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Association of Hemolysin Production, Hemagglutination of Human Erythrocytes, and Virulence for Chicken Embryos of Extraintestinal *Escherichia coli* Isolates

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One hundred forty-two strains of *Escherichia coli* isolated from extraintestinal infections were examined for colicin V (ColV) and hemolysin (Hly) production. For comparison, 20 strains isolated from the feces of normal individuals and 12 enteropathogenic strains of *E. coli* were tested for these properties. Thirty-five to 59% of extraintestinal isolates were Hly^+, but only one fecal strain was Hly^+. Colicin V biosynthesis was found for 12% of blood culture isolates, 7% of urine culture isolates, and 16% of the strains from other extraintestinal infections. None of the fecal isolates was $ColV^+$. Selected strains were tested for virulence in 13-day-old chicken embryos; these same strains were tested for their ability to hemagglutinate chicken or human erythrocytes. Of 22 extraintestinal isolates, 13 (59%) killed ≥60% of the embryos within 72 h. Only one of eight normal fecal isolates and two of three enteropathogenic strains tested were virulent. About 80% of the virulent strains were Hly^+. The most striking finding, however, was the hemagglutination of human erythrocytes by virulent extraintestinal isolates. It seems possible that the hemagglutination property reflects a specific common adherence factor.

Several properties of *Escherichia coli* have been recognized to be often associated with strains isolated from infections and relatively uncommon in *E. coli* of the normal enteric flora. A high proportion of strains from urinary tract infections, appendicitis, and peritonitis are hemolytic (3, 4, 19); *E. coli* from bacteremias of humans and animals commonly produce colicin V (ColV) (13); and, in diarrheal disease, strains may be invasive (6), produce enterotoxin (6, 11, 16) or possess special adhesive properties (8, 16).

Powell and Finkelstein (10) found that differences in virulence among *E. coli* strains could be demonstrated in 13-day-old chicken embryos inoculated allantoically. They also observed a suggestive correlation between hemagglutination of chicken erythrocytes and virulence in these experimental infections.

We initiated this study to survey the incidence of colicin V and hemolysin production in clinical isolates from extraintestinal sources and to assess the effect of these properties on the virulence of *E. coli* for chicken embryos. Furthermore, we wished to extend the observation of the correlation of hemagglutination of chicken erythrocytes with virulence for chicken embryos.

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MATERIALS AND METHODS

Bacteria and media. One hundred forty-two strains of *E. coli* isolated from extraintestinal sources were collected from hospital laboratories in the Seattle area to screen for various phenotypic traits. These cultures were provided by James J. Plorde, Carla R. Clausen, Helen M. Pollock, Marie B. Coyle, Marilyn Murphy, and Enoch Rowland. For comparison, a number of fecal isolates were also tested. These included 20 strains from normal stool specimens furnished by Janice Jernigan of the Laboratory Section of the State of Washington Department of Social and Health Services, 9 strains of standard enteropathogenic *E. coli* serotypes (O119:B14, O128:B12; O55:B5; O111:B4; O126:B16; O125:B15; O26:B6; O127:B8, and O86:B7) provided by Marie B. Coyle of the University Hospital microbiology laboratory, an invasive strain of *E. coli* (3608-58), and enterotoxigenic strains H10407 and B44 (17). *E. coli* K-12 strains 711 and 711 $ColV^+$, used as indicators for colicin production, were obtained from H. Williams Smith. *E. coli* K-12 strain 185 Hly^+ carried a plasmid (Hly) that encoded for hemolysis production (17).

For this study, stock cultures were maintained in soft agar stabs (0.7% agar in nutrient broth [Difco

Laboratories, Detroit, Mich.]); working cultures were maintained on Trypticase soy agar slants (Baltimore Biological Laboratories [BBL], Cockeysville, Md.). Routine culture media were brain heart infusion broth (BBL), Trypticase soy agar, and MacConkey agar (BBL). Hemolysis was observed on blood agar base medium (BBL) containing 5% sheep erythrocytes. Strains producing a clear zone of lysis (beta-hemolysis) after overnight incubation were considered positive (+).

Colicin production. Colicins were detected by a modification of the method of Lewis (9). *E. coli* strains were inoculated onto Trypticase soy agar with sterile toothpicks. After overnight incubation at 35°C, the organisms were killed by treatment with chloroform vapor for 30 min and then allowed to air for at least 30 min with the lids of the petri plates ajar. The medium was next overlaid with soft agar (0.4% agar and 0.5% NaCl in nutrient broth) containing approximately 10^6 cells of viable *E. coli* 711 or *E. coli* 711 ColV$^+$ per ml. After overnight incubation at 30°C, inhibition of the indicator strains by the test strain (colicin production) was recorded. *E. coli* strains that inhibited indicator *E. coli* 711 but not *E. coli* 711 ColV$^+$ were considered colicin V producers.

Hemagglutination of erythrocytes. The slide test for hemagglutination of erythrocytes was performed as described by Powell and Finkelstein (10). Bacteria were harvested from overnight growth on Trypticase soy agar; about 10^9 cells were emulsified in a drop of 0.85% saline on a chilled glass microscopic slide. Blood was collected in sodium citrate (3.8%); the erythrocytes were washed three times and suspended in 0.85% saline at a 3% (vol/vol) concentration. One drop (0.05 ml) of either human type O, Rh$^+$ erythrocytes or chicken erythrocytes suspended in saline was added to the bacterial emulsion. The slide was gently rotated to observe macroscopic hemagglutination.

Virulence assay in chicken embryos. Twelve-day-old embryonated White Leghorn chicken eggs were obtained from Lab Associates, Inc., Kirkland, Wash. The embryonated eggs were kept at 37°C in a humidified incubator for 24 h before inoculation. A 0.1-ml volume of 0.85% saline (for controls) or *E. coli* cell suspension in saline was inoculated allantoically by the method described by Powell and Finkelstein (10). The bacterial inoculum was harvested from overnight growth on Trypticase soy agar into saline (0.85%); the suspension was adjusted to an optical density of 0.35 at 550 nm in a Coleman Junior spectrophotometer and diluted 1:1,000. The cell density of this suspension, determined by dilution plating, was ordinarily 3×10^5 to 10^6 viable organisms per ml. A set of 10 embryonated eggs inoculated with each *E. coli* strain tested and another set of 10 saline-inoculated controls were used in each experiment. Death of the embryos was determined by candling. The embryonated eggs were routinely held for 4 days after inoculation; however, virulent strains ordinarily killed ≥60% of the embryos within 72 h. Strains were classified as virulent when mortality was ≥60% and avirulent when mortality was ≤50%. In the vast majority of cases, however, avirulent strains killed ≤30% of the embryos in 72 h. No deaths were observed in any of the saline-inoculated embryonated eggs.

RESULTS

Hemolysin and colicin production. One hundred forty-two clinical isolates of *E. coli* from a variety of extraintestinal sites were tested for hemolysin and colicin biosynthesis, as were 20 *E. coli* isolated from the feces of normal individuals and 9 known enteropathogenic *E. coli* strains (Table 1). Colicinogeny was a common property in both extraintestinal and fecal *E. coli* isolates. Colicin V biosynthesis, suspected as a virulence factor (13, 15), was demonstrated only in extraintestinal isolates and was found in 7% of urine isolates, 12% of blood isolates, and 16% of the strains isolated from various other extraintestinal infections.

Hemolysis was observed in from 35 to 59% of the extraintestinal isolates (Table 1). In contrast, only 1 hemolytic strain was observed among the 20 tested *E. coli* isolates from stool cultures of normal subjects, and none of the nine enteropathogenic strains tested was hemolytic.

Virulence for chicken embryos. Twenty-two selected strains of extraintestinal origin carrying combinations of colicins and hemolysin were tested for their virulence for 13-day-old chicken embryos. For comparison, three known enteropathogenic strains (one invasive and two enterotoxigenic) and eight fecal isolates from normal subjects were examined. Of the 22 extraintestinal isolates, 13 (59%) killed ≥60% of the embryos within 72 h after allantoic inoculation (Table 2). Two of the three enteropathogenic *E. coli* (the invasive strain and a strain elaborating heat-labile enterotoxin) were also virulent. Only one of the eight fecal isolates from normal subjects showed virulence, and this one strain was hemolytic.

There was clearly a high degree of correlation

TABLE 1. *Incidence of hemolysin and colicin production by isolates of E. coli from various sources*

Source	No. tested	Hemolytic (%)	Colicin[a] positive (%)	Colicin V positive (%)
Blood	51	35	26	12
Urine	59	49	32	7
Sputum	5	40	60	0
Miscellaneous[b]	27	59	5	18
Stool				
EEC[c]	9	0	22	0
Normal	20	5	30	0

[a] Does not include ColV$^+$ strains.

[b] Includes wounds, secretions, drainage, abscesses, and other extraintestinal sources.

[c] EEC, Standard serotypes of enteropathogenic *E. coli* (see text).

TABLE 2. *Association of certain characteristics of E. coli with virulence for 13-day-old chicken embryos*

E. coli strains[a]	Virulence[b]	ColV+	Hly+	Hemagglutination	
				Chicken	Human
Extraintestinal isolates (22)[c]	13 vir	4 (31%)	11 (85%)	4 (31%)	12 (92%)
	9 avir	2	0	3	2
Fecal isolates					
Normal individuals (8)	1 vir	0	1	1	0
	7 avir	0	0	5 (71%)	1
Enteropathogenic (3)[d]	2 vir	0	0	0	0
	1 avir	0	0	0	0

[a] Numbers in parentheses represent numbers of strains tested.

[b] Virulence: Virulent (vir) strains killed ≥60% of (10) embryos; avirulent (avir) strains killed ≤50%.

[c] Sources of extraintestinal isolates: nine blood, eight urine, two sputum, and one each from a decubitus ulcer, duodenostomy drainage, and an unknown source.

[d] Invasive strain 4608-58 and the human toxigenic strain H10407 were virulent, but calf strain B44 was avirulent.

between hemolysin synthesis and virulence for the chicken embryos (13/16 virulent strains). It was of some interest that *E. coli* K-12, which was consistently avirulent for the chicken embryo, was not measurably increased in virulence when a plasmid-mediated hemolysin of porcine origin (14) was introduced (data not shown). Similarly, *E. coli* K-12 ColV+ was avirulent in this experimental model.

Hemagglutinating properties of strains. Powell and Finkelstein (10) reported that the virulence of *E. coli* for the 13-day-old chicken embryo might be correlated with the ability to hemagglutinate chicken erythrocytes. Consequently, we examined this property as well as the ability of cells to hemagglutinate human erythrocytes. The results of these hemagglutination studies are summarized in Table 2.

Among extraintestinal *E. coli* isolates, 4 of the 13 (31%) virulent strains and 3 of 9 (33%) avirulent strains hemagglutinated chicken erythrocytes. In contrast, six of eight (75%) fecal *E. coli* from asymptomatic individuals hemagglutinated chicken erythrocytes; none of the three enteropathogenic strains tested possessed this hemagglutinating property. The most striking finding among this series of strains was that 14 of 22 (64%) *E. coli* isolated from extraintestinal infections hemagglutinated human erythrocytes. Particularly noteworthy was the finding that 12 of the 13 (92%) strains virulent for the chicken embryo possessed this capacity, whereas only 2 of 9 (22%) avirulent extraintestinal isolates were able to hemagglutinate human erythrocytes. None of the enteropathogenic strains and only one of the eight (12%) normal fecal *E. coli* tested hemagglutinated human erythrocytes. The one fecal isolate showing this property was avirulent in the chicken embryo.

DISCUSSION

The gastrointestinal tract is commonly the source of endogenous infection in peritonitis, appendicitis, septic complications after penetrating abdominal wounds or surgery involving the colon, and infections of the urinary tract. *E. coli* is a major component of the normal fecal flora; therefore, it is not surprising that *E. coli* is the most common facultative gram-negative bacillus isolated from these septic conditions. At one time it was thought that the predominant *E. coli* strain resident in the fecal flora of a patient would most likely be implicated in a subsequent infection. It has become increasingly clear, however, that only a relatively limited number of *E. coli* strains possessing "special properties" are most often associated with extraintestinal infection (2–4, 7, 13, 18, 19), just as strains of *E. coli* possessing special properties are often associated with diarrheal disease. The present study supports the thesis that virulent *E. coli* possess a constellation of special genetic properties that include hemolysin production, colicin V biosynthesis, and specific adherence.

Our results, like those previously reported by Smith (13), indicate that ColV+ *E. coli* are found in a small but significant proportion of isolates from human infection. The incidence of ColV+ isolates of *E. coli* from blood cultures in our survey was 12%. These results are comparable to those of Smith (13), who reported that 18% of 38 human bacteremic strains were ColV+. The incidence of ColV+ isolates from human bacteremias is considerably lower than that seen in animal sepsis, however (13). These findings may reflect the very low carriage rate of ColV+ *E. coli* in the fecal flora of humans (9) or the instability of ColV plasmids in human *E. coli*

isolates. In any event, colicin V appears to be less of a contributing factor in human infection than it is in the infection of farm animals.

As early as 1921, Dudgeon et al. (4) suggested that hemolytic activity might be important in the virulence of *E. coli* for the urinary tract. Subsequently, the incidence of hemolytic strains was reported to be 26% by Vahlne (19) and 56% by Cooke and Ewins (3). In our study, 49% of *E. coli* strains isolated from the urinary tract were hemolytic. Moreover, our data clearly indicated that *E. coli* strains isolated from all sites of extraintestinal infection are frequently hemolytic (ranging from 35 to 59%). In contrast, the incidence of hemolytic *E. coli* in the feces of normal individuals has been estimated to be from 8% (2) to 18% (12). We found only 1 hemolytic strain of *E. coli* in 20 randomly selected *E. coli* from the stools of normal food handlers, but we made no effort to quantitate the number of hemolytic cells among the total fecal *E. coli* population in well versus ill individuals. It does seem clear, however, that hemolysin production plays a contributing role in permitting *E. coli* to initiate or sustain extraintestinal infections. The precise role hemolysin may play in the pathogenesis of extraintestinal infections is largely speculative. Cooke and Ewins (3) found that hemolysin production was associated with necrotoxicity in rabbits and cytotoxicity in tissue culture. In this study, all of the hemolytic strains were virulent for chicken embryos. On the other hand, an *E. coli* K-12 strain carrying an Hly plasmid, derived originally from an *E. coli* strain isolated from porcine diarrhea, was avirulent in the chicken embryo model. Smith and Linggood (16) found that plasmid-mediated hemolysin enhanced the pathogenicity of *E. coli* for mice injected intraperitoneally in some instances, but this effect appeared to be plasmid dependent. These data suggest that there may be a fundamental difference in the plasmid-mediated hemolysin and the hemolysin found in extraintestinal *E. coli* isolates. Not enough Hly plasmids of human or animal origin have been examined to give a definitive answer, but our preliminary results (unpublished data) indicate that in most instances the hemolysin in extraintestinal strains is not plasmid mediated.

Colicin V may aid an infecting microorganism to evade the host-defense mechanisms (13, 15), and the infecting cell may possess a cytotoxic hemolysin (3), yet neither of these properties may be of consequence if the organism is unable to establish itself within a host and colonize the appropriate target tissue. The role of specific adherence factors in the pathogenesis of *E. coli* diarrheal disease has been well demonstrated in the last decade (16). Similarly, Svanborg-Edén et al. (18) have reported that *E. coli* from urinary tract infections are able to attach to normal uroepithelial cells and have suggested that this ability may be a virulence factor for strains causing pyelonephritis or cystitis. We believe that the hemagglutination of human erythrocytes by *E. coli* may reflect a specific adhesive factor that is often involved in the pathogenesis of extraintestinal infection. We have recently shown (unpublished data) that the bacterial hemagglutination of human erythrocytes is resistant to inhibition by 0.5% D-mannose, whereas the hemagglutination of chicken erythrocytes is susceptible to inhibition by D-mannose. It should be noted that the hemagglutination of erythrocytes by common pili of *E. coli* is inhibited by D-mannose (5), whereas the hemagglutination of erythrocytes by virulence-associated cellular appendages such as K88 or pili of gonococci is D-mannose insensitive (1, 8).

There is little doubt that the majority of *E. coli* strains isolated from extraintestinal infections possess a constellation of special virulence properties. How these special properties—colicin V, hemolysin, and adherence—contribute to the pathogenic potential of the particular types of *E. coli* isolated from extraintestinal infection is not known. Yet, we may hope to learn the genetic basis and nature of the expression of these factors in *E. coli* to better understand the pathogenesis of extraintestinal *E. coli* infection and the disease process itself.

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