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Hemolysin Production as a Virulence Marker in Symptomatic and Asymptomatic Urinary Tract Infections Caused by *Escherichia coli*

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Potential virulence, as defined by combined levels of adhesion to urinary epithelial cells, serum resistance, and mouse toxicity, was assessed for *Escherichia coli* strains causing symptomatic and asymptomatic urinary tract infections in relation to the carriage of hemolysin and other suspected virulence determinants. Hemolysin production (Hly), associated with certain O (O4, O6, O18, and O75), K (5), and hemagglutination (VI and VII) antigenic types but not colicin V production (Cva), was evident in 83 and 60% of isolates in groups possessing high potential virulence and in only 11 and 6% of those with low virulence. Strains of particular O-types were not more virulent per se, but among the serotypes, specific combinations of virulence factors appeared decisive, e.g., O18 HAVI B/D/G Hly^+ $K5^{+/-}$ and O18 HAIII/IVB/V Hly^- $Cva^{+/-}$ $K1^{+/-}$ strains were, respectively, of high and low potential virulence. Isolates with high potential virulence were found to a similar extent in symptomatic and asymptomatic infections.

Escherichia coli causes about three-quarters of all urinary tract infections (UTI), regardless of whether symptoms are evident, as in cystitis and pyelonephritis, or not evident, as in asymptomatic bacteriuria (20). Infections are assumed to be instigated via the ascending route by bacterial strains present in the host feces, but it now seems clear that such strains exhibit characteristics, for example, adherence to urinary tract epithelial cells and survival of complement action, which appear to contribute to urinary tract virulence and which are not as frequently observed among the normal fecal flora (2, 12, 14, 18, 23, 25, 29).

Many cell surface components have been shown to be associated with *E. coli* strains causing extraintestinal infections, including fimbriae (hemagglutination [HA] antigens) (3, 8, 13, 16, 30) and specific O and K antigens (3, 8, 14, 31), as have the extracellular proteins colicin V (6) and hemolysin (3, 5, 8, 13, 17). Although the effects of these factors on virulence characteristics such as adhesion and serum resistance are in certain cases partially elucidated, it remains unclear to what extent they interact to determine virulence in the strains of *E. coli* isolated from UTI and to what extent they are associated with the establishment of clinical symptoms in the host.

As part of a study of the role of hemolysin in UTI, we examined hemolytic and nonhemolytic *E. coli* causing symptomatic and asymptomatic infections with respect to factors implicated in extraintestinal infection, i.e., adherence to urinary tract epithelial cells (7, 14, 28, 29), resistance to normal human serum (1, 2, 12, 18), and toxicity (10, 17, 27), relating findings to the carriage, particularly in specific combinations, of O, K, and HA antigens and colicin V production.

MATERIALS AND METHODS

Bacteria. A total of 93 strains were isolated by culture of suprapubic aspirates of urine from patients attending the UTI clinic at Charing Cross Hospital, London, England, and identified by standard methods. Infections were either asymptomatic, i.e., detected at screening, or symptomatic, which in this hospital is largely synonomous with cystitis; very few infections develop into pyelonephritis due to early treatment. Strains were O-typed by using antisera (Centers for Disease Control, Atlanta, Ga.) prepared against serotypes O1, O2, O4, O6, O7, O9, O18, and O75. K1 and K5 antigens were detected by the use of specific phage supplied by G. Schmidt, Max-Planck-Institute, Freiburg, West Germany.

HA. HA types were determined with erythrocytes obtained either locally (human, bovine, and guinea pig) or from Flow Laboratories, Bonn, West Germany (chicken or African green monkey). Agglutination, sensitive and resistant to 1% mannose, was assayed in phosphate-buffered saline and classified into types IVB (no agglutination), III, IVA (mannose sensitive hemagglutination [MSHA]), V, VI, and VII (mannose

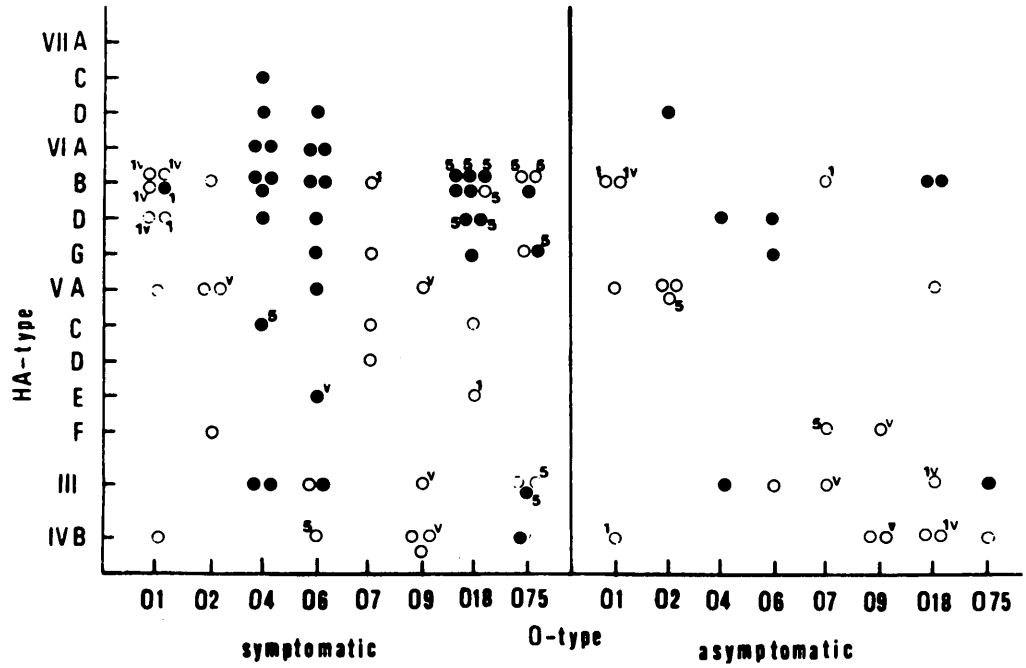

FIG. 1. Association of hemolysin production with other virulence factors among strains causing symptomatic and asymptomatic UTI. V, colicin V production; 1, K1 antigen; 5, K5 antigen; B', agglutination with chicken erythrocytes is sometimes unclear and may include a small number of type VI C. Symbols: ●, Hly$^+$, ○, Hly$^-$.

resistant hemagglutination [MRHA]) as described previously (9).

Hemolysin production. Erythrocyte lysis was detected on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) containing washed sheep erythrocytes and confirmed in liquid assay (15).

Colicin V production. Colicinogenic isolates were identified on BBL antibiotic sensitivity agar by using the colicin-sensitive indicator strain *E. coli* K12 KH 215 and colicin V producers (Cva$^+$) by their action on colicin-insensitive mutant indicator strains (15).

Adhesion to urinary epithelial cells. Adhesion of washed logarithmic-phase bacteria to urinary epithelial cells was measured by the method of Svanborg-Eden et al. (22) with fresh cells collected on the day of the test. Values given (as mean number of bacteria per cell) were the means of two independent tests, with at least 30 cells being counted for each test. Strains were classified as having high, intermediate, or low adhesion if they adhered at >10, 10 to 5, or <5 bacteria per cell, respectively.

Serum sensitivity. Response to normal human serum was assayed as described previously (15) with fresh serum taken from healthy volunteers on the day of the test. The survival of washed logarithmic-phase bacteria (initial inoculum, ca. 3×10^5 ml^{-1}) was measured by viable counts at the beginning and after 1, 2, and 3 h of incubation in undiluted serum. The response was graded (15, 24) from 1 (complete sensitivity; less than 0.1% survival after 1 h) to 6 (complete resistance; unhibited growth over 3 h). Grades 5 and 6 represent high, 1 and 2 low, and 3 and 4 intermediate resistance.

Toxicity. By using a modification of the test described by Van den Bosch et al. (27), the toxicity of 70 of the 93 isolates for mice was assessed. Cells from overnight cultures (enriched nutrient broth) were harvested, washed twice in phosphate-buffered saline, and suspended in this buffer to approximately 10^9 ml^{-1}. Ten NMRI female mice (each weighing ca. 16 g; Central Institute for Laboratory Animals, Hannover, West Germany) were injected via the intraperitoneal route with 0.2 ml of this suspension, and the number of mice dead after 24 h was recorded. Toxicity was designated high (≥5 mice killed) or low (<5).

RESULTS AND DISCUSSION

The eight O-types examined in this study, O1, O2, O4, O6, O7, O9, O18, and O75, cause over 40% of UTI, both symptomatic and asymptomatic, examined at Charing Cross Hospital (20). Strains were selected to allow analysis of virulence factors within all of these O-types and do not therefore reflect the incidence with which these serotypes are isolated from UTI. In this hospital, strains of serotypes O1, O2, O4, O6, O7, O9, O18, and O75 cause approximately 7, 2, 5, 10, 3, 2, 5, and 9% of UTI, respectively. Several characteristics of urinary strains are strongly associated with particular O-types, including hemolysin production (Hly) (predominantly serotypes O6, O75, O18, and O4) (8, 13, 15) and colicin V production (Cva) (serotypes O9 and O1). It also seems (Fig. 1) that MRHA factors are more common in certain O-types than in others; e.g., and O9 isolates examined

here exhibited MRHA (types V, VI, and VII) in only 2 out of 8 cases (both type V), in contrast to 14 (11 type VI) of the 17 O18 strains and 10 of 13 O4 isolates. Strains of O-types O1, O4, O6, O18, and O75, which are the five serotypes most prevalent in UTI (see, e.g., reference 20), were most commonly of MRHA type VI; MRHA type V was found particularly often among O2 strains, and type VII was rare. These data support and extend those of Greene and Thomas (13), who, unlike Vosti (30), found agglutination of human erythrocytes not to be randomly distributed throughout the O-types. Hemolytic isolates were more often $MRHA^+$ and less often HA^- than those which were nonhemolytic. Of 42 Hly^+ isolates, 83% were $MRHA^+$ (64% type VI), 14% showed MSHA (type III), and 2% showed no HA (HA^- type IVB); in contrast, 63% of 51 Hly^- strains were $MRHA^+$, 14% were $MSHA^+$, and 24% were HA^-. In particular, strains of MRHA types VI and VII and MSHA type III were over 60% Hly^+, in contrast to types V (16%) and IVB (HA^-, 8%). This is in general agreement with previous studies (8, 13, 17) of unselected UTI isolates, i.e., not restricted to the eight most important O-types.

Although $K5^+$ strains were often (8 of 15) Hly^+, $K1^+$ isolates were rarely (1 of 13) so; examination of O-types O1 and O9, in which K1 and Cva occur singly and together most frequently, emphasizes the lack of association between Hly and K1 previously noted by Evans et al. (8), who also described the coincidence of specific K antigens with Hly^+ strains of a particular O-type, e.g., K2, and K13 with O6. That they did not note such a correlation among O18 isolates is explained by our data which show that these strains are very frequently $K5^+$ (not tested in previous studies). Hly^+ strains were generally Cva^- (only one of the Cva^+ strains was hemolytic); thus, it is not surprising that Cva^+ strains showed no tendency to be $MRHA^+$. Cva^+ and Cva^- isolates were $MRHA^+$ in 60 and 74% of cases, respectively.

Cumulative virulence in relation to hemolysin production and other virulence factors among isolates from symptomatic and asymptomatic infections. In agreement with reported data (e.g., references 14 and 28), we observed adhesion of isolates to urinary epthelial cells to be strongly correlated with MRHA types V, VII, and especially VI. No $MRHA^+$ strains were nonadhesive (<5 bacteria per cell), and almost all strains exceeding 15 bacteria per cell were $MRHA^+$. As expected from the association between Hly and MRHA, in particular of type VI, Hly^+ strains were generally adhesive. Of the 42 Hly^+ isolates examined, only 2 (5%) were apparently nonadhesive, compared to 24% of the 51 which were Hly^-. As reported in the extensive studies of Svanborg-Eden and co-workers (e.g., reference 23), strains from asymptomatic bacteriuria were more frequently nonadhesive than those isolated from symptomatic infections (21 and 9%, respectively), although our strains (from eight O-types, excluding rough strains) did not demonstrate such a clear difference between levels of adhesion among the different forms of UTI. Varian and Cooke (29) also observed only a significant difference between UTI and fecal isolates but none between strains isolated from the lower and upper urinary tract.

We have recently presented data on the serum sensitivity of the strains examined in this study (15) demonstrating that hemolysin production is significantly correlated with high levels of serum resistance among *E. coli* causing UTI, specifically within O-types O6, O18, and O75. This correlation appears to reflect association of *hly* carriage (among UTI isolates, generally chromosomal [17; unpublished results]), with an unknown factor altering the serum response of these strains. In contrast, the carriage of the K1 antigen, shown to increase resistance to serum in certain instances (11), seems not to be important in determining the serum sensitivity of urinary pathogens. Among the 93 strains tested here, $K1^+$ isolates were overall, and within O-types O1 and O18, more sensitive than their $K1^-$ counterparts (mean serum response, 2.1 and 3.1, respectively). K1 carriage is apparently similar to carriage of R and ColV plasmids in that it may be a decisive factor only in individual cases and not in the majority of strains (15). In agreement with previous reports (2, 12), we found resistance to serum to be higher among isolates from symptomatic infections. This appeared to reflect the higher incidence of certain "types" of isolate, e.g., $MRHA^+$ Hly^+ strains of O-types O6 and O18.

Of the 30 Hly^+ isolates tested, 90% killed five or more mice within 24 h, compared to 40% of their 40 Hly^- counterparts. In all cases in which no mice were killed (or only one or two mice), the test strain was nonhemolytic. There was no striking difference in toxicity between the O (i.e., endotoxin)- or HA-types, independent of hemolysin production. Neither were symptomatic isolates more toxic than those from asymptomatic bacteriuria (63 and 65%, respectively; killed $\geq$5 mice).

The strong association between *hly* carriage and toxicity appears attributable to Hly itself, as genetically manipulated derivatives, either Hly^- mutants or UTI strains carrying recombinant plasmids bearing chromosomally *hly* determinants, show a corresponding change in toxic activity (J. Hacker, C. Hughes, and W. Goebel, manuscript in preparation). This view agrees with other published data (10, 32, 33) and sup-

TABLE 1. Association of hemolysin production and other virulence factors among *E. coli* isolates displaying various degress of serum resistance, toxicity, and adhesion

Group (no. of isolates)	Virulence[a]			Isolates (no.)[b]
	Serum resistance	Toxicity	Adhesion	
I (12)	High	High	High/intermediate	O4 HAIII/VID Hly (2) O6 HAIII Hly O9 HAIII/VA Cva (2) O18 HAVIB/D/G Hly $K5^{+/-}$ (6) O75 HAVIB Hly
II (15)	Intermediate	High	High/intermediate	O1 HAVIB Hly K1 O2 HAVIID Hly O6 HAVIG/VIID/VE Hly $Cva^{+/-}$ (3), HA^- K5 O7 HAVF K5, VI $K1^{+/-}$ (2) O9 HA^- Cva O18 VIB/G $Hly^{+/-}$ $K5^{+/-}$ (3) O75 HAVIB/III Hly $K5^{+/-}$ (2)
III (15)	Low	High	High/intermediate	O1 HAVA K1 HAVIB/D Cva K1 (2) O2 HAVA (2) O4 HAVC Hly K5 HAIII/VIB/D, VIIC Hly (6) O6 HAVIA/D Hly (2) O18 HAVE K1
IV (4)				
a	Intermediate	High	High/low	O7 HAIII Cva O18 HAIII Cva K1
b	High	Low	Intermediate/low	O1 HA^- O18 HAVC
V (15)	Intermediate/low	Low	High/intermediate	O1 HAVA HAVIB Cva $K1^{+/-}$ (2) O2 HAVA $Cva^{+/-}$ (2) O6 HA^- HAIII O7 HAVIIA/VD (2) O9 HA^- (2), HAVF Cva O75 HAVIB/G $Hly^{+/-}$ $K5^{+/-}$ (3)
VI (9)	Low/intermediate	Low	Low	O1 HA^- K1 O9 HA^- O18 HA^- $Cva^{+/-}$ $K1^{+/-}$ (2), VA O75 HA^- $Hly^{+/-}$ (2) HAIII $K5^{+/-}$ (2)

[a] Grades of virulence are defined in the text.
[b] Where no figure is given, only one strain was examined.

ports the view that Hly determines a cytotoxic activity (4). Van den Bosch and co-workers have shown that *hly* carriage is associated with higher virulence of *E. coli* UTI strains in their adult mouse model but reported that such carriage is not decisive in all cases (26). This is consistent with the view that *hly* determinants may promote different levels of toxicity (33; J. Hacker, unpublished data).

The association between surface antigens, extracellular products, and potential virulence characteristics among *E. coli* causing extraintestinal infection described here and elsewhere (3, 7, 13–15, 17, 30, 31) suggests a cumulative virulence among *E. coli* causing UTI. High degrees of adhesion, serum resistance, and toxicity were not independently distributed throughout the strains examined in this study. For example, isolates possessing, respectively, high, intermediate, and low serum resistance were toxic in 86, 71, and 47% of cases. Similarly, 91% of toxic strains showed high or intermediate adhesion, compared to 56% of those having lower toxicity. To assess whether strains with high (cumulative) potential virulence carry typical combinations of virulence factors, the isolates were grouped according to their combined levels of adhesion, serum resistance, and toxicity (Table 1).

There were no striking differences between O-types per se, whereas hemolysin and, to a lesser extent MRHA, were clearly associated with the groups of higher potential virulence. Of isolates in groups I and II (high potential virulence), 83

and 60%, respectively, were Hly$^+$, compared to 7 and 11% in groups V and VI (low potential virulence). The equivalent percentage of MRHA$^+$ strains in these groups were 75, 80, 73, and 11%. Of particular interest was the different frequency of specific combinations of virulence factors among the groups. Most marked in this respect were strains of the O18 serotype; O18 HAVI Hly$^+$ strains, in many cases K5$^+$, accounted for one-third of the 27 strains of groups I and II. In contrast, the remaining O18 isolates, all Hly$^-$ K5$^-$ but frequently K1$^+$, Cva$^+$, or HA$^+$ (III or V) belonged to groups V or VI. It seems likely from the data of Evans et al. (8) that most of the hemolytic O4 and O6 isolates which, like the O18 Hly$^+$ strains, almost all belonged to groups I to III, carried K2 or K13 (06) or K13 or K12 (O4) antigens. Strains of low potential virulence (groups V and VI) did not possess such constellations of the virulence factors tested as were evident in isolates of groups I and II.

Our data and those of others (8, 13) indicate that a relatively limited number of "aggresive" strains are well adapted to extraintestinal, e.g., urinary tract, invasiveness, and, as previously suggested for other *E. coli* infections (19, 21), these strains may represent descendants of a small number of clones.

Strains of groups I, II, III, IV, and V were isolated from symptomatic infections in 75, 73, 60, 77, and 33% of cases, respectively. Although indicating, with data presented earlier, that strains of very low potential virulence (Hly$^-$ MRHA$^-$) are not as likely to be associated with symptoms, the data show that *E. coli* virulence factors do not directly govern the development of symptoms. We regard this as a reaffirmation of the importance of host factors, about which little is known, in dictating the course of UTI.

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