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著者 Auther(s)	Ozaki, H.; Yonehara, K.; Murase, T.
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Virulence of *Escherichia coli* isolates obtained from layer chickens with colibacillosis associated with pericarditis, perihepatitis, and salpingitis in experimentally infected chicks and embryonated eggs

H. Ozaki,[AB] K. Yonehara, [A] T. Murase [ABC]

[A] Laboratory of Veterinary Microbiology, Faculty of Agriculture, Tottori University, 4-101 Koyama, Tottori, 680-8553, Japan.

[B] Avian Zoonosis Research Center, Faculty of Agriculture, Tottori University, 4-101 Koyama, Tottori, 680-8553, Japan.

[C] Corresponding author. E-mail: murase@muses.tottori-u.ac.jp

Short title: Virulence of avian pathogenic *Escherichia coli*

SUMMARY. To evaluate the virulence of avian pathogenic *Escherichia coli* (APEC) isolates obtained from colibacillosis cases associated with pericarditis, perihepatitis, and salpingitis, the embryo lethality assay and experimental infection model in chicks were used in this study. According to the established criteria based on mortality in the embryo lethality assay for evaluating the virulence of *E. coli* isolates, 23 of the 26 APEC isolates associated with pericarditis and perihepatitis and 8 of the 20 isolates associated with salpingitis were found to be virulent. Isolate D137 that had been obtained from a case with pericarditis and perihepatitis and had an embryo mortality of 92% and isolate D445 that had been obtained from a case with pericarditis and perihepatitis and had an embryo mortality of 17% were used for the experimental infection. Four of the five 11-day-old chickens inoculated through the air sac with isolate D137 died 1 day post-inoculation, and the challenge strain was recovered from the air sac, pericardial sac, or liver; however, colibacillosis lesions were found in only one of the five birds postmortem. All five chicks inoculated with isolate D445 survived for 7 days post-inoculation and exhibited airsacculitis or pericarditis lesions at 7 days post-inoculation; the challenge strain was not recovered from the lesions postmortem. The results obtained in this study suggest that the different APEC isolates tested cause illness in chickens through distinct pathogenesis.

Key words: avian pathogenic *Escherichia coli*, chicken, colibacillosis, embryo lethality assay, infection model, virulence

Abbreviations: APEC=avian pathogenic *Escherichia coli*; DHL= desoxycholate hydrogen sulfate; PBS=phosphate-buffered saline; PFGE=pulsed-field gel electrophoresis; TSA=tryptosoya agar

INTRODUCTION

Colibacillosis in chickens is referred to as either localized or systemic infection with *Escherichia coli*, and the *E. coli* strains causing colibacillosis are designated as avian pathogenic *E. coli* (APEC) (10). Although airsacculitis, pericarditis, and perihepatitis associated with septicemia resulting from APEC infection though the respiratory route are most common in broiler chickens (4), cases of such diseases and salpingitis caused by APEC infection in laying hens have also been found (15, 16, 19). Putative virulence genes suggested to play a role in the pathogenesis of colibacillosis have been found in APEC (4, 5, 18). Genotypes including combinations of virulence genes or phenotypes are variable among the strains; for example, different clinical *E. coli* isolates have different serotypes (3, 8). Moreover, *E. coli* can infect as either a primary or a secondary pathogen (2, 14, 18). To assess the virulence of APEC strains, chicken infection models, wherein the infection is induced through the intra air sac and subcutaneous inoculation or aerosol are most often used (1). However, Wooley *et al*. (21) showed that the embryo lethality assay can differentiate between virulent and avirulent *E. coli* isolates and that the results of the assay are consistent with those obtained using the intravenous and subcutaneous challenge models (6, 7).

We obtained APEC isolates from cases of colibacillosis in laying hens associated with pericarditis and perihepatitis, which were considered to be closely related to each other based on the pulsed-field gel electrophoresis (PFGE) analysis (19), indicating that these isolates were found to be primary pathogens. In other cases with salpingitis (15), PFGE patterns of the salpingitis isolates varied to each other and these isolates might have behaved as secondary pathogens. However, this speculation was only based on indirect evidence. Therefore, the aim of the present study was to evaluate the virulence of APEC isolates from different origins using the embryo lethality assay and the experimental infection model.

MATERIALS AND METHODS

Bacterial isolates. Twenty-six *E. coli* isolates obtained from cases of colibacillosis in laying hens associated with pericarditis and perihepatitis (19) and 20 isolates obtained from cases with salpingitis (15) were used in this study. The pulsed-field gel electrophoresis (PFGE) patterns of these isolates have been analyzed previously (15, 19). These patterns were previously distinguished by more than 7-band differences between isolates and re-designated as patterns A through J according to the established criteria for bacterial typing using PFGE (20). Isolates belonging to patterns A and J are further distinguished by one- to three-band differences and designated as A1 through A4 and J1 through J3, respectively (Fig. 1). We also used four isolates (21001, 21023, 21034, and 21062) obtained from fecal samples of healthy broiler chickens (14), and the presence of putative virulence genes in these isolates was examined by a PCR assay using previously designed primer pairs (5).

For the embryo lethality assay and experimental infection, the bacterial isolates were cultured overnight on tryptosoya agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) (TSA) plates and then washed three times with phosphate-buffered saline (PBS), suspended, and diluted in PBS to obtain the desired viable cell counts. Enumeration of the viable cell counts was performed by spread-plating 0.1 mL of the dilution onto TSA plates. The actual numbers of *E. coli* inoculated are described below.

Embryo lethality assay. The embryo lethality assay was performed as described previously (11) with slight modifications. Briefly, 100-300 colony-forming units (CFU) in 0.1 mL of PBS were inoculated into the allantoic cavity of twelve 12-day-old embryonated eggs. Twelve PBS-inoculated embryos were used as controls. The eggs were candled daily, and deaths were recorded for 2 days post-inoculation. Based on mortality rates, the

pathogenicity was determined according to the criteria established previously by Wooley *et al*. (21).

Experimental infection model. Ten-day-old layer chicks were obtained from a commercial source (N.G.C., Inc., Hyogo, Japan) and raised to 11 days of age in isolation units with ad libitum access to food and water. The birds received a 0.1-mL inoculum of 1 × 10^7 CFU of *E. coli* strains isolated from cases with perihepatitis (D137) and salpingitis (D445) and a fecal strain 21034; the inoculum was administered into the right thoracic air sacs. Clinical symptoms and mortality were recorded 6, 12, 24, 36, and 48 h post-inoculation and subsequently on a daily basis. When the chickens died, they were examined for the presence of gross lesions typical of colibacillosis, including airsacculitis, pericarditis, and perihepatitis (9). Samples for bacteriological examinations were taken from the air sacs, pericardial sac, and liver (9). Recovery of the challenge *E. coli* strain from direct streaking onto desoxycholate hydrogen sulfate (DHL) agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) plates was determined using the PFGE patterns of the chromosomal DNA of the isolates after digestion with *Xba*I (Takara Bio Inc., Japan). Chickens surviving 7 days post-inoculation were killed by cervical dislocation. Gross pathologic and bacteriological examinations were performed as described above. Differences in the mortality and the proportion of the presence of the lesions between birds inoculated with each of the isolates were evaluated by application of the Fisher's exact test. Differences were considered significant at $P < 0.05$. All of the procedures performed on the birds were approved by the Animal Research Committee of Tottori University (approval no. 10-T-4).

RESULTS AND DISCUSSION

The mortality associated with the isolates at 2 days post-inoculation in the embryo

lethality assay varied from 0% to 92% (Fig. 1). Variation in the ability of APEC isolates to cause mortality has also been reported in previous studies (6, 12). A mortality rate of >29%, which is considered to be virulent according to the criteria described by Wooley *et al*. (21), was observed in 23 of the 26 *E. coli* isolates obtained from cases associated with pericarditis and perihepatitis in our previous study (19). Of the 20 isolates associated with salpingitis (15), eight were found to be associated with a mortality rate of >29%. None of the fecal *E. coli* isolates obtained from the healthy broilers was found to cause mortality in the embryos. Of the four fecal isolates, strain 21034 harbored the *astA* gene and the other three strains had no putative virulence genes.

The results of the experimental infection are summarized in Table 1. The challenge *E.coli* isolates D137, D445, and 21034 showed a mortality rate of 92%, 17%, and 0%, respectively, in the embryo lethality assay (Fig. 1) and were considered to be virulent, moderately virulent, and avirulent, respectively (21).

All five chickens inoculated with isolate D137 exhibited depression and anorexia for 12 h post-inoculation, and four chickens died 1 day post-inoculation (Table 1). Although the challenge *E. coli* strain was recovered from the air sac, pericardial sac, or liver samples of the dead birds, airsacculitis lesions were observed only in one chicken. The other bird survived for 7 days and did not show colibacillosis lesions; *E. coli* was also not recovered at necropsy in this bird. These results suggest that isolate D137 caused mortality in most of the chickens through acute or peracute respiratory-origin colisepticemia before the lesions developed, because the birds died 1 day post-inoculation and the challenge *E. coli* strain was recovered from the visceral organs. To understand the molecular pathogenesis of *E. coli*, the further analysis of the occurrence of specific virulence determinants and mortality or lesions in the animal challenge is necessary.

All five chickens inoculated with isolate D445 exhibited depression and anorexia for 6 h post-inoculation (Table 1). After 1 day post-inoculation, these symptoms were not

observed in any of the birds, and they survived for 7 days. At necropsy, typical colibacillosis lesions, including airsacculitis and pericarditis lesions, were observed in all birds inoculated with isolate D445. However, *E. coli* strains were not recovered from all of the birds. All of the birds appeared to be clinically normal at the end of the experiment. Therefore, it was suggested that the birds developed transient respiratory infection and were under recovery at day 7 of infection. Moreover, the birds were likely to have transient bacteremia, because a pericarditis lesion was found in one of the birds. In the five chickens inoculated with isolate 21034, anorexia or depression was observed for 6 h post-inoculation (Table 1). After 12 h post-inoculation, these symptoms were not observed in any of the birds, and they survived for 7 days. Colibacillosis lesions were not observed in these birds, and *E. coli* was not recovered. The mortality in the birds inoculated with isolate D137 and the proportion of the presence of the lesions in the birds inoculated with isolate D445 were significantly ($P < 0.05$) higher, respectively, than the birds of the other experimental groups.

In a previous study, (19) isolated strains with the PFGE pattern A1, including strain D137, from birds in multiple chicken houses and therefore suggested that these strains might have been solely associated with the pathogenesis of colibacillosis in the commercial layer farm. In this previous study, the commercial layer chickens from which isolate D137 was isolated exhibited pericarditis and perihepatitis resulting from colisepticemia. The progress after infection with these strains in this farm may have been slower than that observed in the experimental infection models in the present study because the affected birds were much older than those used in this study (approximately 20 weeks) and the infectious dose may have been much less than that in the present study. In another study, additional *E. coli* isolates with PFGE patterns different from that of isolate D445 were recovered from the reproductive tract of the affected bird from which isolate D445 was obtained (15). Therefore, we hypothesized that isolate D445 that was obtained

from a commercial layer chicken associated with salpingitis may be a moderate or secondary pathogen. Further studies using layer chicken models of experimental infection are necessary to understand the role of isolate D445 in the pathogenesis of salpingitis. Pors *et al*. (17) recently developed an experimental model in chickens for ascending infection of the reproductive tract, and the same group revealed considerable variation in the virulence of different strains of *E. coli* (13).

In summary, in the present study, we used the embryo lethality assay and showed that the mortality associated with different *E. coli* isolates isolated from cases with colibacillosis associated with pericarditis, perihepatitis, and salpingitis is different. Our findings indicate that two representative isolates with different embryonic mortality cause illness in chickens through distinct pathogenesis. Although only a limited number of isolates was used in the present study, the results of the embryo lethality assay were consistent with those obtained using the experimental infection models, similar to the findings of previous studies (6, 7). Thus, chicken embryos may be useful to evaluate the virulence of isolates of various origins, including affected birds, feces, and dust, in chicken farms to understand the etiologic aspects of colibacillosis.

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Figure legends

Fig. 1. Mortality rates of embryos inoculated with *Escherichia coli* strains from cases with colibacillosis in the embryo lethality assay.

Isolates with pulsed-field gel electrophoresis (PFGE) patterns A1, A2, A3, A4, B, and C were isolated from chickens associated with pericarditis and perihepatitis (19), and those with patterns D, E, F, G, H, I, J1, J2, and J3 were from chickens associated with salpingitis (15). The dashed line represents the threshold differentiating virulent isolates (21). Strains D137 (arrowhead) and D445 (arrow) were used for experimental infection in the chickens (see Materials and Methods).

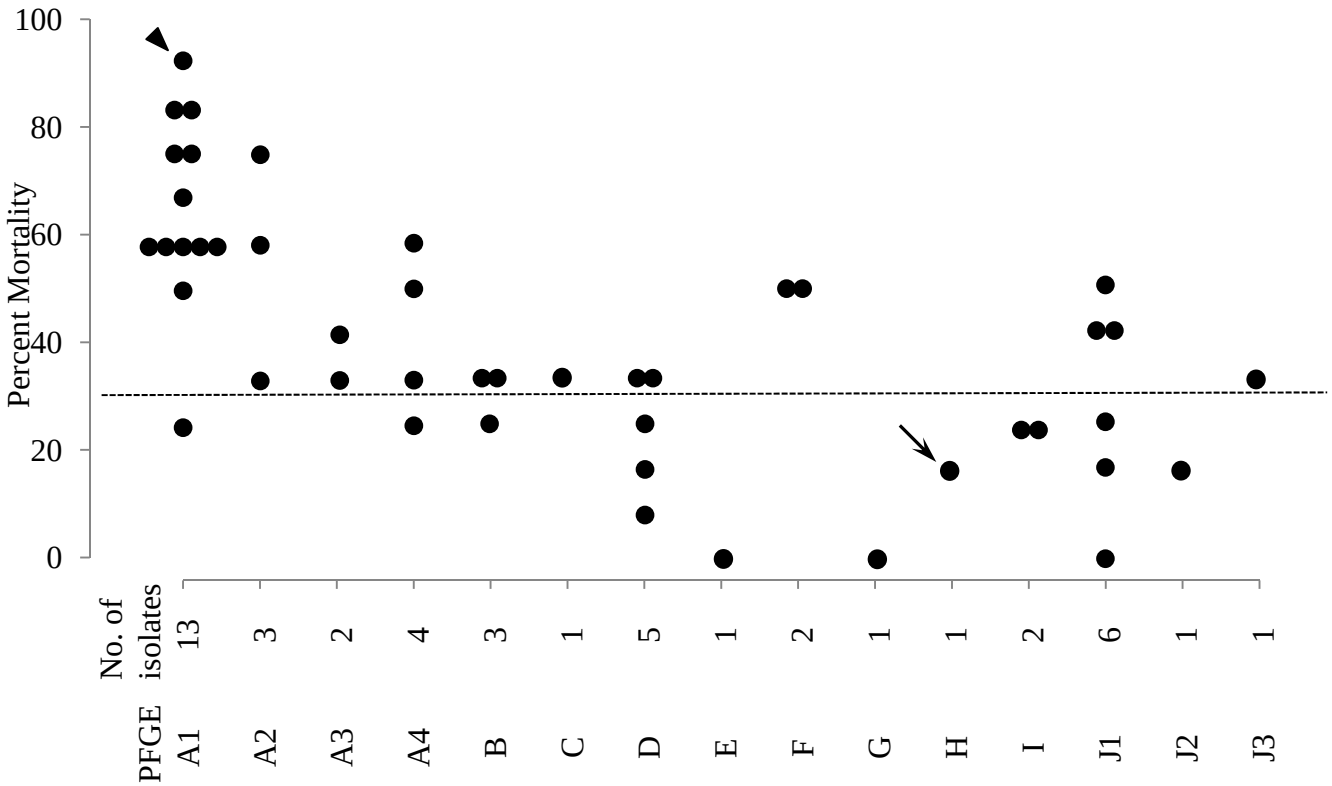
Percent Mortality
100
80
60
40
20
0
No. of isolates
PFGE
13
A1
3
A2
2
A3
4
A4
3
B
1
C
5
D
1
E
2
F
1
G
1
H
2
I
6
J1
1
J2
1
J3

Table 1. Clinical symptoms, lesions, and mortalities in chickens inoculated with *Escherichia coli* isolates D137, D445, and 21034.

		Clinical symptoms at hours post-inoculation[A]										Gross lesions[B]			Recovery of *E. coli*[C]
Isolate	Bird	6	12	24	36	48	72	96	120	144	168	Airsacculitis	Pericarditis	Perihepatitis	
D137	1	A	A	D	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-	+
	2	A	A	D	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-	+
	3	A	A	D	n/a	n/a	n/a	n/a	n/a	n/a	n/a	-	-	-	+
	4	A	A	D	n/a	n/a	n/a	n/a	n/a	n/a	n/a	+	-	-	+
	5	A	A	N	N	N	N	N	N	N	N	-	-	-	-
D445	6	A	B	N	N	N	N	N	N	N	N	+	-	-	-
	7	A	B	N	N	N	N	N	N	N	N	+	-	-	-
	8	A	B	N	N	N	N	N	N	N	N	+	-	-	-
	9	A	B	N	N	N	N	N	N	N	N	+	+	-	-
	10	A	B	N	N	N	N	N	N	N	N	+	-	-	-
21034	11	A	N	N	N	N	N	N	N	N	N	-	-	-	-
	12	B	N	N	N	N	N	N	N	N	N	-	-	-	-
	13	A	N	N	N	N	N	N	N	N	N	-	-	-	-
	14	A	N	N	N	N	N	N	N	N	N	-	-	-	-
	15	B	N	N	N	N	N	N	N	N	N	-	-	-	-

[A] A, depression and anorexia; B, depression or anorexia; D, death; N, normal; n/a., not applicable.

[B] Results of post-mortem examinatons for dead birds 1 day post-inoculation or birds killed 7 days post-inoculation.

[C] Recovery from the air sacs, pericardial sac, or liver samples during post-mortem examinations.