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Production of Toxins (Enterotoxins, Verotoxins, and Necrotoxins) and Colicins by *Escherichia coli* Strains Isolated from Septicemic and Healthy Chickens: Relationship with In Vivo Pathogenicity

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Since the mechanism of virulence of *Escherichia coli* strains pathogenic to birds is not fully understood, the prevalence of toxic factors produced by *E. coli* strains pathogenic to other animals was investigated. A total of 625 *E. coli* strains isolated from visceral organs of chickens with colisepticemia and from feces of healthy chickens in Spain were tested for production of enterotoxins (heat labile [LT] and heat stable [STa]), verotoxins (VT1, VT2, and VT2v), cytotoxic necrotizing factors (CNF1 and CNF2), α-hemolysin (Hly), enterohemolysin (EntHly), colicin V (Col V) and other types of colicins, and necrotic and lethal activities. Only 45 (7%) of avian *E. coli* strains were toxigenic: 20 strains produced a cytotoxic response in HeLa but not in Vero cells, indicating the production of a cytotoxin not related to the VTs; 16 were EntHly$^+$; 5 produced a new cytotonic product that causes the appearance of whitish vacuola in Vero and HeLa cells; 3 synthesized soluble factors that cause lethal activity in mice; and 1 elaborated LT. None of 625 avian *E. coli* strains was positive for production of VTs or CNFs. In contrast, colicinogenicity occurred in 335 (73%) of the 458 septicemic strains and 97 (58%) of 167 fecal isolates ($P < 0.01$), and this property was correlated with in vivo pathogenicity of strains. Thus, 80% ($P < 0.001$) and 66% ($P < 0.001$) of strains producing Col V and other types of colicins were characterized as being of high pathogenicity, whereas only 15% of the noncolicinogenic strains were classified as highly pathogenic. Our results clearly support the special pathogenicity theory, because 60% of the *E. coli* strains belonging to 18 serogroups (O1, O2, O5, O8, O12, O14, O15, O18, O20, O53, O78, O81, O83, O102, O103, O115, O116, and O132) most frequently identified among clinical septicemic strains were classified as highly pathogenic in in vivo assays, whereas only 24% of the strains with O serogroups less prevalent among diseased chickens were considered highly pathogenic ($P < 0.01$).

Toxigenic *Escherichia coli* strains that cause infections in human and domestic animals have been classified into three categories: enterotoxigenic *E. coli* (ETEC), verotoxigenic *E. coli* (VTEC), and necrotoxigenic *E. coli* (NTEC). The ETEC strains may synthesize thermolabile (LT) and/or thermostable (STa and STb) enterotoxins and cause diarrhea in children, calves, lambs, and piglets (7, 10, 12, 25). The VTEC strains may produce three main types of verotoxins (VT1, VT2, and VT2v) that are similar to Shiga toxin synthesized by *Shigella dysenteriae* type 1. VTEC (VT1$^+$ and/or VT2$^+$) strains are recognized as a cause of hemorrhagic colitis and the hemolytic-uremic syndrome in humans who contract infection following the consumption of contaminated foodstuffs such as meat and unpasteurized milk. Epidemiological investigation revealed that cattle frequently excrete VTEC (VT1$^+$ and/or VT2$^+$) strains in their feces and thus may represent a source of infection (12–14). Furthermore, VTEC strains producing VT2v are responsible for the edema disease in pigs (25, 26). We have recently reported that NTEC strains can produce the cytotoxic necrotizing factors CNF1 and CNF2 (8, 16). CNF1 is produced by α-hemolysin-positive (Hly$^+$) *E. coli* strains that cause extraintestinal infections in humans (5, 8) and are isolated from stools of healthy cats (9), whereas CNF2 is synthesized by strains of bovine origin (8, 12). A new type of Hly, enterohemolysin (EntHly), which is different from Hly produced by ETEC strains of porcine origin and *E. coli* strains that cause extraintestinal infections in humans, was recently discovered in VTEC strains (4).

Colisepticemia is responsible for significant economic losses in aviculture in many parts of the world, although the mechanism of pathogenicity of *E. coli* strains that are potentially pathogenic to birds is not fully understood (1, 3, 18, 27, 28, 37, 41, 46–48). Chicken-lethal toxin obtained from avian *E. coli* O2, O45, and O109 was described by Truscott (42), but this toxin has not been observed in the majority of avian *E. coli* strains. However, recent studies indicate that *E. coli* strains isolated from chickens with septicemia produce other toxins that could be implicated in in vivo pathogenicity (15, 23, 24). In the present study, we have investigated the production of different types of toxins in avian *E. coli* strains. To the authors' knowledge, this is the first survey that describes the prevalence of ETEC, VTEC, and NTEC strains among isolates from septicemic and healthy chickens. Furthermore, we have studied the in vivo pathogenicity of strains, a property that was correlated with the synthesis of colicins and the O serogroups of strains.

MATERIALS AND METHODS

Bacterial strains. A total of 458 *E. coli* strains isolated from clinically affected broiler chickens and 167 strains isolated from healthy controls between 1992 and 1993 were collected in numerous commercial hatcheries in the province of Orense (Galicia, Northwest Spain). All strains isolated from diseased chickens

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were recovered from confirmed cases of colisepticemia in which bacteria were obtained in profuse cultures from both heart and liver tissue. Control strains were isolated from the cloacal contents of 100 healthy chickens. From each fecal sample, five colonies of *E. coli* were randomly chosen and serotyped. When one healthy chicken yielded colonies with different serogroups, only one of each serogroup was selected for the study. Isolation and identification of *E. coli* were performed by standard bacteriological methods. Strains were stored at room temperature in nutrient broth with 0.75% agar. Reference *E. coli* strains used as positive and negative controls were m452-C_1 (O63:K−:H−, LT$^+$ STa$^+$), 933 (O157:H7, VT1$^+$ VT2$^+$), H19 (O26:H11, VT1$^+$ EntHly$^+$), MR48 (O75:K95, CNF1$^+$ Hly$^+$), S5 (O15:K?:H21, CNF2$^+$) and K-12-185 (nontoxigenic strain).

Production and detection of toxins. One loopful of each *E. coli* strain was inoculated into a 50-ml Erlenmeyer flask containing 5 ml of tryptone soy broth (pH 7.5) (Oxoid, Basingstoke, United Kingdom) and incubated for 20 h at 37°C in an orbital shaker (200 rpm). Filtrates of cultures treated with mitomycin, extracellular fluids, and sonic extracts were obtained as previously described (6). Filtrates of cultures treated with mitomycin were inoculated on Vero and HeLa cells for the detection of LT, VTs, CNF1, and CNF2. Extracellular fluids were assayed for STa enterotoxin by the infant mouse test, and sonic extracts were tested for necrosis in the rabbit skin test and for lethality in the mouse intraperitoneal test. All cited assays have been described in previous papers (6, 12, 16). Briefly, Vero and HeLa cell assays were performed with cell monolayers grown nearly to confluence in plates with 24 wells. At the time of assay, the growth medium was changed (0.5 ml per well), and 75 μl of undiluted filtrate of culture treated with mitomycin was added. Vero and HeLa cells were incubated at 37°C in a 5% CO_2 atmosphere, and the morphological changes in cells were observed under a phase-contrast inverted microscope after 24 and 48 h of inoculation. The infant mouse test was performed by oral inoculation of 0.1 ml of undiluted extracellular fluid containing Evans blue dye. For necrosis, 0.1 ml of undiluted sonic extract was intradermally injected into three rabbits. Twenty-four hours after inoculation, the rabbits were killed and the existence of necrosis was evaluated. Finally, lethality in the mouse intraperitoneal test was recorded during a period of 7 days following intraperitoneal administration of 0.5 ml of the undiluted sonic extract. For detection of Hlys, bacteria were grown (37°C, 48 h) on blood agar base medium (Merck, Darmstadt, Germany) supplemented with 10 mM $CaCl_2$ and 5% defibrinated washed sheep blood in phosphate-buffered saline.

PCR for amplification of VT1 and VT2 genes. DNA to be amplified was released from whole organisms by boiling. The primer pairs selected to amplify VT1 and VT2 gene segments (5′→3′) (VT1, CAGTTAATGTGGTGGCGAAG and CTGCTAATAGTTCTGCGCATC; VT2, CTTCGGTATCCTATTCCCGG and GGATGCATCTCTGGTCATTG) have been used previously for PCR of VTEC (34). The PCR was performed with a thermal cycler (Pharmacia, Uppsala, Sweden) at 94°C for 2 min for 1 cycle, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The amplified product was visualized by standard submarine gel electrophoresis as previously described (11).

Colicin production. Tryptone soy medium (Oxoid) with 1.2% agar was inoculated with *E. coli* strains. After incubation for 24 h at 37°C, the organisms were killed by treatment with chloroform vapor (Merck) for 30 min and then exposed to air for 1 h. Next, the medium was overlaid with soft agar (0.4% agar and 0.5% NaCl in nutrient broth; Difco) containing *E. coli* K-12-711 or *E. coli* K-12-711-Colicin V$^+$ at 10^6 CFU/ml. After incubation for 24 h at 37°C, inhibition of the indicator strains by the test strain was scored. Strains that inhibited indicator strain K-12-711 but not strain K-12-711-Colicin V$^+$ were considered to be colicin V (Col V) positive. Strains that inhibited both indicator strains were considered to produce non-V colicins.

In vivo pathogenicity. The relative pathogenicity of *E. coli* strains was evaluated in mice. Four BALB/C mice (25 to 30 g each) per isolate were inoculated intraperitoneally with 0.2 ml of a 24-h Mueller-Hinton broth culture containing 10^7 CFU of *E. coli*. The number of mice that died during a week was scored. *E. coli* strains were grouped according to their degree of pathogenicity as follows: (i) highly pathogenic strains produced mortality in more than 50% of the mice inoculated (three or four dead mice), (ii) intermediate-pathogenicity strains were lethal to 50% of mice (two dead mice), and (iii) low-pathogenicity strains produced mortality in less than 50% of mice (one or zero dead mice).

Serogrouping. Identification of O antigens was carried out by a microtechnique described by Guinée et al. (29) and modified by Blanco et al. (8) with all available O (O1 to O171) antisera. All antisera were absorbed with the corresponding cross-reacting antigens to remove nonspecific agglutinins.

Statistical analysis. Results were compared by χ^2 test with Yate's correction for continuity.

RESULTS

Phenotypic production of toxins. As can be seen in Table 1, only 45 (7%) of the 625 avian *E. coli* strains assayed were toxigenic. Twenty strains produced a cytotoxic response in HeLa cells but not in Vero cells, indicating the production of a cytotoxin not related to the VTs; 16 were EntHly$^+$; 5 produced a new cytotonic product that causes the appearance of whitish vacuola in Vero and HeLa cells; three synthesized soluble factors that cause lethal activity in mice; and 1 elaborated LT. None of 625 avian *E. coli* strains was positive for production of VTs or necrotoxins.

TABLE 1. Toxins produced by avian *E. coli* strains in this study

Type of toxin(s) or toxicity	No. of toxigenic *E. coli* strains/ strains assayed		Serogroup(s) of toxigenic strains (no. of strains)
	Septicemic isolates	Fecal isolates	
LT	1/458	0/167	NT[c] (1)
STa	0/30[a]	—[b]	
VT1 and VT2	0/458	0/167	
CNF1 and CNF2	0/458	0/167	
Cytotoxicity on HeLa cells	16/458	4/167	O8 (1), O18 (9), O20 (1), O24 (1), O49 (1), O53 (1), O102 (1), O103 (1), NT (4)
Vacuolizant	3/458	2/167	O8 (1), O36 (1), NT (3)
Hly	0/458	0/167	
EntHly	2/458	14/167	O2 (2), O3 (1), O24 (2), O36 (1), O90 (2), O102 (1), O103 (3), O114 (2), O119 (1), NT (1)
Necrotic activity in rabbit skin	0/30[a]	—	
Lethal activity in mice induced with cell-free culture filtrates	3/30[a]	—	O1 (1), O15 (1), O78 (1)

[a] The 30 *E. coli* strains tested with animals belonged to 30 different O serogroups more frequently detected among septicemic isolates.
[b] —, not tested.
[c] NT, not typeable.

Genotypic detection of VT1 and VT2 genes. The detection of VT1 and VT2 genes was performed by PCR with 51 representative *E. coli* strains: 20 were cytotoxic in HeLa cells, 16 were EntHly$^+$, and 15 belonged to O serogroups (O111, O119, O128, O138, and O157) previously associated with VTEC strains. All strains assayed were negative for both VT1 and VT2 genes.

Colicinogenicity. Of the 458 *E. coli* strains isolated from chickens with septicemia, 101 (22%) produced Col V, and 234 (51%) produced other types of colicins. Among the fecal isolates, 12 (7%) of 167 strains elaborated Col V, and 85 (51%) were positive for other colicins. The production of Col V was found significantly more frequently ($P < 0.001$) in isolates from septicemic chickens than in those from healthy birds.

Pathogenicity. The in vivo pathogenicity of 140 representative *E. coli* strains was studied with mice: 105 strains were obtained from septicemia and 35 were obtained from feces. Among 105 *E. coli* strains isolated from clinically affected birds, 66 (63%) were characterized as highly pathogenic strains, 14 (13%) were characterized as of intermediate pathogenicity, and 25 (24%) were characterized as of low pathogenicity. Among 35 isolates from feces of healthy chickens, 10 (29%) showed high pathogenicity, 7 (20%) showed intermediate pathogenicity, and 18 (51%) showed low pathogenicity. Thus, the highly pathogenic strains were significantly more frequent in septicemic isolates (63%) than in isolates from healthy chickens (29%) ($P < 0.001$).

Relationship between pathogenicity and colicinogenicity. There was a clear correlation between the production of colicins and in vivo pathogenicity. Thus, 80% ($P < 0.001$) and 66% ($P < 0.001$) of strains producing Col V and other types of colicins, respectively, were characterized as being of high path-

TABLE 2. Relationship between colicinogenicity and in vivo pathogenicity

Pathogenicity	No. (%) of strains[a]		
	Col V$^+$ (n = 30)	Other than Col V$^+$ (n = 70)	Noncolicinogenic (n = 40)
High	24 (80)	46 (66)	6 (15)
Intermediate	5 (17)	10 (14)	6 (15)
Low	1 (3)	14 (20)	28 (70)

[a] Of the 140 *E. coli* strains tested for in vivo pathogenicity, 105 (27 Col V$^+$, 53 other than Col V$^+$, and 25 noncolicinogenic) strains were isolated from clinically affected birds, whereas 35 (3 Col V$^+$, 17 other than Col V$^+$, and 15 noncolicinogenic) strains were isolated from healthy chickens.

ogenicity, whereas only 15% of the noncolicinogenic strains were classified as highly pathogenic (Table 2).

Pathogenicity by serogroup. Table 3 shows pathogenicity according to O serogroup. Seventy-one (60%) of 119 *E. coli* strains belonging to 18 serogroups most frequently identified among septicemic strains were classified as highly pathogenic. In contrast, only 5 (24%) of 21 strains with O serogroups less prevalent were considered highly pathogenic. This difference was also statistically significant ($P < 0.01$). However, strains belonging to the O81, O103, and O116 serogroups did not show high pathogenicity.

Colicinogenicity by serogroup. Interestingly, the production of Col V was detected especially among *E. coli* strains belonging to four serogroups (O2, O5, O8, and O18) most frequently identified in isolates from septicemic chickens. Thus, 66 (45%) of those 148 strains were Col V$^+$, whereas only 47 (10%) of the remaining 477 isolates were positive for Col V ($P < 0.001$). All 29 O18 strains were Col V$^+$ (Table 3).

DISCUSSION

Very little information about production of toxins by avian *E. coli* strains exists, although 13 years ago, Truscott (42) described a chicken-lethal toxin in avian *E. coli* O2, O45, and O109. However, the results obtained in four recently published reports (15, 23, 24, 30) suggest that toxin production could be a virulence factor of avian septicemic *E. coli* strains. Thus, the results of studies performed by DeRosa et al. (15) indicate that soluble factors produced by *E. coli* during incubation induce hematologic, cytologic (air sac lavage), and morphologic (severe heterophilic airsacculitis) changes similar to those induced by cultures of *E. coli*, suggesting that the initial response of chickens to *E. coli* infection may be attributed to a substance(s) present within cell-free culture filtrate. Emery et al. (23) identified two distinct heat-labile cytotoxins: (i) 24 (6%) of the 420 septicemic turkey isolates and 6 (7%) of the 80 septicemic chicken isolates produced a heat-labile toxin that was cytotoxic for both Vero and Y-1 cells; (ii) in contrast, 48 (11%) of the turkey isolates and 18 (22%) of the chicken isolates produced a distinct heat-labile toxin that was cytotoxic only for Vero cells, indicating the possible production of VTs. Fantinatti et al. (24) assayed 17 avian septicemic *E. coli* strains, and the toxin production was correlated to the highest levels of in vivo pathogenicity established in 1-day-old-male chickens. Of the 17 strains assayed for the production of LT and STa enterotoxins as well as for VTs by Fantinatti et al. (24), only three were able to produce cytotoxic activity on Vero cells. However, the cytotoxic activities on Vero cells observed by Emery et al. (23) and Fantinatti et al. (24) were not sufficiently characterized to determine whether these avian strains really produce VT1, VT2, or VT2v. Results obtained in our study indicate that *E. coli* strains isolated from visceral organs of chickens with colisepticemia and from feces of healthy chickens do not commonly produce enterotoxins (LT and STa), VTs (VT1, VT2, and VT2v), and necrotoxins (CNF1 and CNF2). The results of the present study were comparable to those of previous reports, in which no cytotoxic activity was detectable on Vero cells (31, 36, 40, 45). Neither were Vidotto et al. (45) able to detect toxic factors in 45 avian *E. coli* strains studied under different growth conditions.

Poultry can harbor a variety of different food-borne pathogens, especially *Salmonella* and *Campylobacter* strains. VTEC strains of serotype O157:H7 have been recovered from retail fresh poultry products in Wisconsin by Doyle and Schoeni (19) and in Washington by Samadpour et al. (38). In the past few years, VTEC strains have been associated with many outbreaks and cases of human hemorrhagic colitis and hemolytic-uremic syndrome (14, 32). VTEC strains were not detected in any of the 100 cloacal samples from the broiler chickens selected in our study. This is consistent with other work conducted by Irwin et al. (31), in which 500 chicken cloacal swabs were negative in the Vero cell assay. Under experimental conditions, VTEC O157:H7 is capable of colonizing chicken ceca, followed by prolonged fecal shedding (2). This finding has led to the proposal that chickens may be a reservoir of the organism, although our results do not support this.

Several studies of strains of *E. coli* recovered from avian colibacillosis have identified a number of virulence properties:

TABLE 3. Relationship between O serogroup and colicinogenicity and in vivo pathogenicity of *E. coli* strains

Serogroup	No. of strains tested		No. (%) of strains tested for in vivo pathogenicity	
	Total (S/F)[a]	CV/OC/NC[b]	CV/OC/NC	High pathogenicity/no. tested
O1	15 (13/2)	0/11/4	0/4/3	5/7
O2	48 (34/14)	16/22/10	3/3/2	7/8
O5	28 (23/5)	10/17/1	3/3/1	7/7
O8	43 (33/10)	11/20/12	4/4/1	5/9
O12	6 (6/0)	0/5/1	0/5/1	4/6
O14	8 (8/0)	0/6/2	0/5/0	5/5
O15	7 (5/2)	1/6/0	1/5/0	3/6
O18	29 (29/0)	29/0/0	8/0/0	7/8
O20	11 (8/3)	0/10/1	0/6/1	3/7
O53	9 (9/0)	3/4/2	2/4/1	4/7
O78	21 (20/1)	0/21/0	0/9/0	8/9
O81	13 (13/0)	0/0/13	0/0/6	0/6
O83	8 (6/2)	5/1/2	5/1/1	5/7
O102	12 (11/1)	5/0/7	2/0/4	2/6
O103	11 (6/5)	0/5/6	0/2/4	0/6
O115	20 (19/1)	0/20/0	0/5/0	4/5
O116	15 (14/1)	0/0/15	0/0/5	0/5
O132	14 (13/1)	0/11/3	0/3/2	2/5
Subtotal[c]	318 (270/48)	80/159/79	28/59/32	71/119 (60%)
Other[d]	307 (188/119)	33/160/114	2/11/8	5/21[e] (24%)

[a] S/F, septicemic/fecal *E. coli* strains.
[b] CV/OC/NC, Col V$^+$/other than Col V$^+$/noncolicinogenic.
[c] Sum of results obtained with strains belonging to previously cited 18 serogroups most frequently identified among isolates from chickens with septicemia.
[d] Sum of results with strains belonging to less-prevalent O serogroups, including 168 typeable strains belonging to 67 different serogroups and 139 not typeable strains.
[e] Strains belonging to serogroups O4 (two strains) O7, O9, O11, O21, O41, O51, O62 (two strains), O70, O77, O84, O88, O90, O91 (two strains), O114, O117, O131, O141.

adhesive properties associated with pili or fimbriae (17, 20, 21, 27, 44), aerobactin production (33), serum resistance (22, 48), and Col V production (45, 49, 50). The production of Col V mediated by Col V plasmid is a characteristic associated with the invasion and pathogenicity of *E. coli*, and its importance has been demonstrated by elimination of Col V plasmids of pathogenic strains (39). However, it has been emphasized that Col V activity itself is not essential for virulence enhancement of *E. coli*. The Col V plasmids also may codify outer membrane proteins that are responsible for serum resistance and the siderophore aerobactin that enables *E. coli* to grow under iron-limited conditions in tissues and is associated with invasive *E. coli* (43, 45, 50). In the present study, a substantially higher percentage of avian septicemic strains (73%) than fecal isolates (58%) produced colicins, but there was a significant difference in the production of Col V only (22 to 7%) ($P < 0.001$). However, our results indicate that both Col V production and the synthesis of other colicins are clearly correlated with in vivo pathogenicity of avian *E. coli* strains. Interestingly, the production of Col V was detected especially among *E. coli* strains belonging to four serogroups (O2, O5, O8, and O18) more frequently identified in isolates from septicemic chickens. Thus, 45% of those strains were Col V^+, whereas only 10% of the remaining isolates were positive for Col V ($P < 0.001$) (Table 3).

Two theories have been presented to explain the emergence of extraintestinal infections with a fecal strain (35). The prevalence theory postulates that serogroups frequently recovered in extraintestinal infections represent phenotypes that are generally abundant in the fecal reservoir, whereas the special pathogenicity theory postulates that some serogroups are specially adapted pathogens possessing specific virulence factors that permit involvement in extraintestinal infections. Our results support the hypothesis that avian septicemic *E. coli* strains represent special groups of pathogenic clones. Thus, 60% of *E. coli* strains belonging to 18 serogroups most frequently identified among septicemic strains were classified as highly pathogenic, whereas only 24% of strains with other less-prevalent O serogroups were considered highly pathogenic in in vivo assays ($P < 0.01$) (Table 3). Furthermore, in this study, the higher percentage (83%) of highly pathogenic strains was detected among isolates belonging to six serogroups (O2, O5, O8, O18, O78, and O115) most commonly found in avian septicemic *E. coli*. Therefore, our results clearly support the special pathogenicity theory and suggest that *E. coli* strains belonging to O serogroups frequently detected in cases of extraintestinal infections must possess specific virulence factors which confer on them their special pathogenicity in vivo. However, among the virulence factors of clinical avian *E. coli* strains, the production of enterotoxins, VTs, and necrotoxins is not usually present.

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