JOURNAL OF CLINICAL MICROBIOLOGY, June 2002, p. 2163–2168 Vol. 40, No. 6
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.6.2163–2168.2002

Random Amplified Polymorphic DNA and Amplified Fragment Length Polymorphism Analyses of *Pasteurella multocida* Isolates from Fatal Fowl Cholera Infections

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Received 5 September 2001/Returned for modification 29 December 2001/Accepted 9 March 2002

Fowl cholera, a disease caused by *Pasteurella multocida*, continues to be a major problem for the poultry industry. The sources of pathogenic organisms responsible for most sporadic epidemics remain unconfirmed, although attenuated vaccines that retain a low level of virulence have occasionally been implicated in outbreaks of the disease. One of the vaccines most commonly used to prevent fowl cholera is the M-9 strain. In the present study, 61 clinical isolates from turkeys that died of fowl cholera from 1997 to 1999 on 36 Utah farms were analyzed and compared to the M-9 vaccine strain. Genetic analyses of the isolates were done by random amplified polymorphic DNA (RAPD) analysis and amplified fragment length polymorphism (AFLP) fingerprinting. The results of these genetic analyses were correlated with the vaccination status of the flock, isolate serotype, and geographic location. Although both genetic techniques effectively identified similar subtle genomic differences, RAPD analysis provided only 77% of the detail provided by AFLP analysis. While a relationship between genetic profile and serotype was evident, no significant relationship indicating geographic influence was found ($P = 0.351$). Interestingly, organisms isolated from vaccinated flocks were significantly closer genetically to the M-9 vaccine strain than isolates from unvaccinated birds were ($P = 0.020$). Statistical analyses revealed that this relationship could not have been determined by serotyping alone ($P = 0.320$), demonstrating the value of AFLP and RAPD analyses in the characterization of disease-causing strains.

Deaths from fowl cholera, a disease caused by *Pasteurella multocida*, continue to be a major concern for the poultry industry, especially turkey growers. Cholera costs the turkey industry millions of dollars annually due to deaths, condemnation losses, and vaccination and medication costs (3, 10, 20). *P. multocida* has a broad host range, and many animals could serve as persistent reservoirs of the organism and sources of infection (21). However, the source of pathogenic isolates responsible for most sporadic epidemics remains unidentified. Once introduced into a flock, *P. multocida* can increase in virulence and spread rapidly, leading to high rates of mortality among the turkeys in the flock (17). Chronic respiratory infection and an acute septicemia are the most common hallmarks of the disease (25).

Typing of *P. multocida* is principally accomplished via passive hemagglutination and gel diffusion precipitin assays. Isolates are classified into five groups according to their capsular antigens and into 16 serotypes on the basis of their lipopolysaccharide antigens. Often, for reasons not fully understood, isolates cross-react with multiple antisera. Such isolates are considered distinct serotypes and are classified individually.

The vaccine used most often in Utah to prevent outbreaks of fowl cholera consists of the M-9 attenuated strain, which most commonly reacts as serotype 3,4. This mutant retains some virulence and has occasionally been implicated in outbreaks of disease associated with vaccination (2, 4, 7, 10, 14, 18, 25). However, because of antigenic complexity, it is difficult to accurately determine by serotyping whether an outbreak is caused by the vaccine strain or by a related strain of *P. multocida*. For this reason, molecular techniques are increasingly being used to characterize the relatedness of isolates that cause fatalities.

In recent years numerous biochemical methods that reveal the genetic diversity of similar organisms have arisen. Random amplified polymorphic DNA (RAPD) analysis is a fast, PCR-based method of genetic typing based on genomic polymorphisms. More recently, amplified fragment length polymorphism (AFLP) analysis has been used for DNA fingerprinting of microorganisms. AFLP analysis is based on selective amplification of DNA restriction fragments (24, 26). It is technically similar to restriction fragment length polymorphism analysis, except that only a subset of the fragments are displayed and the number of fragments generated can be controlled by primer extensions. Previous studies have shown that AFLP analysis has greater discriminatory power than RAPD analysis and other genomic fingerprinting methods (1, 13). Neither AFLP nor RAPD analysis requires previous detailed knowledge of the DNA to be analyzed, and both have been shown to be reliable methods of distinguishing small genomic differences. However, comparison of the data generated by RAPD analysis in different laboratories has been problematic (6, 19, 23).

In the study described here, genetic and serological analyses

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were performed with *P. multocida* isolates from turkeys with fatal infections to determine whether vaccination plays a role in pathogenesis. Results from AFLP and RAPD analyses were compared, and the results of these genetic analyses were correlated with serotype and geographic location.

MATERIALS AND METHODS

Organisms and DNA preparations. Sixty-one *P. multocida* isolates were taken from turkeys that died of fowl cholera from 1997 to 1999. Turkey carcasses were presented to the veterinary unit of Moroni Feed Company (Moroni, Utah), and at necropsy the organisms were isolated from the organ of primary infection in each bird. Samples of the affected organs were initially streaked onto tryptose agar and the plates were incubated at 37°C for 18 to 24 h. Individual colonies were inoculated into 10 ml of tryptone broth and incubated again at 37°C for 18 h. The broth culture was subjected to biochemical tests and Gram stained to verify that the organism was *P. multocida*. DNA was isolated by alkaline lysis extraction. Briefly, turbid liquid cultures were pelleted by centrifugation and resuspended in TE (Tris-EDTA) buffer. Lysis was accomplished by the addition of sodium dodecyl sulfate and proteinase K and incubation at 37°C. The remaining debris was removed by the addition of 5 M NaCl and hexadecyltrimethylammonium bromide. This was followed by chloroform and phenol-chloroform extractions. DNA was recovered from the resulting supernatant by isopropanol precipitation.

RAPD analysis. The RAPD assay was performed with the Ready-To-Go RAPD analysis kit (Amersham Pharmacia Biotech, Piscataway, N.J.). This kit included six RAPD analysis primer sets (primer sets AP1 to AP6) and Ready-To-Go RAPD analysis beads with thermostable polymerases (AmpliTAQ and Stoffel fragments), lyophilized buffer (10 mM Tris, 30 mM KCl, and 3 mM $MgCl_2$ [pH 8.3] in a 25 μM reaction volume), and bovine serum albumin (2.5 μg). DNA amplification was performed by the addition of 25 pmol of primer, H_2O to a final volume of 25 μl, and one RAPD analysis bead to 10 ng of template DNA. All six primer sets (primer sets AP1 to AP6) were run in triplicate for each isolate.

Samples were placed in a thermocycler and subjected to the following cycle profile: 1 cycle of 95°C for 5 min, followed by 45 cycles at 95°C for 1 min, 36°C for 1 min, and 72°C for 2 min. The amplified fragments were separated by electrophoresis in a 2% agarose gel at 9 V/cm in 0.5× TBE (Tris-borate-EDTA) buffer. The bands were visualized after staining with ethidium bromide (10 μg/ml) and photographed under UV illumination. The sizes of the bands were determined by extrapolation against size standards (PCR marker containing bands of from 50 to 2,000 bases; Sigma, St. Louis, Mo.). *Escherichia coli* C1a DNA, provided in the analysis kit, was used as a positive control. Negative controls contained all elements except template DNA. Data matrices were generated manually by scoring all major RAPD bands in the range of 275 to 3,000 bp. The number of bands present ranged between one and seven for each run.

AFLP analysis. DNA (100 ng) was digested with *Eco*RI and *Mse*I, ligated to double-stranded adapters, and amplified as described by Keim et al. (12). The initial PCR product using primers with no extensions (+0/+0) was diluted 20-fold, and 3 μl was used in the selective amplification. Selective amplification was performed with the following five 1-base (+1/+1) primer extension combinations: *Eco*RI-C with *Mse*I-G, *Eco*RI-G with *Mse*I-C, *Eco*RI-T with *Mse*I-A, *Eco*RI-A with *Mse*I-A, and *Eco*RI-C with *Mse*I-C. The *Eco*RI primers were tagged with the fluorescent dye 6-carboxyfluorescein. These selective amplifications were performed in a total volume of 20 μl. The cycling profile consisted of one cycle of 94°C for 30 s, 65°C for 30 s, and 72°C for 1 min. The annealing temperature was then lowered by 1°C per cycle until it reached 56°C (nine cycles). An additional 26 cycles at an annealing temperature of 56°C followed.

Following the selective amplification, 0.5 to 1.0 μl of each of the AFLP products was added to 0.75 μl of a mixture of internal lane size standards consisting of Genescan-500 (Applied Biosystems Inc., Foster City, Calif.) and MapMarker-400 (BioVentures Inc., Murfreesburo, Tenn.) labeled with *N,N,N,N*-tetramethyl-6-carboxyrhodamine. After a 2-min heat denaturation at 90°C, the reaction mixtures were loaded onto a Long Ranger 5% acrylamide DNA sequencing gel (FMC BioProducts, Rockland, Maine) and fractionated on an ABI 377 automated fluorescent sequencer (Applied Biosystems Inc.). Each sample was run on a total of three separate gels. Genescan analysis software (Applied Biosystems Inc.) was used to determine the sizes of the sample fragments by use of the internal lane size standards containing a total of 24 markers of between 100 and 500 AFLP map units. Only fragments from the triplicate runs that were between 100 and 500 map units in size and that had a minimum absorbance of 50 were used in the analysis. The number of bands for a run ranged between 38 and 99.

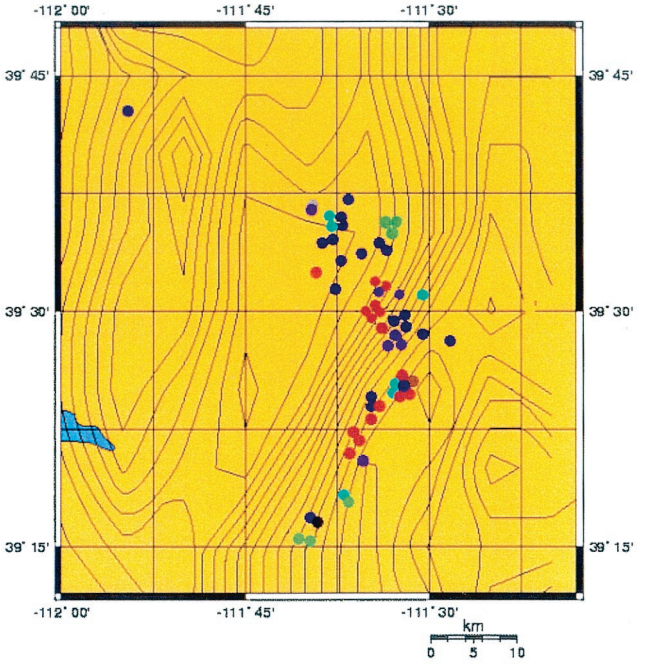

FIG. 1. Contour map of Sanpete County, Utah, showing the geographic distributions of isolates color coded by serotype. See Fig. 3 for serotype key.

Serotyping. Serotyping was performed by a slight modification of the technique described by Heddleston et al. (8).

Antigen preparation. A sample from a turbid broth culture of each isolate was streaked as a lawn onto a tryptose agar plate. The tryptose agar plate was incubated at 37°C for 18 to 24 h. The bacteria were harvested with a sterile cotton swab and suspended in a 0.85% NaCl solution containing 0.3% formalin. The bacterial cells were heated in a water bath at 94°C for 60 min and then centrifuged (7,000 × *g* for 30 min) until a clear supernatant was obtained. The supernatant was used as the antigen in the gel diffusion precipitin test.

Antiserum preparation. Antisera (serotypes 1 to 16) provided by National Veterinary Services Laboratory (Ames, Iowa) were reconstituted with 1 ml of sterile water and were then diluted according to the instructions on the label.

Gel diffusion precipitin test. Approximately 10 to 12 ml of 0.9% Noble agar in 0.85% NaCl was placed in the lid of a sterile petri dish (100 by 15 mm) and left to solidify. A pattern of eight wells (seven wells peripherally located around a center well 3.5 mm in diameter and separated approximately 5.5 mm from center to center) was cut in the agar gel. Antigens were placed in the outer wells, while antiserum was placed in the center well. This arrangement provided for the testing of six different antigens against one antiserum (six unknown and one positive control antigen in the seven outer wells). The lids were placed in a covered, high-humidity chamber and incubated at 37°C for 24 to 48 h. The development of precipitation lines was observed by the use of oblique light.

Statistical analysis. (i) RAPD and AFLP analyses. For each organism, data from analyses with all five primer sets used for AFLP analysis were concatenated prior to the production of genetic distances. Data from analyses with the six primer sets used for RAPD analysis were similarly concatenated. We refer to these as the raw AFLP and RAPD analysis data, respectively. Two methods of combining data for the three AFLP and RAPD analysis runs were investigated: consensus rule and simple concatenation. By the consensus rule method, a band was considered present only if it appeared in all three runs. The second method for combining data was used to evaluate only the AFLP analysis data and was simple concatenation in which all of the data were analyzed. Additionally, the collapsed AFLP and RAPD analysis data were concatenated to produce a data set labeled the combined AFLP and RAPD analysis data.

Neighbor-joining analyses under the distance criterion were performed with the software program PAUP* 4.0 (D. L. Swofford 2000; test version β4a) to estimate genetic similarities between the clinical isolates and the M-9 vaccine strain. In all analyses, the M-9 vaccine strain was designated the outgroup. Distances based on the concatenated AFLP analysis data, collapsed AFLP anal-

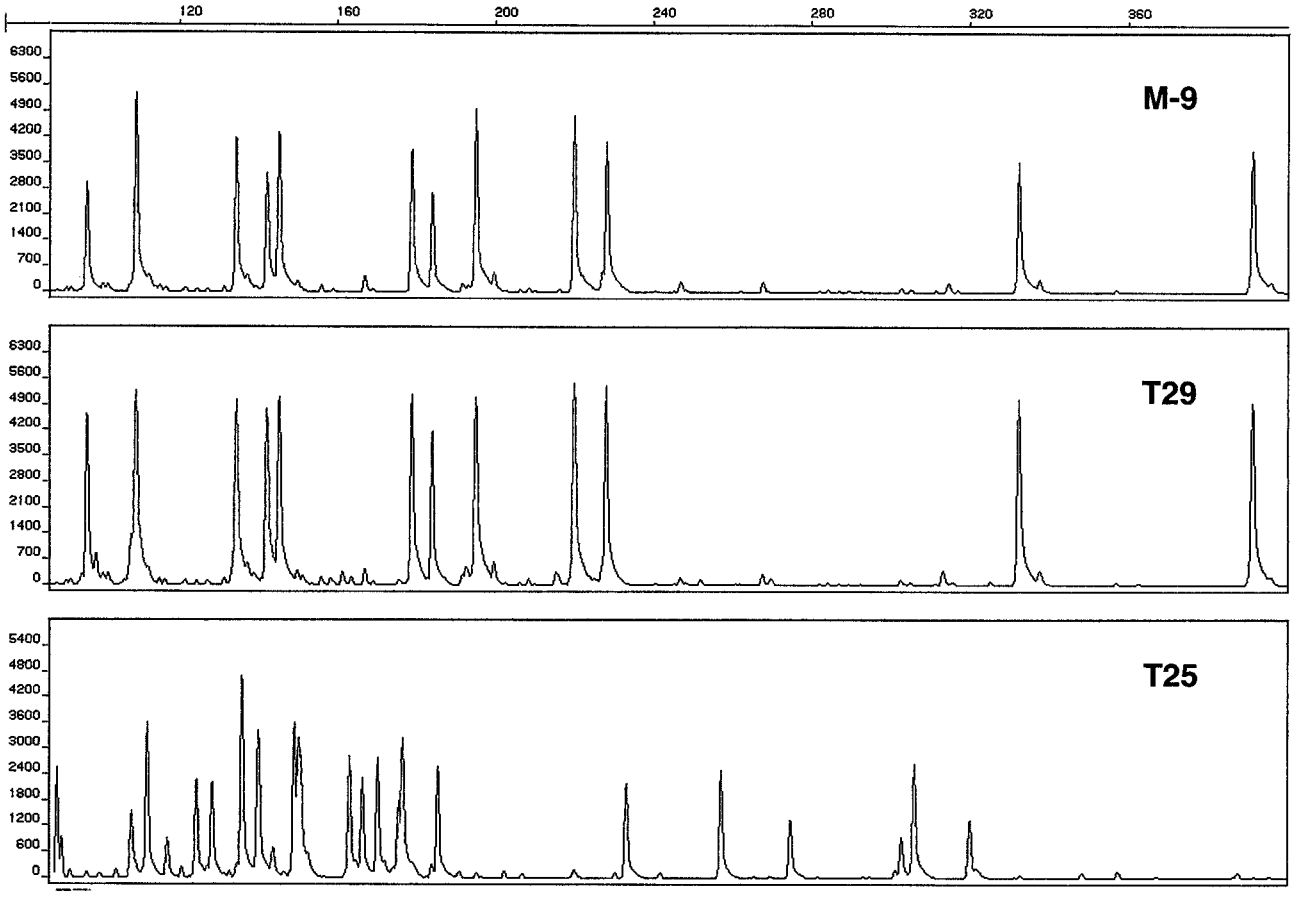

FIG. 2. Portions of three electropherograms generated from AFLP analysis of three *P. multocida* isolates. The M-9 vaccine strain and isolate T29 are genetically similar. Isolate T25 is genetically dissimilar to the M-9 vaccine strain.

ysis data, collapsed RAPD analysis data, and the combined AFLP and RAPD analysis data were generated.

The freely available *R* statistical environment (http://www.R-project.org) was used for all statistical analyses. Correlations of the distances between the four data sets were analyzed, and scatterplots illustrating these correlations were produced. A standard contingency table analysis was performed to examine relationships between vaccination status and serotype. Permutation tests were performed in the *R* statistical environment on both the RAPD and AFLP distance data as described below (5).

(ii) Vaccination status. The influence of vaccination on the genetic relatedness of clinical isolates to the M-9 vaccine strain was evaluated by subtracting the mean genetic distance for the isolates from vaccinated turkeys to the M-9 vaccine strain (V) from the mean genetic distance for the isolates from unvaccinated turkeys to the M-9 vaccine strain (U). Since the distribution of distances (D) was unknown, a permutation test was run to evaluate the significance of the test statistic value. As 27 of the 61 isolates used to obtain the original data were from vaccinated turkeys, the test was performed by randomly assigning 27 of the distances to be "vaccinated" and the rest to be "unvaccinated." The test statistic $D = U - V$ was calculated for each sample, and this test was repeated 100,000 times.

(iii) Serotype analysis. Each sample was categorized according to vaccination status and serotype. These data were entered into a contingency table. A chi-square test of independence was performed to determine whether or not serotype and vaccination status were independent. Serotypes represented by only one organism (serotypes 4,7 and 4,12 and that for the untypeable organism) were excluded, yielding a total of 58 observations.

(iv) Geographic clustering. To test for a geographic trend, farm locations obtained by the use of a Global Positioning System unit were divided into four approximately equally sized geographic regions. A Kruskal-Wallis rank sum test was performed by use of the ranked distance data. This test was chosen because the estimated genetic distances were nonnormally distributed.

RESULTS AND DISCUSSION

Serotype analysis. Of the 61 clinical isolates, 7 were of serotype 1, 7 were of serotype 3, 21 were of serotype 3,4, 6 were of serotype 4, 1 each was of serotypes 4,7 and 4,12, and 17 were of serotype 5. Even after repeated testing, one isolate was untypeable. The M-9 vaccine strain reacted as serotype 3,4.

Analysis of the relationship between serotype and vaccination status showed that the two were independent ($P = 0.32$). Comparison of the serotype results with vaccination status showed that serotyping did not provide sufficient information to determine any involvement of the vaccination process in disease. Since the majority of *P. multocida* isolates that cause fatalities are characterized only by serotyping, little useful information is commonly collected for these isolates.

Geographic clustering. Figure 1 shows the geographic distributions of *P. multocida* isolates from Sanpete County, Utah, that caused fatal infections; the isolates are color coded by serotype. Visual inspection shows limited clustering that might suggest some correlation between location and serotype. However, statistical analysis showed that there was no significant geographic clustering with respect to either serotype or genetic profile. The spatial distribution of vaccination status was also random and showed no significant clustering (data not shown). In addition, the P value by the Kruskal-Wallis rank sum test

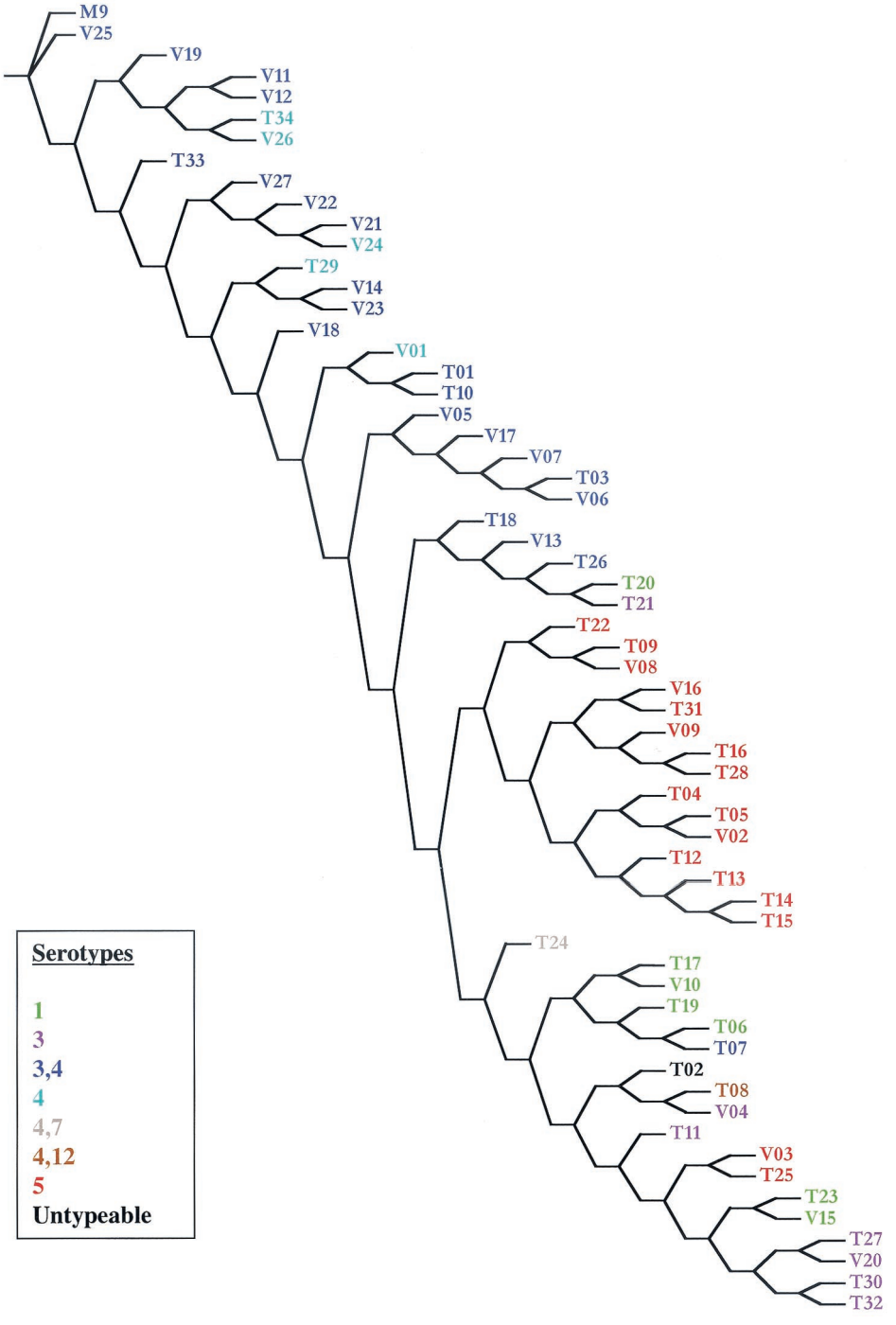

FIG. 3. Cluster analysis tree of the concatenated AFLP analysis data generated by the neighbor-joining method. Isolates from vaccinated turkeys are identified by a V prefix, while unvaccinated turkeys are identified by a T prefix. Isolates are color coded according to serotype (see key).

with vaccinated and unvaccinated samples was 0.35. Thus, with correction for vaccination status, the true mean genetic distance was the same for all four regions, indicating no statistically significant influence of geographic location on genetic profile.

RAPD and AFLP analyses. Similar results were obtained by the RAPD and AFLP techniques. Distances based on neighbor-joining analysis calculated with the collapsed and concatenated AFLP analysis data were virtually identical (correlation = 0.998). The combined AFLP and RAPD analysis data yielded distances identical to those obtained with the concatenated AFLP analysis data (correlation = 1.000), indicating that the RAPD analysis data contained no additional information. In fact, RAPD analysis provided only 77% of the detail provided by AFLP analysis. This was expected, since the mean number of bands generated by AFLP analysis was much

greater than the mean number generated by the RAPD technique.

Figure 2 shows portions of electropherograms obtained by AFLP analysis for three representative isolates. These three isolates consist of the M-9 vaccine strain, one isolate that caused a fatality and that is genetically similar to the M-9 vaccine strain, and one isolate that caused a fatality and that is genetically dissimilar to the M-9 vaccine strain. Differences and similarities in band patterns are clearly evident, showing the discriminatory value of AFLP analysis in the genetic typing of these isolates.

Because of the relatively long primers (~16 bases), amplification of polymorphisms by AFLP analysis was much more stringent and reproducible than that by RAPD analysis. In addition, the use of internal lane size markers and polyacrylamide gels allowed more accurate and more precise measurements of the polymorphisms generated by AFLP analysis. Bands within the gels used for AFLP analysis were sufficiently defined to permit automated gel analysis. Although RAPD analysis was quicker and less labor intensive, the smaller, random primers were more permissive and more time was required to achieve optimization by RAPD analysis than by AFLP analysis. In addition, although RAPD analysis is reliable and the results are reproducible in the same laboratory, difficulties have been reported when results from different laboratories have been compared (6, 19, 23). In summary, RAPD analysis was quicker and less technical, but AFLP analysis was more consistent and easier to optimize.

Vaccination status. Previous studies of the nature of the present study have used the unweighted pair group method with arithmetic averages (UPGMA) as a cluster analysis algorithm (1, 12, 13, 14, 15, 16, 22). However, neighbor-joining analysis was selected over UPGMA for use in this study because it does not assume ultrametric distances in the data. Ultrametric distances are distance values that fit a rooted tree with a constant molecular clock (9). By not invoking a constant molecular clock, the assumption that all lineages in this study have diverged in equal amounts is eliminated and the genetic variations of the isolates can be studied more realistically (22).

Neighbor-joining analysis was used to create a distance-based tree for the concatenated AFLP analysis data (Fig. 3). The difference in the mean genetic distance from the M-9 vaccine strain between unvaccinated and vaccinated isolates, calculated by using the AFLP analysis data, was 0.0182. The permutation test, based on 100,000 runs, yielded a distribution of differences in mean distances from which significance could be evaluated. Isolates from vaccinated turkeys were significantly closer genetically to the M-9 vaccine strain than those from unvaccinated turkeys were ($P = 0.02$). RAPD analysis data showed a similar statistical link between genetic distance from the M-9 vaccine strain and vaccination status. Figure 3 also showed notable clustering of some serotypes, indicating a distinct relationship between genetic differences and the antigenic compositions of the isolates. Most prominent is the individual clustering of serotype 3,4 and serotype 5.

The exact role that vaccination plays in fatalities caused by *P. multocida* or whether or not the M-9 vaccine strain is directly involved cannot be determined from the existing data. Further studies, including the analysis of sequence data, are needed to identify precisely how vaccination contributes to this disease. Some possible explanations are considered below.

Although the M-9 vaccine strain maintains a low level of virulence and could possibly kill birds experiencing a concomitant stress, this scenario is not consistent with the data herein since only 12 of the 27 isolates (44%) were of the same serotype as the vaccine strain. It is conceivable that both the M-9 vaccine strain and a wild strain are involved in the genesis of fatal disease. Conditions commonly found on turkey farms such as heat and overcrowding, the presence of predators, and the presence of other pathogens operating within the flock can stress and compromise turkeys. The vaccination process itself may weaken birds sufficiently to make them susceptible to agents with lower levels of virulence. It is possible that these factors could increase the risk of fatal disease by strains that happen to share genetic similarities with the M-9 vaccine strain.

In summary, this work has shown that pathogenic isolates from vaccinated turkeys are more genetically similar to the M-9 vaccine strain than isolates from nonvaccinated turkeys are. This relationship could not have been detected by the use of serotyping data alone, even though there was a significant association between the serotype and the genetic profile. This illustrates the importance of genetic analyses in these types of epidemiological investigations. No link between geographic location and the genetic profile of an isolate was observed, indicating the lack of any obvious specific disease reservoir. Future research involving the identification of specific genomic regions will likely result in a better understanding of the role that vaccination plays in fowl cholera epidemics.

ACKNOWLEDGMENTS

This work was supported by grants from the Utah Turkey Marketing Board and Brigham Young University.

We thank Adam Gregory, Scott Hawkins, Douglas Huber, and John Smart for work on this project.

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