AVIAN DISEASES 43:756–762, 1999

Spatial Heterogeneity of Escherichia coli DNA Fingerprints Isolated from Cellulitis Lesions in Chickens

Randall S. Singer,[AE] Joan S. Jeffrey,[B] Tim E. Carpenter,[A] Cara L. Cooke,[C] Richard P. Chin,[D] E. Rob Atwill,[B] and Dwight C. Hirsh[C]

[A]Department of Medicine and Epidemiology
[B]Department of Population Health and Reproduction
[C]Department of Pathology, Microbiology, and Immunology
[D]California Veterinary Diagnostic Laboratory System, Fresno Branch,
School of Veterinary Medicine, University of California, Davis, CA 95616

Received 12 April 1999

SUMMARY. Avian cellulitis in broiler chickens is characterized by subcutaneous lesions that result in economic losses because of the partial or complete condemnation of the carcasses at processing. *Escherichia coli* is the primary causative agent of this condition. Previous research with a biotyping system found that the *E. coli* of cellulitis origin were unique to each ranch, suggesting that these *E. coli* were endemic within the ranch environment. The objective of our study was to analyze the genetic variability of *E. coli* isolates associated with cellulitis. We analyzed the genetic relatedness of the isolates in relation to the houses, ranches, and complexes in which the broilers were grown. This analysis enabled us to assess the spatial heterogeneity, or genetic diversity on a spatial scale, of the isolates. Forty-nine broilers with cellulitis lesions were necropsied. These broilers came from six houses on four ranches on three complexes that had been placed with chicks from the same hatchery within a 2-wk period. Isolates of *E. coli* from the lesions were DNA fingerprinted by pulsed-field gel electrophoresis. Relatedness among isolates was determined with the Dice coefficient and an unweighted pair group method with average linkages cluster analysis. The complexes possessed isolates with a variety of DNA fingerprints, yet each complex appeared to have isolates with a unique set of DNA fingerprints. Isolates from the same complex tended to form clusters with similarity coefficients greater than 90%. Isolates from different complexes were genetically distinct. This heterogeneity at the level of the complex suggests that isolates were not disseminated from a source common to the complexes. The spatial heterogeneity of the *E. coli* isolates in this study implies an endemic population of cellulitis-associated *E. coli* exists in the broiler house environment.

RESUMEN. Heterogeneidad espacial de las huellas DNA de Escherichia coli aislada de lesiones de celulitis de pollos.

La celulitis aviar en pollos de engorde se caracteriza por lesiones subcutáneas que producen pérdidas económicas debido a los decomisos parciales o completos de las canales en la planta de procesamiento. El agente causal primario de esta condición es *Escherichia coli.* Algunas investigaciones anteriores que han utilizado un sistema de tipificación biológica encontraron que las cepas de *E. coli* aislada de los casos de celulitis eran únicas para cada granja sugiriendo que estas cepas de *E. coli* son endémicas dentro del medio ambiente de cada granja. El objetivo de este estudio fue el de analizar la variabilidad genética de las cepas de *E. coli* asociadas con celulitis. Se analizaron las cepas aisladas con relación a los galpones, granjas y complejos avícolas en los cuales los pollos habían sido criados. Este análisis permitió determinar la heterogeneidad espacial o la diversidad genética de las cepas en una escala espacial. Se examinaron 49 pollos de engorde con lesiones de celulitis. Estos pollos provinieron de seis galpones de cuatro granjas de 3 complejos avícolas que habían recibido pollitos de la misma incubadora en un período de dos semanas. Se obtuvieron huellas DNA de las cepas aisladas de las lesiones, utilizando geles de electroforesis. La relación entre las cepas fue

[E]Present address: Department of Veterinary Pathobiology, University of Illinois, 2001 S. Lincoln Avenue, Urbana, IL 61802.

determinada utilizando el coeficiente Dice y un método de grupo de pares no pesados con análisis de grupos por conexiones promedio (UPGMA). Los complejos avícolas tuvieron cepas con una variedad de huellas DNA, cada complejo avícola mostró tener cepas con un grupo único de huellas DNA, las huellas del mismo complejo avícola tendieron a formar grupos con coeficientes de similaridad mayores de 90%. Cepas de diferentes complejos avícolas fueron genéticamente diferentes. Esta heterogeneidad a nivel de complejos avícolas sugiere que la diseminación de las cepas no ocurrió mediante una fuente común a los complejos. La heterogeneidad espacial de las cepas de *E. coli* en este estudio implica que existe una población endémica de *E. coli* asociada con celulitis en el medio ambiente de los galpones de pollos de engorde.

Key words: cellulitis, *Escherichia coli,* pulsed-field gel electrophoresis, genetic relatedness, molecular epidemiology

Abbreviations: EDTA = ethylenediaminetetraacetic acid; PFGE = pulsed-field gel electrophoresis

Avian cellulitis in broiler chickens is characterized by a diffuse inflammatory reaction in the subcutaneous tissue that results in the complete or partial condemnation of the carcass at processing (4,12). The incidence of cellulitis in U.S. broilers continues to increase every year. In 1991, U.S. losses were estimated to be $20 million (13,14). Current U.S. losses have been estimated at greater than $80 million per year (17). The economic impact of this disease is due to a combination of losses from 1) those carcasses totally condemned, 2) the carcasses partially condemned, and 3) the slowing of the processing line to remove affected carcasses. Numerous investigators have causally linked the presence of *Escherichia coli* with cellulitis (7,9,19,20). The *E. coli* of avian cellulitis origin are extremely diverse in terms of serotype, biochemical, antimicrobial susceptibility, and virulence factor profiles (6,16,21).

In early reports of this condition, two types of cellulitis were described. Type 1 cellulitis was considered to be initiated at the hatchery, whereas Type 2 cellulitis was considered to be associated with management and environmental factors affecting the birds during growout (13,14). On the basis of recent studies (7,11,19), most researchers and industry professionals now believe that most, if not all, of the cellulitis lesions observed at processing are initiated at some time during growout (18). Although the lesions do not appear to be initiated at the hatchery, the hatchery may still be the source of the *E. coli* affecting the birds during the growout period.

The sequence of events that precipitate the development of cellulitis lesions involves a break in the skin defense followed by infection with *E. coli* (5,7,19,20). Several studies reported that the most common cause of the breach in the skin surface appeared to be a scratch from another bird (2,19,20). Because cellulitis appears to result secondary to a scratch, the inoculated pathogen is likely living in the litter environment. In one cellulitis study, all of the cellulitis *E. coli* biotypes matched the *E. coli* isolated from the litter of the broiler house (3). A previous study in Canada used a biotyping system to characterize *E. coli* of cellulitis origin and found that ranches had their own unique populations of *E. coli* associated with cellulitis (21). These researchers speculated that the populations of *E. coli* strains that caused cellulitis were endemic on the farms and were not disseminated from a source common to the ranches in the study. However, the farms in that study did not receive birds from the same hatchery; therefore, the hatchery could not be evaluated as the potential source of the *E. coli* associated with cellulitis.

This current study was designed so that all birds in the study originated from the same hatchery. In addition, the litter and feed were obtained from the same source. Ranches were enrolled over a short temporal period in order to increase the probability that if *E. coli* associated with cellulitis were being disseminated from the hatchery with the chicks, then all of the ranches in our study would receive similar isolates. The objectives of this study were to 1) assess the spatial heterogeneity of cellulitis-associated *E. coli* from a single integrated broiler company and hatchery over a short temporal period and 2) use the genetic structure and se-

rotype information of the isolates to infer possible disseminating sources of these bacteria. We hypothesized that the *E. coli* from cellulitis lesions are endemic in the broiler environment. This hypothesis would be supported by an increased genetic heterogeneity of the isolates when compared across complexes. However, because ranches on a complex and houses on a ranch have the same workers as well as other similar resources, we hypothesized that *E. coli* isolates would have more genetic similarity within the complex.

MATERIALS AND METHODS

Sampling of *E. coli* from broilers. The broilers originated from three complexes, with one to two ranches enrolled per complex and one to two houses enrolled per ranch (Fig. 1). For this study, a complex was defined as a facility approximately 0.5 × 0.5 km across and containing between two and four ranches. All ranches on a complex had the same laborers and resources, and workers moved among ranches daily. Workers did not move among complexes. A single ranch had between 10 and 16 houses, usually of similar construction. The complexes all had litter (rice hulls) and feed from the same sources. The houses were completely cleaned with new litter provided prior to the placement of the flocks studied. The chicks came from different parent flocks.

Birds with suspected cellulitis lesions were collected from the processing line prior to evisceration so the lesions would be intact and uncontaminated. Forty-nine broilers with cellulitis lesions were sampled by convenience and necropsied. Between 5 and 10 broilers with cellulitis lesions were sampled per house. Each cellulitis lesion was cultured onto MacConkey and blood agar plates. A single isolated lactose-positive colony was then randomly selected from the MacConkey plate (22) and identified as *E. coli* by standard biochemical techniques (10). All isolates were serotyped at the *E. coli* Reference Center (Pennsylvania State University, State College, PA).

Pulsed-field gel electrophoresis (PFGE) of *E. coli* from broilers. DNA fingerprinting was performed on all isolates by PFGE. For extraction of genomic DNA, we utilized the CHEF Bacterial Genomic DNA Plug Kit (BioRad Laboratories, Hercules, CA) as per manufacturer's instructions. Briefly, 62.5 μl of an overnight brain–heart infusion broth culture was centrifuged and washed with TE buffer (10 mM Tris, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0). The cells were resuspended in a cell resuspension buffer (BioRad Laboratories), and an equal volume of molten 2% low-melting-point agarose was added. The combination was mixed and pipetted into the plug mold. Solidified plugs were then treated with a lysozyme solution (BioRad Laboratories) for 1 hr at 37 C followed by digestion in a proteinase K solution (BioRad Laboratories) at 50 C overnight. The plugs were then washed four times in 1× wash buffer (BioRad Laboratories); the second wash contained 1 mM phenylmethylsulfonyl fluoride (PMSF). Plugs were then washed in 0.1× wash buffer for 1 hr followed by 1× restriction enzyme buffer for 1 hr. The plugs were digested with 20 U of restriction endonucleases *Not*I and *Xba*I in separate digestions (New England BioLabs, Beverly, MA) at 37 C overnight.

PFGE was performed with a 1.2% agarose gel on a CHEF III apparatus (BioRad Laboratories) in 0.5× TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8.3) at 14 C and 200 V. Linearly ramped switching times of 5–50 sec and 1–40 sec were used for *Not*I and *Xba*I, respectively, and were applied over 22 hr. Three lanes with molecular size markers were included on every gel. The DNA size standards used were bacteriophage lambda ladder (BioRad Laboratories) consisting of concatemers of 48.5-kb fragments.

After PFGE, the gel was stained with ethidium bromide (0.2 μg/ml) and photographed under ultraviolet transillumination. The gel was then digitized into a computer by the Gel Doc 1000 (BioRad Laboratories) and the software program Molecular Analyst v. 1.4.1 (BioRad Laboratories). The computerized image was analyzed with Molecular Analyst v. 1.12 software (BioRad Laboratories). Only PFGE fragments larger than 100 kb were evaluated in order to eliminate the potential influence of large plasmids.

Genetic similarity between each pair of isolates was assessed with the Dice coefficient of similarity (1). The similarity between pairs of isolates was calculated by: $S_D = 2n_{AB}/(n_A + n_B)$, where n_{AB} is the number of bands common to isolates A and B, n_A is the total number of bands for isolate A, and n_B is the total number of bands for isolate B. This index excludes negative matches from the analysis. Indices for each pair of isolates were based on the banding patterns produced by both *Not*I and *Xba*I digestions. Dendrograms based on the similarity of isolates were created by the unweighted pair group method with average linkages clustering. This allowed the similarity structure of isolates to be assessed quantitatively and graphically.

Linear spatial analyses were conducted to determine whether isolates on the complexes were significantly clustered. A nonparametric test for randomness of runs (15) was used to test the null hypothesis that isolates from complexes A and B were randomly distributed along the dendrogram. This test determined the number of runs of isolates from complexes A and B along the line. If there were fewer runs than expected under the null hypothesis of a random distribution, then the distribution along the line was

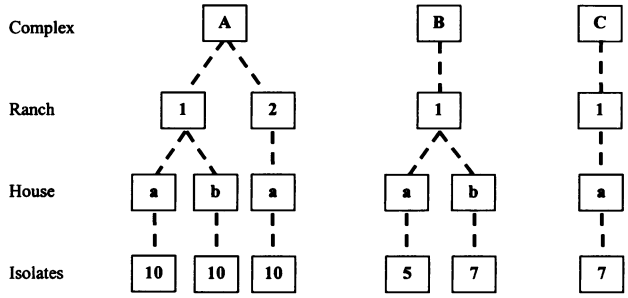

Fig. 1. Hierarchical sampling structure for *E. coli* of avian cellulitis origin in broiler chickens. Spatial information is denoted by complex, ranch, and house. The number of isolates collected from each house is provided in the isolates row.

considered to be clustered. A standard formula was used to determine the 95% confidence interval for the expected number of runs if isolates were randomly distributed. Isolates were considered to be clustered if the observed number of runs was less than the lower limit of the 95% confidence interval.

RESULTS

We analyzed a total of 49 *E. coli* isolates from cellulitis lesions. Six different O serogroups were represented: O78 (n = 10), O11 (n = 8), O36 (n = 6), O115 (n = 4), O65 (n = 2), and O132 (n = 1). Thirty-one of the 49 isolates (63%) were typeable by O serogrouping. We were able to DNA fingerprint 43 of the 49 isolates (88%) by PFGE (Fig. 2). Six of the isolates (12%) did not yield bands after repeated attempts. On the basis of results of other studies, this condition is likely due to the degradation of the DNA during PFGE by endogenous endonucleases (8). Four of these isolates were serogroup O11 and two were O78.

Using the DNA fingerprint data, we constructed a dendrogram based on the Dice sim-

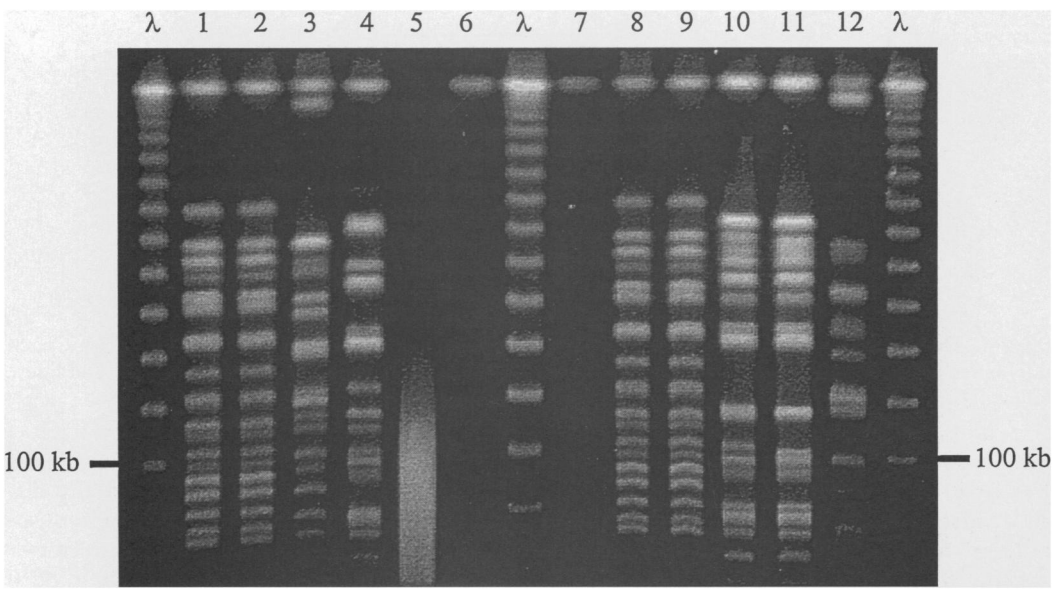

Fig. 2. Pulsed-field gel of DNA obtained from cellulitis *E. coli* that was digested with *Not*I. Lanes with λ represent bacteriophage lambda DNA molecular size markers. Only bands larger than 100 kb were considered in the analysis. Isolates 1, 2, 8, and 9 have identical fingerprints as do isolates 10 and 11. Isolates 6 and 7 had no or few cuts with this enzyme, resulting in large fragments that did not migrate through the gel. Isolate 5 could not be fingerprinted after repeated attempts because of degraded DNA.

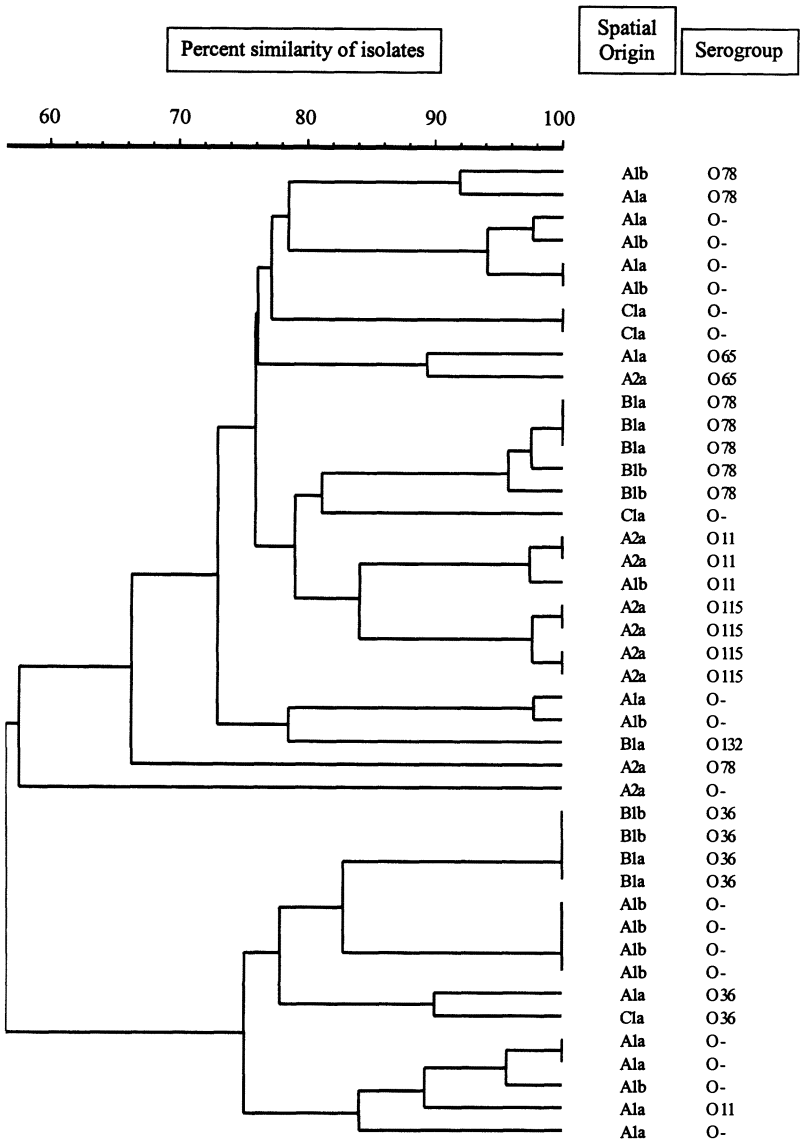

Fig. 3. Dendrogram of *E. coli* isolates from cellulitis lesions based on Dice coefficient of similarity. Each node represents a different isolate. The spatial origin of each isolate is denoted by complex, ranch, and house designation (see Fig. 1). The O serogroup information of each isolate is also provided.

ilarity coefficients for each pairwise comparison of isolates. The DNA fingerprint data used in this dendrogram are a combination of the *Not*I and *Xba*I digestions for each isolate. The dendrogram was then combined with the spatial origin and O serogroup information for each isolate (Fig. 3). Isolates from the same complex tended to form clusters with similarity coefficients greater than 90%. Each of the 10 clusters having a Dice coefficient greater than 90% consisted of isolates from a single complex. Isolates from different complexes were genetically distinct. The closest relationship between two isolates from different complexes was between two isolates of O36 that were approximately 88% similar. Typically, though, isolates from different complexes were less than 80% similar. Isolates from different ranches on the same complex as well as different houses on the same ranch were genetically similar and did not form

unique clusters. Significant clustering ($P < 0.001$) of isolates from complexes A and B along the line of the dendrogram was observed (Fig. 3).

We assessed the relationship of the DNA fingerprint data and the O serogroup information with the dendrogram (Fig. 3). Only isolates that could be typed by both PFGE and serotyping were included. For every cluster that was formed by isolates with greater than 85% similarity, we assessed the diversity of O serogroups within that cluster. A single O serogroup was consistently represented within each cluster. However, multiple different clusters of DNA fingerprints belonged to the same serogroup.

DISCUSSION

We have used a combination of serogroup and DNA fingerprint information in order to assess the spatial heterogeneity of *E. coli* associated with avian cellulitis in broiler chickens. A diversity of O serogroups was represented in the 49 isolates analyzed in this study, a finding supported by other researchers (16,21). However, by including the use of PFGE, we were able to assess the similarity of isolates with a much finer degree of resolution. No instance was found in which isolates belonging to multiple O serogroups had the same DNA fingerprint. However, we did observe isolates with different DNA fingerprints that had the same O serogroup. Fewer isolates were untypeable by PFGE ($n = 6$) as compared with serogrouping ($n = 18$), and PFGE facilitated a more discriminatory assessment of *E. coli* relationships.

The major genetic differences between *E. coli* isolates occurred at the level of the complex. Although the complex possessed isolates with a variety of DNA fingerprints, each complex appeared to have isolates with a unique set of DNA fingerprints. *Escherichia coli* isolates from different ranches on the same complex were not genetically unique. In addition, different houses on the same ranch had genetically similar isolates of *E. coli* associated with avian cellulitis. These findings could be because ranch workers move among all ranches on a single complex and could be carrying *E. coli* from house to house and ranch to ranch.

One possible explanation for the genetic relationships that we observed is that the *E. coli* associated with avian cellulitis were not disseminated from the hatchery or from a source common to the complexes in this study. Because we used complexes that were placed with broiler chicks from the same hatchery within a narrow time frame as well as received litter and feed from the same sources, we might expect any isolates disseminated from these sources to be the same across the complexes. However, if the litter, feed, or hatchery were the disseminating source but had a rapidly changing flora, then the complexes in the study may have initially received different *E. coli.*

Another possible explanation for the observed genetic relationships is that the isolates were disseminated from a common source and experienced genetic drift. Each house could exert its own unique selection pressures on the population of *E. coli* in the environment, and, thus, we would expect some house-specific population differentiation. However, many of the clusters from different complexes were of different serogroups, implying that the clusters were unique and were not originally derived from the same clone. In addition, the band-sharing similarity coefficients that we calculated in this study were based on 20–30 bands for each isolate. If two isolates were 80% similar and each had 20 bands, this would mean that a total of eight bands were not shared. That these two isolates were derived from the same clone would be highly unlikely. Clearly, an estimate of the rate of genetic drift of *E. coli* isolates in the broiler environment is needed.

The spatial heterogeneity of the *E. coli* isolates in this study implies that an endemic population of *E. coli* exists in the broiler environment. To further support the conclusion of endemicity, we are currently using PFGE to assess whether cellulitis-associated *E. coli* isolates persist in successive flocks that use the same house.

REFERENCES

1. Dice, L. R. Measures of the amount of ecological association between species. Ecology 26:297–302. 1945.

2. Elfadil, A. A., J. P. Vaillancourt, and A. H. Meek. Impact of stocking density, breed, and feathering on the prevalence of abdominal skin scratches in broiler chickens. Avian Dis. 40:546–552. 1996.

3. Elfadil, A. A., J. P. Vaillancourt, A. H. Meek, and C. L. Gyles. A prospective study of cellulitis in broiler chickens in southern Ontario. Avian Dis. 40: 677–689. 1996.

4. Elfadil, A. A., J. P. Vaillancourt, A. H. Meek, R. J. Julian, and C. L. Gyles. Description of cellulitis lesions and associations between cellulitis and other categories of condemnation. Avian Dis. 40:690–698. 1996.

5. Glunder, G. Dermatitis in broilers caused by Escherichia coli: isolation of Escherichia coli from field cases, reproduction of the disease with Escherichia coli O78:K80 and conclusions under consideration of predisposing factors. Zentralbl. Veterinaermed. Reihe B 37:383–391. 1990.

6. Gomis, S. M., R. Goodhope, L. Kumor, N. Caddy, C. Riddell, A. A. Potter, and B. J. Allan. Isolation of Escherichia coli from cellulitis and other lesions of the same bird in broilers at slaughter. Can. Vet. J. 38:159–162. 1997.

7. Gomis, S. M., T. Watts, C. Riddell, A. A. Potter, and B. J. Allan. Experimental reproduction of Escherichia coli cellulitis and septicemia in broiler chickens. Avian Dis. 41:234–240. 1997.

8. Izumiya, H., J. Terajima, A. Wada, Y. Inagaki, K. I. Itoh, K. Tamura, and H. Watanabe. Molecular typing of enterohemorrhagic Escherichia coli O157: H7 isolates in Japan by using pulsed-field gel electrophoresis. J. Clin. Microbiol. 35:1675–1680. 1997.

9. Jeffrey, J. S., R. P. Chin, and R. S. Singer. Assessing cellulitis pathogenicity of Escherichia coli isolates in broiler chickens using an in vivo inoculation model. Avian Dis. 43:491–496. 1999.

10. MacFaddin, J. F. Enterobacteriaceae and other intestinal bacteria. In: Biochemical tests for identification of medical bacteria. J. F. MacFaddin, ed. Williams and Wilkins, Baltimore. pp. 439–464. 1980.

11. McMurtrey, B. L., R. A. Norton, K. S. Macklin, and S. F. Bilgili. Investigations into the role of Escherichia coli in yolk sac and cellulitis infections. Poult. Sci. 76:145. 1997.

12. Messier, S., S. Quessy, Y. Robinson, L. A. Devriese, J. Hommez, and J. M. Fairbrother. Focal dermatitis and cellulitis in broiler chickens: bacteriological and pathological findings. Avian Dis. 37: 839–844. 1993.

13. Morris, M. P. Cellulitis in broilers. Broiler Industry September:32–40. 1991.

14. Morris, M. P. Broiler cellulitis update. Broiler Industry March:36–39. 1994.

15. Naus, J. I. The distribution of the size of the maximum cluster of points on a line. J. Am. Stat. Assoc. 60:532–538. 1965.

16. Ngeleka, M., J. K. P. Kwaga, D. G. White, T. S. Whittam, C. Riddell, R. Goodhope, A. A. Potter, and B. Allan. Escherichia coli cellulitis in broiler chickens—clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infect. Immun. 64:3118–3126. 1996.

17. Norton, R. A. Old principles and new research. Broiler Industry February:28–33. 1998.

18. Norton, R. A., and S. Bilgili. Type-1 IP: does it really exist? Broiler Industry December:40–46. 1996.

19. Norton, R. A., S. F. Bilgili, and B. C. McMurtrey. A reproducible model for the induction of avian cellulitis in broiler chickens. Avian Dis. 41: 422–428. 1997.

20. Peighambari, S. M., R. J. Julian, J. P. Vaillancourt, and C. L. Gyles. Escherichia coli cellulitis—experimental infections in broiler chickens. Avian Dis. 39:125–134. 1995.

21. Peighambari, S. M., J. P. Vaillancourt, R. A. Wilson, and C. L. Gyles. Characteristics of Escherichia coli isolates from avian cellulitis. Avian Dis. 39: 116–124. 1995.

22. Singer, R. S., W. O. Johnson, J. S. Jeffrey, R. P. Chin, T. E. Carpenter, E. R. Atwill, and D. C. Hirsh. A statistical model for assessing sample size for bacterial colony selection: a case study of Escherichia coli and avian cellulitis. J. Vet. Diagn. Invest. In press. 1999.

ACKNOWLEDGMENTS

This work was supported by a Division of Agricultural and Natural Resources competitive grant and by the Center for Food Animal Health, University of California, Davis. We thank Lori Hansen, Michelle Ganci, and Vera Perez for their technical assistance and Dr. Walter Boyce for invaluable contributions. We thank the California Poultry Industry Federation for its support.