calculation of index of discrimination. The index of discrimination (D) indicates the probability that the fingerprinting method will identify two unrelated bacterial strains as different fingerprint types. Statistical analysis to determine the discriminating power of rep-PCR and AFLP was performed with the discriminatory index (8).

Concordance analysis of fingerprinting methods. Concordance between subtyping techniques was calculated by comparing all possible pairs of 44 isolates (1,081 pairwise comparisons) and classifying each pair of isolates for whether both isolates were in the same or different subtype and whether they matched or mismatched in another subtype. The sum of the same somatic type match rep-PCR or AFLP types and of different somatic types mismatch rep-PCR or AFLP types represents the percent agreement of the pairwise comparisons of isolates. The statistical analysis of concordance was performed with the G test of independence (22).

DNA fingerprinting of avian isolates of *P. multocida* with rep-PCR. Cluster analysis assigned the 47 avian isolates of *P. multocida* into 17 distinct fingerprint patterns (Table 1 and Fig. 1). The average number of bands per rep-PCR fingerprint of *P. multocida* was 12.4 ± 1.3 (range, 10 to 14). Repeated PCRs for each isolate revealed identical rep-PCR fingerprint patterns. The index of discrimination of rep-PCR for differentiation of the *P. multocida* isolates was 0.89. At an arbitrary 75% similarity cutoff level, the 47 isolates could be further grouped into three major clusters, designated A through C (Fig. 1). It is noteworthy that all of the isolates (20 of 20) in cluster B (rep-PCR patterns 1, 2, and 3) were recovered from turkeys. On the other hand, only 7.69% (1 of 13) of the isolates recovered from turkeys were found in rep-PCR patterns 7, 8, and 9 (cluster C) (Fig. 1).

DNA fingerprinting of avian isolates of *P. multocida* with AFLP. (i) AFLP fingerprints of *P. multocida*. The fingerprints obtained by AFLP with primer FAM-*Eco*RI-A:MseI-O were highly polymorphic. The average number of bands per isolate was 20.6 ± 2.9, ranging from 16 to 25 bands. A dendrogram generated by computer-assisted analysis of 47 AFLP fingerprints is shown in Fig. 2. AFLP characterized the 47 *P. multocida* isolates into 20 distinct AFLP patterns (Table 1). The reproducibility of AFLP fingerprinting was evaluated by generating at least two separate AFLP reactions for each isolate, and the results showed that the same AFLP fingerprint patterns were generated for each isolate at a cutoff value of 90% similarity. The index of discrimination of AFLP was 0.93, which was slightly higher than that obtained by rep-PCR (0.89).

(ii) Concordance analysis of somatic types, rep-PCR types, and AFLP types. The concordance between somatic types, rep-PCR types, and AFLP types is shown in Table 2. The overall percent agreement between somatic types and rep-PCR and AFLP types was 79.60% (kappa value [K] = 0.32) and 76.53% (K = 0.17), respectively, and these values were statistically significant (G test of independence, G = 104.8, df = 1, $P < 0.001$, and G = 37.9, df = 1, $P < 0.001$, respectively).

Forty-seven isolates were included for concordance analysis between rep-PCR types and AFLP types (Table 3). Isolates of the same AFLP patterns typically had identical rep-PCR patterns. From the total of 1,081 possible pairwise comparisons, 5.82% of the isolates of the same rep-PCR matched in AFLP patterns and 87.79% of the isolates with different rep-PCR had different AFLP patterns. On the other hand, only 1.39% of isolates with different rep-PCR patterns matched in AFLP patterns, and 5% of isolates with the same rep-PCR patterns

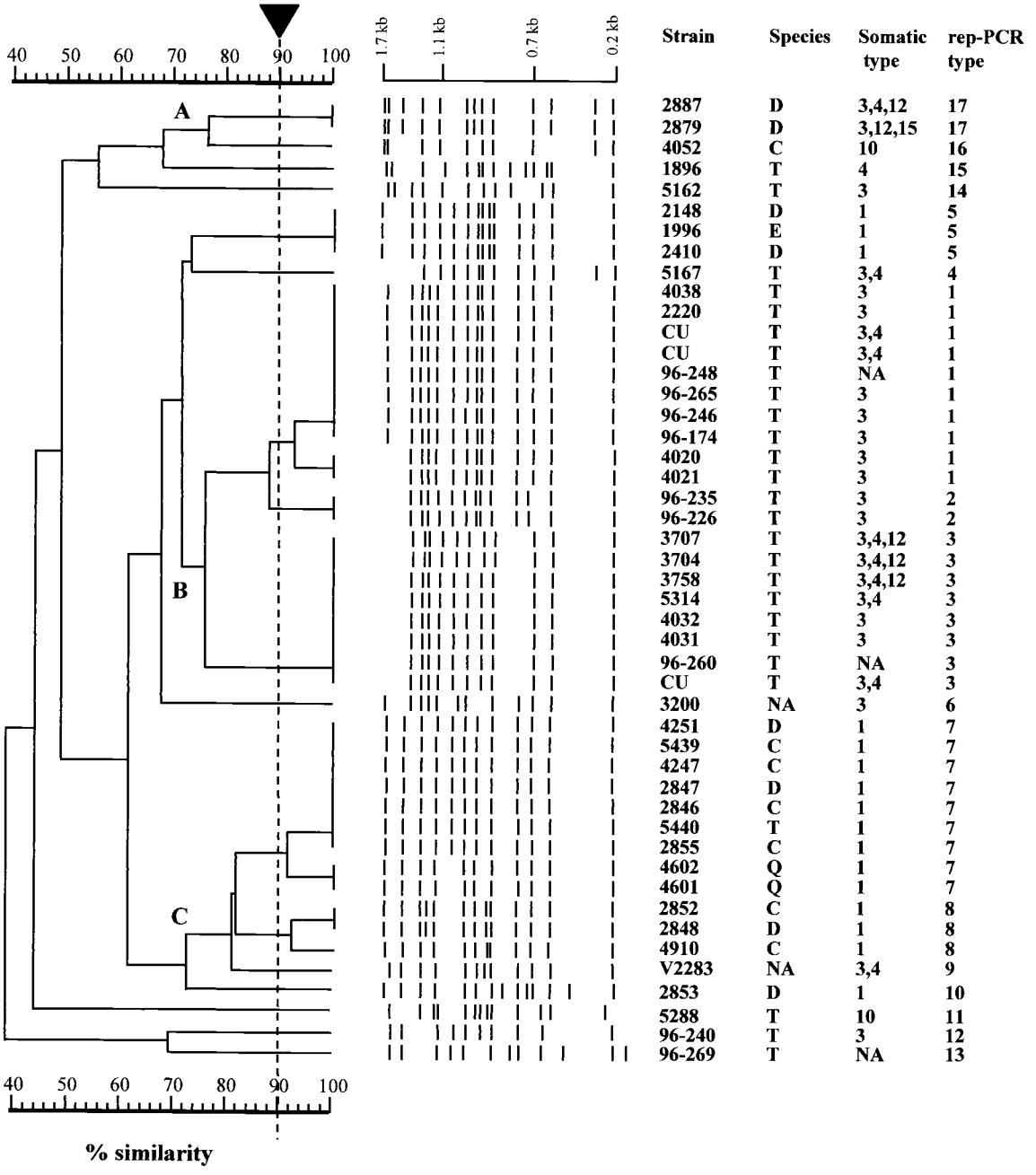

FIG. 1. UPGMA dendrogram of genetic relatedness of 17 rep-PCR types (1 to 17) of 47 *P. multocida* isolates recovered from avian sources. The scale represents percent similarity generated by computer-assisted comparison of rep-PCR fingerprint profiles. The arrow indicates the cutoff value (90%) for cluster analysis. Species: D, duck; C, chicken; T, turkey; E, bald eagle; Q, quail. NA, not available; CU, Clemson University.

differed in AFLP patterns. The overall concordance was 93.61% ($K = 0.61$) and was highly significant ($G = 244.18$, df $= 1$, $P < 0.001$), indicating a strong correlation between rep-PCR and AFLP fingerprints (Table 3).

Investigation of fowl cholera outbreaks with rep-PCR and AFLP fingerprinting. Nine field isolates of *P. multocida* recovered from recent fowl cholera outbreaks on turkey farms in Minnesota (Table 1) were characterized by rep-PCR and AFLP, and a total of five distinct fingerprint patterns were identified (Fig. 3 and 4). At a 90% cutoff value, rep-PCR results showed that four isolates from farm A (96-265, 96-248, and 96-246) and B (96-174) were classified into the common rep-PCR pattern 1 that included two vaccine strains. Two isolates from farm C (96-226 and 96-235) were classified into pattern 2, which differs from pattern 1 by two bands. One isolate (farm D), 96-260, was assigned to pattern 3, which differs from pattern 1 by three bands. However, two isolates, 96-240 and 96-269 (from farms E and F, respectively), displayed unique patterns, 12 and 13, respectively. These results show the ability of rep-PCR to identify epidemiologically related strains as the same pattern and suggest that the recent fowl cholera outbreaks in Minnesota resulted from infection

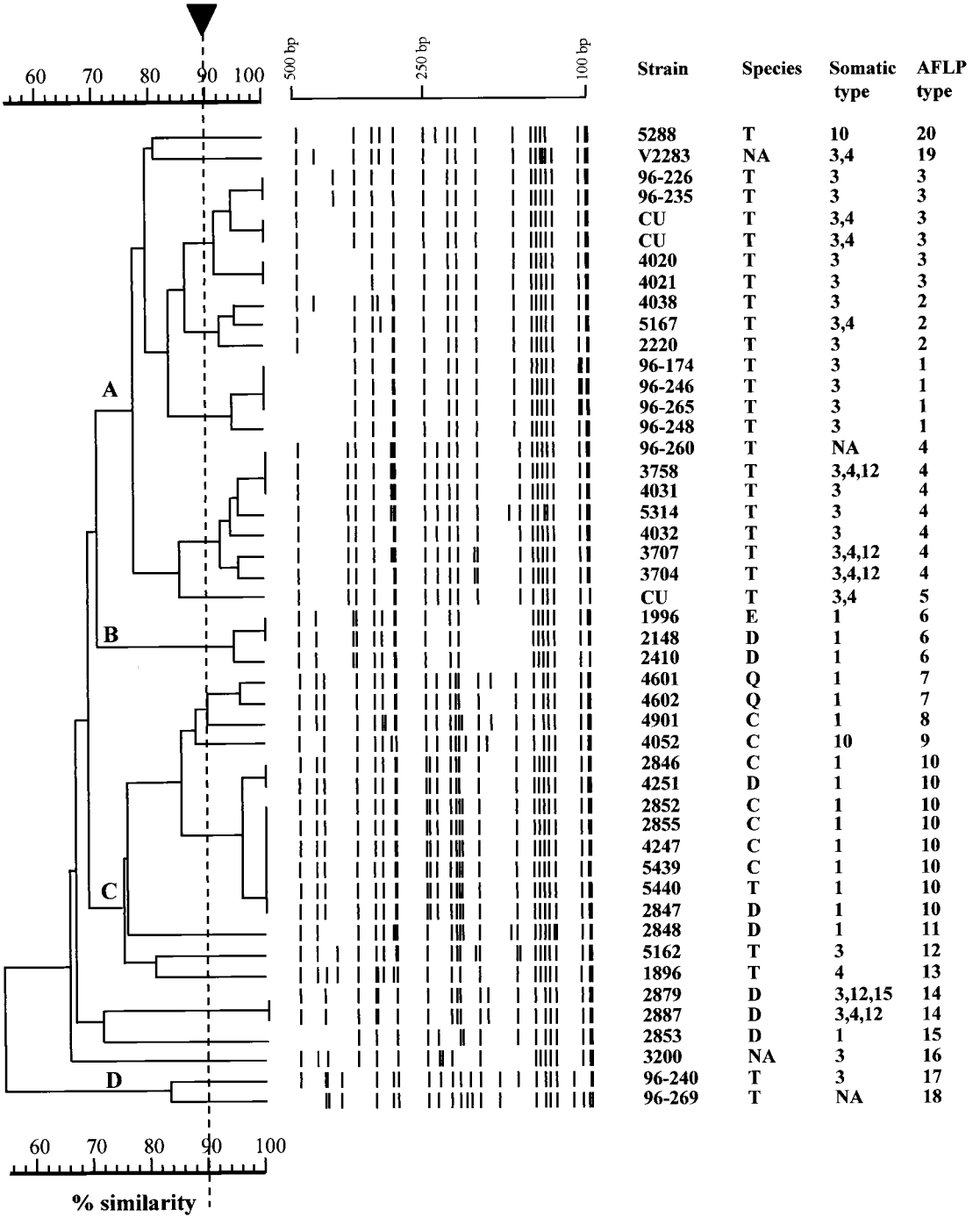

FIG. 2. UPGMA dendrogram of the genetic relatedness of 20 AFLP types from 47 *P. multocida* isolates from avian sources. The scale represents percent similarity generated by computer-assisted comparison of AFLP fingerprint profiles. The arrow indicates the cutoff value (90%) for cluster analysis. Species: D, duck; C, chicken; T, turkey; E, bald eagle; Q, quail. NA, not available; CU, Clemson University.

with closely related ($S_D > 0.75$) strains of patterns 1, 2, and 3 (cluster B).

It is interesting that three vaccine strains that have been described previously also belong to these common clones. AFLP analyses of the outbreak strains showed similar results, indicating that both rep-PCR and AFLP fingerprinting techniques will be useful for epidemiological investigations of fowl cholera outbreaks.

rep-PCR and AFLP fingerprinting of *P. multocida* isolates. Traditional epidemiologic investigations of fowl cholera outbreaks have been based primarily on a thorough examination of case histories and detailed characterization of bacterial isolates by tests such as biochemical profiling, antimicrobial sensitivity profiling, and serotyping. However, not all isolates are typeable by these methods. For example, although serotyping has proved to be a useful tool for classifying isolates of *P.*

TABLE 2. Concordance analysis of somatic type, rep-PCR, and AFLP fingerprints[a]

Somatic type	No. (%)			
	rep-PCR type		AFLP type	
	Match	Mismatch	Match	Mismatch
Same	70 (7.40)	163 (17.23)	40 (4.23)	193 (20.40)
Different	30 (3.17)	683 (72.20)	29 (3.07)	684 (72.30)
Concordance	753 (79.60)		724 (76.53)	

[a] The sum of the same somatic types matching rep-PCR types or AFLP types and different somatic types mismatching rep-PCR types or AFLP types represents the percent agreement for the total of 946 pairwise comparisons of isolates.

multocida, it has been difficult to conduct epidemiologic investigations and assess the relationships of *P. multocida* isolates involved in fowl cholera outbreaks by serotyping because of the existence of considerable genetic variation within serotypes (3, 31, 32).

Therefore, the application of direct genetic methods of testing, especially PCR-based techniques, has gained widespread acceptance, and they are used in the investigation of putative disease outbreaks. An important advantage of PCR-based methods is that, in many instances, they enable the sensitive detection and typing of pathogens, as the tests require only a small amount of target DNA. Moreover, since these methods do not depend on the expression of bacterial gene products, all isolates are typeable.

DNA fingerprinting of bacterial isolates involves the generation of distinct "signature" profiles that enable bacterial strain identification. These fingerprints are usually represented by band patterns that result from the migration of different sizes of bands in a gel matrix. PCR-based typing methods include randomly amplified polymorphic DNA (RAPD) (30) and rep-PCR (28). rep-PCR has an advantage over RAPD analysis in that it involves specific rather than arbitrary amplification of target DNA, since oligonucleotide primers used in the amplification reactions are based on conserved repetitive elements that are dispersed throughout the bacterial genome. Thus, in our hands, and as described previously (1), this technique yields reliable and easily reproducible fingerprint patterns.

For instance, our analyses have shown that both AFLP and rep-PCR fingerprints were stable in testing an isolate of *Mycobacterium avium* over time (10 passages) and in testing 10 different colonies from the same isolate, indicating high reproducibility of the two techniques (A. Amonsin and V. Kapur,

TABLE 3. Concordance analysis of rep-PCR and AFLP fingerprints[a]

Rep-PCR type	No. (%) of AFLP type	
	Match	Mismatch
Same	63 (5.82)	54 (5.00)
Different	15 (1.39)	949 (87.79)
Concordance	1,012 (93.61)	

[a] The sum of the same rep-PCR types matching AFLP types and different rep-PCR types mismatching AFLP types represents the percent agreement for the total of 1,081 pairwise comparisons of isolates.

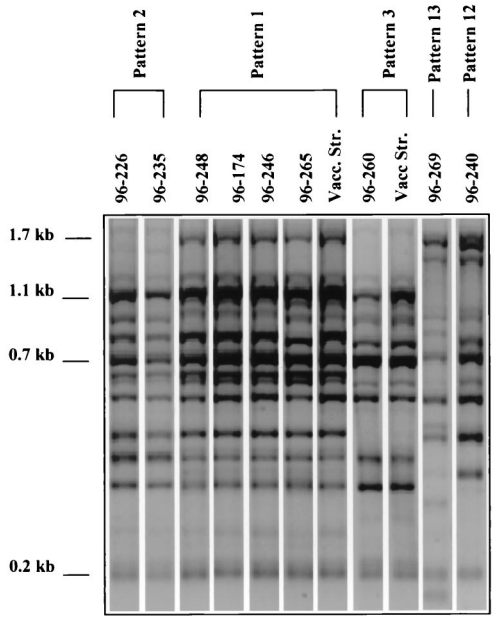

FIG. 3. Identification of clinical *P. multocida* isolates recovered from fowl cholera outbreaks in Minnesota by comparing their rep-PCR patterns with fingerprint patterns obtained from reference vaccine strains.

unpublished data). DNA fingerprinting of *P. multocida* recovered from poultry, pigs, and cattle with rep-PCR has also been reported previously (7, 9, 23, 24, 25, 26).

AFLP is a relatively recently described PCR-based DNA fingerprinting method that can be used for the characterization and comparison of DNA samples regardless of their origin or complexity (29). This method of genotyping has been used for a number of different bacterial species (5, 6, 11, 12, 13, 15, 27). For instance, the use of AFLP to trace the source of infection for a nosocomial outbreak of gentamicin-resistant *Klebsiella pneumoniae* has recently been documented and resulted in controlling of the disease outbreak (27). AFLP has been shown to have several advantages compared to various other typing methods, including increased discriminatory power, reproducibility, and easy compatibility with computerized analysis of the banding patterns.

Hence, the present investigation was conducted to assess the utility of two rapid PCR-based approaches, rep-PCR and AFLP, for the differentiation of *P. multocida* isolates recovered from multiple avian species and fowl cholera outbreak investigations in turkey farms. The results of our investigations clearly show that rep-PCR has a high discriminatory ability ($D = 0.89$) when used for the differentiation of *P. multocida* isolates recovered from avian sources. In addition, the method is rapid, reproducible, and easy to perform. Together, the data suggest that rep-PCR has great utility for investigating the epidemiology of fowl cholera and conducting outbreak investigations.

We have standardized AFLP to characterize *P. multocida* isolates from multiple avian sources, and the results show that the FAM-*Eco*RI-A and *Mse*I-O primers yield highly polymorphic fingerprints for this bacterium. Overall, AFLP was found to be a powerful tool for epidemiological investigations of fowl

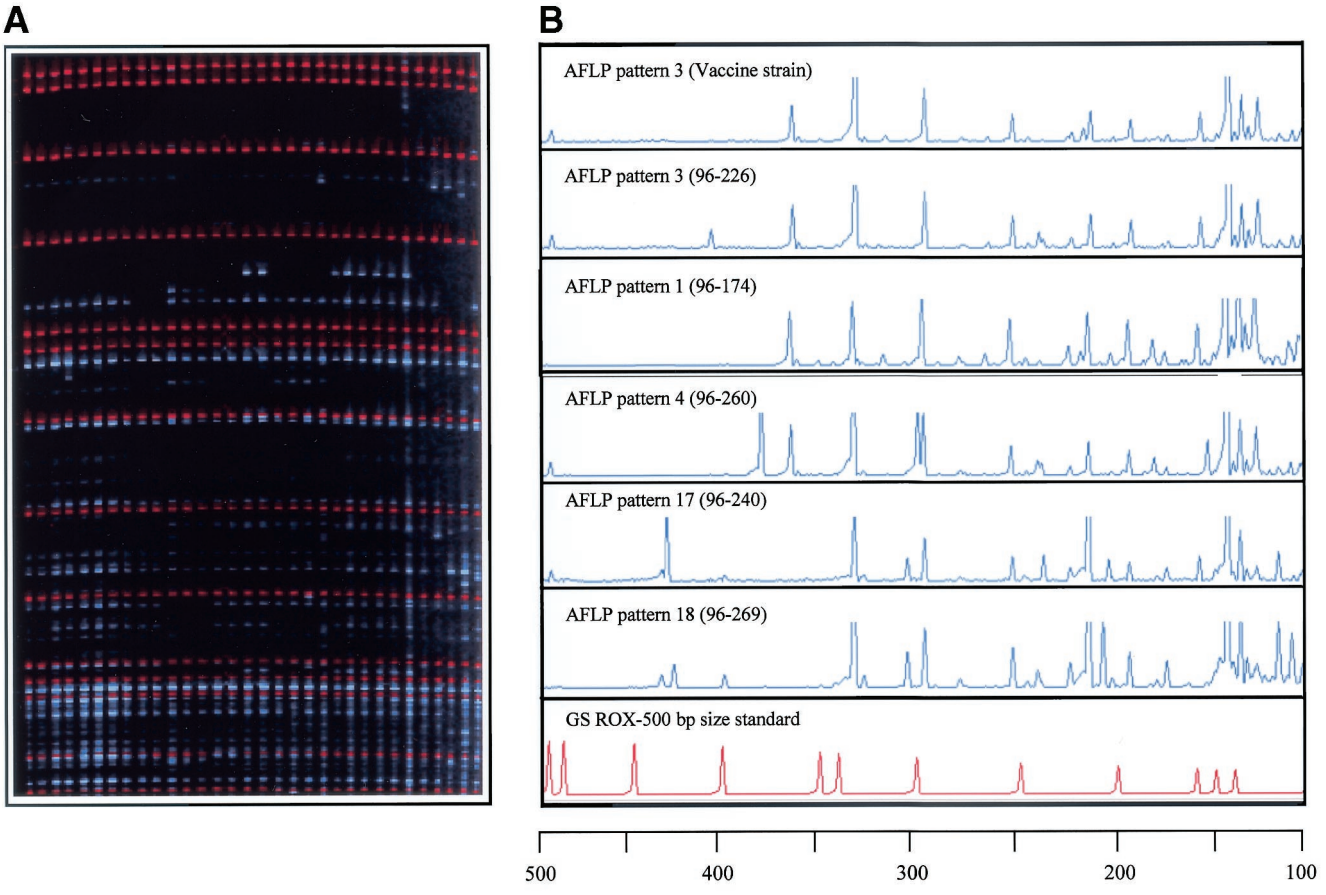

FIG. 4. (A) AFLP gel image of *P. multocida* isolates generated with primers FAM-*Eco*RI-A and *Mse*I-O. Blue bands represent primer-generated DNA fragments. Red bands represent the internal size standard (GS-500 ROX) included in each lane to help correct interlane variation and provide accurate band sizing. (B) Electrophoretogram of clinical isolates and vaccine strains of *P. multocida*.

cholera outbreaks in our study, and the index of discrimination among the *P. multocida* isolates obtained by AFLP was 0.93, slightly higher than that obtained from rep-PCR fingerprints (0.89), and some *P. multocida* isolates that were indistinguishable by rep-PCR were identified as distinct clones by AFLP. More importantly, the results of the AFLP analysis correlated well with those of rep-PCR.

In contrast, the concordances between somatic types and rep-PCR types or somatic types and AFLP types were relatively low, indicating that somatic serotypes, in general, do not correlate well with molecular subtyping systems. These results indicate that although there was fair agreement between the somatic serotype and rep-PCR or AFLP type of the isolates examined in this investigation, somatic serotypes are, in general, poor indicators of overall genetic relatedness among isolates of *P. multocida* recovered from avian sources. These findings reflect the fact that the somatic antigens are likely to be adaptive traits and hence subject to strong evolutionary pressure and therefore poor indicators of genetic (rather than phenotypic) similarity. As has been shown for other species of gram-negative and gram-positive bacteria (14, 19), *P. multocida* somatic antigens may be horizontally transferred among genetically divergent clones of the bacterium.

The results of these studies show that rep-PCR and AFLP are useful techniques for indexing genetic variation in this bacterium. There are two major advantages of AFLP. First, the internal size standard included in each lane obviates ambiguities due to interlane variation in electrophoretic mobility, allowing facile intergel comparisons. Second, a large number of isolates can be examined in a short period of time. However, this method has the limitation of being more expensive than rep-PCR in terms of reagent and equipment costs. Overall, our studies show that depending on the scope of the study, investigators have two powerful molecular genetic methods, rep-PCR and AFLP, at their disposal for investigating fowl cholera outbreaks.

The occurrence of host and disease specificity among bacterial clones has been well described for a variety of pathogenic bacteria (19). For instance, host specificity is found among isolates of *Bordetella bronchiseptica*, for which clones or clone families are strongly associated with either pigs or dogs (19). A similar host specificity is seen among clones of *Staphylococcus aureus*, in which certain clones are preferentially associated with either humans or cows (10).

The results of the present investigation show that some *P. multocida* clones may colonize several avian species. For instance, clones belonging to rep-PCR type 7 and AFLP type 10 were recovered from chickens, ducks, and turkeys (Table 1).

These data suggest that certain clones of *P. multocida* are able to colonize a wide variety of avian species. However, a striking example of host specificity of *P. multocida* isolates is seen among in cluster B (Fig. 1), in which all four clones (representing 20 isolates) were recovered only from turkeys. Hence, the results of this investigation are fully consistent with the general concept of host specificity among clones, and we hypothesize that the distinctive host range of a bacterial clone is due to innate differences in the ability to successfully colonize a specific host. This hypothesis needs to be tested by constructing a population genetic framework for a larger sample of *P. multocida* isolates recovered from several avian species and from different geographic areas.

In conclusion, the results of our investigation show that rep-PCR and AFLP fingerprinting techniques are useful for the rapid DNA fingerprinting of *P. multocida* isolates. These techniques enable straightforward genetic typing of *P. multocida* isolates and provide a facile means of conducting molecular epidemiologic analyses and outbreak investigations. In addition, the data also provide evidence for host specificity of certain *P. multocida* clones.

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