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# Effect of Normal Intestinal Flora of Chickens on Colonization by Virulent Colicin V-Producing, Avirulent, and Mutant Colicin V-Producing Avian Escherichia coli

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SUMMARY. Colonization of the intestinal tracts of newly hatched chicks with *Escherichia coli* was attempted by swabbing test organisms onto the air-shell of 19-day-old embryos. Test organisms consisted of two virulent *E. coli* isolates, one avirulent isolate, and one laboratory-derived mutant of the avirulent isolate carrying a recombinant plasmid coding for Colicin V production. Chicks were cultured weekly for 3 weeks for total *E. coli* and for the test organisms using selective media. Control chicks were sampled on weeks 1 and 5, and the normal *E. coli* intestinal microflora were examined for the production of colicins. The two virulent *E. coli* isolates maintained colonization of the chicks for the 3-week test period, with titers decreasing from $10^1$ to $10^2$ colony-forming units (CFU)/g of intestine. The avirulent isolate and laboratory mutant did not consistently colonize the intestinal tracts. The majority of intestinal samples taken from the control chicks at 1 and 5 weeks had colicin-producing *E. coli* that were inhibitory to the test organisms.

RESUMEN. Efecto de la flora intestinal normal de pollos sobre la colonización por cepas virulentas de Escherichia coli productoras de colicina-V, cepas avirulentas y cepas mutantes productoras de colicina-V.

Se intentó la colonización por *Escherichia coli* del tracto intestinal de pollitos recién nacidos mediante la inoculación de los microorganismos en estudio en la cámara de aire de embriones de 19 días de edad. Los organismos en estudio consistieron de dos cepas virulentas de *E. coli,* una cepa avirulenta y una cepa mutante avirulenta de laboratorio obtenida de una cepa avirulenta portadora de un plásmido recombinante que codifica para la producción de colicina-V. Durante tres semanas se hicieron cultivos semanales de los pollos para recuento total de *E. coli* y para los organismos en estudio, usando medio selectivo. A las semanas 1 y 5 se tomaron muestras de flora intestinal normal de *E. coli* de los pollos controles y estas fueron examinadas para la producción de colicinas. Las dos cepas virulentas de *E. coli* mantuvieron la colonización de los pollos durante un período de 3 semanas con títulos que disminuyeron de $10^1$ a $10^2$ unidades formadoras de colonias/g de intestino. La cepa avirulenta y la cepa mutante de laboratorio no colonizaron consistentemente los tractos intestinales. La mayoría de las muestras intestinales que fueron tomadas de los pollos controles a la primera y quinta semana contenían cepas de *E. coli* productoras de colicina que fue inhibitoria para los microorganismos en estudio.

Under natural conditions, newly hatched chicks have sterile gastrointestinal tracts (12). The emerging chick is then exposed to intestinal microorganisms from other chicks, feces on the eggshell, litter, feed, and other sources (12). The lower intestinal tract of adult chickens (colon and cecum) contains between $10^6$ and $10^9$ colony-forming units (CFU) of *Escherichia coli* per cm of intestine (5,11,14) or $10^5$ to $10^7$ CFU/g of intestine (16).

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When fed to young chickens, normal chicken microflora markedly inhibit colonization of the chicken with pathogenic *E. coli* and *Salmonella* (21,22,26,27), inhibit colonization of antibiotic-resistant *E. coli* present in the chickens' environment (17), and inhibit translocation of pathogenic *E. coli* from intact intestine to the bloodstream in treated birds (26).

Normal intestinal microflora may affect colonization by other bacteria through competition for nutrients (15) and through competition for common receptor sites (15,21,27). Another factor that may contribute to this exclusion is the production of colicins by the normal flora. Colicins are bactericidal proteins, between 50 and 80 kilodaltons, produced by *E. coli* and other Enterobacteriaceae. About 20 different colicins have been demonstrated (8), all acting against *E. coli* or closely related bacteria (3). Colicins may be encoded by plasmid or chromosomal genes (10,13,25). Modes of colicin action against susceptible bacteria include inactivation of RNA and protein synthesis, degradation of DNA, inhibition of oxidative phosphorylation, and alterations in the membrane permeability of susceptible bacteria (2,13,18,20). Strains of *E. coli* can be resistant to several colicins, and multiple resistances are often found. A bacterium may resist the effects of colicins through loss of colicin receptors, by binding the colicin, or by producing a plasmid-encoded immunity protein (10).

Colicin V (ColV) plasmids, found primarily in virulent, enteric bacteria, encode several virulence-related properties, including ColV production, plasmid transfer-related functions, the aerobactin iron uptake system, increased serum survival, phagocyte resistance, motility, hydrophobicity, and intestinal epithelial cell adherence (25).

The purpose of the present study was to determine the feasibility of colonizing the intestinal tracts of newly hatched chicks by swabbing the organism on the eggshell before hatching, to determine the effect of ColV production on an organisms' ability to colonize the chick intestine, and to determine the inhibitory effect of colicins produced by normal intestinal *E. coli*.

## MATERIALS AND METHODS

**Test organisms.** The test organisms were derived from two virulent avian *Escherichia coli* isolates (V-1 and V-2) (28) and one avirulent isolate (Av) (28). Nalidixic-acid-resistant (NaR) variants of V-1 (V-$1^{NaR}$) and V-2 (V-$2^{NaR}$) were selected for use following multiple passages of the organisms in media containing increasing concentrations of this antibiotic. Strains V-$1^{NaR}$ and V-$2^{NaR}$ both were ColV producers. Av was transformed with the recombinant plasmid pHK11 (6,9,19) to form Av+pHK11. Plasmid pHK11 consists of a 9.4-kb fragment of pColV-K30, which codes for ColV production, immunity, and export, cloned into the vector plasmid pBR322 (9). Acquisition of pHK11 by Av endowed it with the ability to produce ColV.

**Colicin activity.** Colicin production was detected by overlaying chloroform-killed colonies of the test organisms with colicin-sensitive *E. coli* K-12 strains ATCC 23559 and ATCC 23561. Plates were incubated at 37 C for 18 hours and observed for colicin-specific inhibition patterns of bacterial growth of the agar-overlay indicator organisms around the test isolates (7).

**Embryo inoculation and colonization.** Eighty 19-day-old embryonated eggs were divided into five groups: four test groups of 15 eggs each, and one control group of 20. Eggs in the test groups each received 0.2 ml of a 24-hour brain-heart infusion (BHI) broth culture of one of the test organisms (Av, V-$1^{NaR}$, V-$2^{NaR}$, or Av+pHK11; approximately $10^8$ CFU) swabbed onto the shell surface above the air-cell. Eggs in the control group were each swabbed with 0.2 ml of sterile BHI broth. The eggs were allowed to hatch in Horsfall units and were examined daily. Five chicks were killed from each of the four test groups at weeks 1, 2, and 3 postinoculation; intestinal tracts were removed and cultured on appropriate media. Five chicks from the BHI-inoculated control group were also killed at weeks 1, 2, 3, and 5, and the intestinal tracts were examined. To ensure that the chicks did not contain naturally acquired *E. coli* with antimicrobial resistance patterns identical to the test isolates, five uninoculated newly hatched controls were also cultured.

**Bacterial reisolation methods.** The selective media consisted of MacConkey agar containing antibiotics specific for the test organisms. Selective agar media used were ampicillin (50 μg/ml) plus nalidixic acid (Na) (30 μg/ml) for V-$1^{NaR}$ and Av+pHK11, tetracycline (15 μg/ml) plus Na (30 μg/ml) for V-$2^{NaR}$, and Na (30 μg/ml) for Av. MacConkey agar was used to determine total *E. coli* counts. The intestinal tracts were removed from the chicks, minced, weighed, and placed into sterile plastic bags. A known amount of sterile phosphate-buffered saline solution was added to the intestinal contents and homogenized to produce a slurry in a blender (Stomacher Lab-Blender 80; Tekmar Co., Cincinnati, Ohio) (23). Tenfold dilutions of the intestinal contents were inoculated onto the appropriate agar plates and incubated overnight at 37 C; colonies were identified by antibiotic resistance profiles (4) and by plasmid isolation and analysis (1). Bacterial counts of the test organisms and

Table 1. Comparison[A] of the mean $\log_{10}$ colony-forming units (*Escherichia coli* per gram of intestine) for various *E. coli* at 1, 2, and 3 weeks post-hatch isolated on either selective media or MacConkey agar.

| Medium | Organisms | $\log_{10}$ CFU at week[B] 1 | 2 | 3 |
|---|---|---|---|---|
| Selective media | BHI-controls | $0^{a}$ | $0^{a}$ | $0^{a}$ |
| | Av | $4.63^{a}$ | $0^{b}$ | $0^{b}$ |
| | V-$1^{NaR}$ | $4.31^{a}$ | $3.58^{ab}$ | $2.53^{b}$ |
| | V-$2^{NaR}$ | $4.97^{a}$ | $0^{c}$ | $2.15^{b}$ |
| | Av + pHK11 | $2.77^{a}$ | $0^{b}$ | $0^{b}$ |
| MacConkey agar | BHI-controls | $7.23^{a}$ | $6.97^{a}$ | $5.29^{b}$ |
| | Av | $7.80^{a}$ | $6.88^{b}$ | $5.61^{c}$ |
| | V-$1^{NaR}$ | $7.32^{a}$ | $6.81^{a}$ | $6.56^{a}$ |
| | V-$2^{NaR}$ | $7.27^{a}$ | $6.00^{b}$ | $6.90^{a}$ |
| | Av + pHK11 | $6.65^{a}$ | $5.62^{a}$ | $6.26^{a}$ |

[A]Tukey multiple comparison test.
[B]Within a row, means with the same superscript are not significantly different ($P > 0.05$).

total *E. coli* titers were recorded as CFU/g of intestine (28).

**Antibiotic resistance profiles.** Antibiotic resistance profiles of the test and reisolated organisms were determined by the disc diffusion method (4).

**Plasmid isolation.** Plasmid DNA from the test and reisolated organisms was isolated by the method of Birnboim and Doly (1) from overnight BHI broth cultures.

**Reciprocal colicin-sensitivity tests.** The ability of the colicinogenic *E. coli* or the normal microflora to inhibit the test organisms and the ability of the test organisms to inhibit these colicinogenic intestinal *E. coli* were evaluated as follows. The two predominant colicin-producing *E. coli* isolated from the normal chicks' intestinal tracts—Colicin I (ColI) and untypable colicin—were used in the overlay agar of the colicin-production assay in order to evaluate the ability of test organisms to inhibit the ColI and untypable colicin-producing intestinal *E. coli* isolates. A zone of inhibition in the overlay indicated that the organism being tested (Av, V-$1^{NaR}$, V-$2^{NaR}$, Av+pHK11) was inhibitory to the normal, colicin-producing intestinal *E. coli*. In a similar manner, the inhibitory effect of the two intestinal colicin-producing *E. coli* isolates (ColI and untypable) on the test organisms was tested by overlaying the intestinal isolates with Av, V-$1^{NaR}$, V-$2^{NaR}$, or Av+pHK11. Zones of inhibition in the overlay indicated that the normal flora inhibited the test organisms.

**Transformation.** The wild-type avirulent *E. coli* (Av) was made competent for transformation (19) and was transformed (6,24) with recombinant plasmid pHK11 using the Gene Pulser Transfection Apparatus and Pulse Controller (BioRad Laboratories, Richmond, Calif.) set at 25 μF (Farad), 200 Ω, and 2.5 kV (24). Mutants were selected on the basis of the acquisition of ampicillin (Ap) resistance, which was determined by plating the transformants on MacConkey agar plates containing Ap (50 μg/ml) and Na (30 μg/ml).

**Colicin activity of *E. coli* isolated from normal flora.** Three hundred *E. coli* isolates were randomly selected from control MacConkey agar plates at week 1, and 200 isolates were selected at week 5. Colicin activity of these isolates was determined as described above (7).

**Biostatistics.** For each organism within each medium, CFUs/g of intestine were compared across weeks of isolation using an analysis of variance and a Tukey multiple comparison test. Percentages of colicins produced by normal *E. coli* isolated from the intestines of normal chicks were compared at 1 week and 5 weeks of age using a *Z*-test of proportions.

## RESULTS AND DISCUSSION

The total *E. coli* colony counts in the BHI-control group decreased from $10^{7.23}$ CFU of *E. coli*/g of intestine by the end of week 1 to $10^{6.97}$ at week 2 and $10^{5.29}$ by the end of week 3. The total *E. coli* counts for the principal groups ranged from $10^{7.80}$ CFU/g to $10^{5.61}$ over the 3-week sampling period. Av and Av+pHK11 were isolated on selective media at week 1 but not in subsequent weeks. V-$1^{NaR}$ was isolated from chicks at weeks 1, 2, and 3. V-$2^{NaR}$ was isolated at weeks 1 and 3 but not at week 2 (Table 1).

Colicin activity of normal intestinal *E. coli* is shown in Table 2; 431 of the 500 isolates produced colicins. Colicin production of the intestinal *E. coli* was classified into four groups: ColV, ColI, untypable but with similar activity, and nonproducers. Of the 300 *E. coli* isolated

Table 2. Comparison[A] of the percent colicins produced by normal intestinal *Escherichia coli* isolates from chicks at 1 and 5 weeks of age.

| Colicin | % positive reactions | | Z | Significance level |
|---|---|---|---|---|
| | 1 week[B] | 5 weeks[C] | | |
| ColV | 0 | 12.5 | 6.28 | 0.0000 |
| ColI | 40.3 | 50.5 | 1.68 | 0.09 |
| Untypeable | 46.0 | 23.0 | 5.22 | 0.0000 |
| None | 13.7 | 14.0 | 0.10 | 0.92 |

[A]*Z*-test of proportions.
[B]Sample includes 300 *E. coli* colonies.
[C]Sample includes 200 *E. coli* colonies.

at the end of the first week, 121 (40.3%) produced ColI and 138 (46.0%) were untypable; none produced ColV. Forty-one (13.7%) had no colicin activity. A total of 172 of the 200 isolates at week 5 produced colicins, with 25 (12.5%) producing ColV and 101 (50.5%) producing ColI; 46 (23.0%) were untypable. Twenty-eight (14%) of the 200 isolates had no colicin activity.

Test isolates Av, V-1$^{NaR}$, and V-2$^{NaR}$ did not inhibit overlays of a ColI producer and untypable colicin producer isolated from the normal flora. Test strain Av+pHK11 did inhibit the overlays of a ColI producer and untypable colicin producer isolated from the normal flora. The ColI-producing isolates from the intestinal *E. coli* inhibited the growth of Av, V-1$^{NaR}$, V-2$^{NaR}$, and Av+pHK11. Other intestinal *E. coli* producing untypable colicins were not inhibitory to Av, V-1$^{NaR}$, V-2$^{NaR}$, or Av+pHK11.

The population of intestinal *E. coli* found in the normal and test-inoculated chicks developed rapidly following hatching and then declined in numbers over time. An increasing number of intestinal *E. coli* from the control chicks elicited colicin activity from week 1 to week 5. The colicins produced by some of these intestinal *E. coli* inhibited the growth of the test organisms *in vitro,* and some of the intestinal *E. coli* were resistant to the colicin produced by the test strain Av+pHK11. If *in vitro* results can be extrapolated to *in vivo* situations, then the production of colicins by the intestinal microflora might be a factor in preventing colonization of the chick intestine by the test isolates. As predicted by the *in vitro* assays, the two avirulent isolates were unable to persistently colonize the chick. Such results might suggest that colicin interactions between invading bacteria and normal intestinal *E. coli* are complex and that these interactions as well as other factors may affect the invading organisms' colonization of the host. These interactions may need to be considered in competitive-exclusion protocols.

This study indicates that colicin production by normal flora, introduced from the environment, may be another method involved in bacterial exclusion of organisms inoculated orally into newly hatched chicks. This study also showed the feasibility of swabbing organisms onto the air-shell of embryos before hatching as a method of introducing microorganisms into newly hatched chicks.

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