

## Serum Resistance Among *Escherichia coli* Strains Causing Urinary Tract Infection in Relation to O Type and the Carriage of Hemolysin, Colicin, and Antibiotic Resistance Determinants

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The sensitivity to normal human serum of 91 smooth strains of *Escherichia coli* isolated from urinary tract infections was determined. Production of hemolysin, which was common and associated primarily with the types O4, O6, O18, and O75, was significantly correlated with high levels of serum resistance, both within the total population and within individual O types. In contrast, serum resistance was not significantly associated with antibiotic resistance (whether transmissible or not), with colicinogeny in general, or with colicin V production in particular. This indicates that the carriage of R and ColV plasmids, shown previously to be capable of conferring increased levels of serum resistance on individual strains of *E. coli* isolated from other sources, does not play an important part in determining the serum sensitivity of the *E. coli* population involved in urinary tract infection.

Many smooth strains of gram-negative bacteria are resistant to the bactericidal action of human and animal sera, and both epidemiological and experimental data indicate that this may be an important factor in certain infections (7, 15, 21, 26). In urinary tract infection, resistance to serum is related to the site of infection (12, 32) and to the severity of symptoms (22). Investigations into the basis of serum resistance have until recently focused upon chromosomally encoded cell surface antigens. Much evidence now supports the view that the O side chain moiety of lipopolysaccharide (LPS) plays a central role but does not alone determine complete resistance (8, 29). Suggestions that acidic polysaccharide K antigens are able to confer complete protection from serum action (10) are not generally applicable (17, 29, 30, 31), although the K1 antigen may in certain instances exert a strong influence (9). Experiments with both *Escherichia coli* and *Neisseria gonorrhoeae* have demonstrated that conversion to serum resistance may be associated with specific changes in the polypeptide components of the cell envelope (14, 33). It therefore seems likely that complete resistance to serum results from the accumulation of several distinct components at or near the cell surface.

Recent studies (31) indicate that such compo-

nents may, in certain cases, be determined by plasmid-borne genes. Increased resistance to serum has been observed after introduction of a number of antibiotic resistance (R) plasmids, including R1 and R100, into a partially resistant wild-type *E. coli* strain, and a multicopy mutant of R100 demonstrated a gene dosage effect. Subsequent work on the related plasmid R6-5 has demonstrated that such resistance is determined by the *traT* gene (19). A further factor contributing to *E. coli* serum resistance is encoded by the *iss* gene of the ColV.I-K94 plasmid (1). Of additional interest is the production of hemolysin (Hly), which may also be determined by genes carried on transmissible plasmids (11, 27). Many studies have noted the relatively high incidence of the Hly<sup>+</sup> character among isolates from urinary tract and other extraintestinal infections (2, 3, 18, 35), but it remains unclear what specific advantage it confers on these strains. An influence on serum sensitivity would be one possibility.

To assess the degree to which the carriage of antibiotic resistance, colicin, and hemolysin determinants is associated with serum resistance among *E. coli* strains causing urinary tract infections, we examined 91 such strains isolated over a period of 8 years at the Charing Cross Hospital, London. The isolates belong to types O1, O2, O4, O6, O7, O9, O18, and O75, which cause about one-half of the urinary tract infections detected at this (24) and other (13, 25) hospitals.

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## MATERIALS AND METHODS

**Bacteria.** All organisms were isolated by culture of suprapubic aspirates of urine from outpatients attending the urinary infection clinics at Charing Cross Hospital, London, between 1972 and 1979. To facilitate statistical analysis, isolates were chosen to achieve, as near as possible, an equal number of antibiotic-resistant and antibiotic-sensitive strains within each O type. The isolation dates and characteristics of the strains precluded the multiple isolation of a particular clone or resistance plasmid from a limited epidemic. The isolates were identified as *E. coli* by standard methods (4) and maintained on nutrient agar (Oxoid no. 2) slopes at 4°C. The isolates were grouped by using antisera prepared against the *E. coli* serotypes O1, O2, O4, O6, O7, O9, O18, and O75. These sera were supplied by the Biological Reagents Section, Centers for Disease Control, Atlanta, Ga., and reacted with the homologous organisms at titers of 1 in 1,280 or greater, and none showed a heterologous reaction at titers of 1 in 1,000 or greater.

**Antibiotic sensitivity.** After Oxoid DST agar was flooded with a diluted overnight culture, antibiotic sensitivities were determined against impregnated disks containing sulfafurazole (Su, 500 µg), kanamycin (Km, 30 µg), ampicillin (Ap, 25 µg), cephaloridine (Cr, 25 µg), streptomycin (Sm, 10 µg), tetracycline (Tc, 10 µg), chloramphenicol (Cm, 10 µg), and nalidixic acid (Nal, 30 µg).

**Colicinogeny.** Colicin production was detected by the overlay method, using the colicin-sensitive indicator KH215 (*E. coli* φ). Allotment of colicins to groups A and B was determined by action on the mutants KH410 (*tolA*) and KH850 (*tonB*) (16). To identify colicin V, colicins of group B were further tested against KH1039, a mutant made resistant to all colicins except V (5). Colicinogenic strains used as controls were M1247(ColK<sup>+</sup>), M1252(ColE1) (both group A), KH932(ColV-B188), and KH576(ColV-K30). All colicin tests were performed on seed agar (BBL Microbiology Systems) as Cva<sup>+</sup> controls did not produce inhibition zones on other media such as nutrient agar. (This has also been observed by Davies et al. [5].)

**Transfer ability.** The presence of a transfer factor in antibiotic-resistant isolates was detected as the ability to transfer one or more resistance determinants to *E. coli* K-12 M560 (*hsdM hsdR*). Overnight cultures of potential donors were diluted, grown to mid-logarithmic phase, and mixed with an overnight culture of M560 (ca.  $5 \times 10^7$ /ml each). After overnight incubation, mixtures were diluted 100-fold and incubated an additional 5 h. Diluted (saline) and undiluted mixtures were spread on appropriate selection media, and progeny were checked for other markers. No strain was designated *tra*<sup>+</sup> unless it failed to transfer in at least two independent matings (using, where possible, more than one antibiotic to select for transfer), including one performed on solid nutrient agar (11).

**Hemolysin production.** Oxoid blood agar base containing washed erythrocytes was stabbed with overnight cultures and examined after 20 h for clear zones of erythrocyte lysis. Cell-free hemolysin was detected as follows. Overnight cultures grown in alkaline extract broth were diluted 1:1,000 and two samples were taken during logarithmic growth. Bacteria were removed by centrifugation, and the supernatant was

incubated (40 min at 40°C) with washed bovine erythrocytes suspended in 0.9% NaCl–0.02 M CaCl<sub>2</sub>. After removal of erythrocytes by centrifugation, hemolytic activity was assayed as free hemoglobin (absorbance at 420 nm).

**Serum sensitivity.** Serum was obtained from healthy volunteers on the day of each test. An inoculum (0.5 ml) of early-log-phase cells, washed and suspended in 0.9% NaCl (ca.  $10^6$  cells per ml), was added to 1.5 ml of undiluted serum. Viable counts were obtained at the beginning and after 1, 2, and 3 h of incubation at 37°C. Each strain was tested at least three times, and the mean results were expressed as percent inoculum. Responses were graded from 1 to 6 as follows (individual examples of each grade are shown in Fig. 1): grade 1, viable counts (VC) at 1 and 2 h were <10% of the inoculum and at 3 h were <0.1% (Fig. 1, strain O4357); grade 2, VC at 1, 2, and 3 h were <100% of the inoculum, at 1 h were 10 to 100%, and at 3 h were <10% (Fig. 1, O1434); grade 3, VC at 1 h were >100% of the inoculum and at 2 and 3 h were <100% (Fig. 1, O6362); grade 4, VC at 1 and 2 h were >100% of the inoculum and at 3 h were <100% (Fig. 1, O9319); grade 5, VC at 1, 2, and 3 h were >100%, but viable counts fell at some time during the 3-h period (Fig. 1, O18459); grade 6, VC at 1, 2, and 3 h were >100% and rising through the 3-h period (Fig. 1, O6398).

**Statistical analyses.** The significance of differences of the means was assessed by using the Wilcoxon sum or ranks (T) test (36) including, when the number in any group exceeded 20, a Z transformation. The significance of differences in distribution in  $2 \times 2$  contingency tables was determined either by a chi-square ( $\chi^2$ ) test, with correction for continuity if the total number was greater than 50, or by the exact probability test for smaller totals.

## RESULTS

**Association of antibiotic resistance, colicinogeny, and hemolysin production with O type.** The 91 isolates belonging to the O types most commonly causing urinary tract infection were not randomly selected but rather chosen to facilitate statistical comparison between antibiotic-resistant and antibiotic-sensitive strains, this being dictated by three factors. Strains of the eight O types do not occur with equal frequency (Table 1); e.g., in this hospital, those of types O6 and O75 were isolated from 10 and 9% of urinary tract infections, respectively (24), compared with those of O2 and O9, which were each present in only 2% of cases. Within the O types there is an unequal distribution of antibiotic resistance, with strains of types O4, O9, and O18 being frequently resistant, whereas those of O1, O7, and O75 are rarely so (15a, 23). In addition, it has been suggested that qualitative differences in the O side chain of the somatic antigen influence serum response, thus causing a non-random distribution of serum-resistant strains throughout the O types (28). Our data showed that antibiotic resistance spectra also varied with O type; e.g., resistant O4 isolates were in

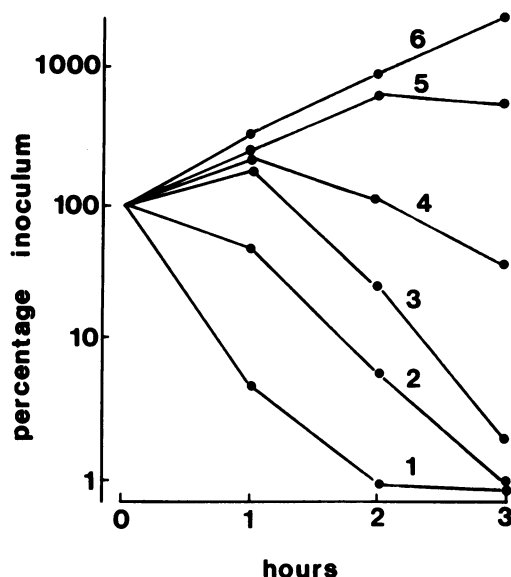


FIG. 1. Grades of response to normal human serum of *E. coli* urinary tract isolates. Responses shown are those of strains described in the text.

most cases resistant to 5 or 6 antibiotics, whereas the rare resistant isolates of O75 and O7 were usually resistant to combinations of tetracycline, streptomycin, and sulfonamide, these resistances being in most cases nontransmissible. Colicinogeny and hemolysin production were also associated with certain O types. Colicinogenic strains were particularly common among O types O1 (7/12, Col<sup>+</sup>), O7 (7/8), and O9 (5/8), with O1 and O9 being also the main source of Cva<sup>+</sup> strains (5/13 and 5/8, respectively). Strains of the remaining O types, O2, O4, O6, O18, and O75, were Col<sup>+</sup> in 1/8, 3/13, 5/14, 6/16, and 1/12 cases, respectively. Hemolytic isolates were most common among serotypes O4 (13/13, Hly<sup>+</sup>), O6 (11/14), O18 (9/16), and O75 (5/12), whereas strains of the O types O1 (1/13), O2 (1/8), O7 (0/8), and O9 (0/8) were generally nonhemolytic. Strains designated as hemolytic were clearly positive on blood agar plates and, in practically all cases (>90%), strongly positive in cell-free assays. The few which were only weakly positive in the latter were included in the study as they did not differ in any other way from the majority of the Hly<sup>+</sup> isolates.

**Serum sensitivity. (i) Relation to O type.** Of the 91 strains (Table 1), 17 were resistant to serum (grades 5 and 6), 26 displayed intermediate (delayed) sensitivity (grades 3 and 4), and 48 were completely sensitive (grades 1 and 2). Isolates of O types O1, O2, O4, and O7 were only rarely resistant (3/41, grade 5 or 6; 8/41, grade 3 or 4), whereas those of O18 were very frequently so

(7/16, grade 5 or 6; 6/16, grade 3 or 4). The mean serum response of the O types ranged from 2.0 (O2) to 4.0 (O18), and the difference between the mean serum response of the O18 isolates was significantly different ( $P < 0.01$ ) from those of the remaining population and also from those of types O1, O2, and O4.

**(ii) Relation to antibiotic resistance and colicinogeny.** Although many of the isolates showing grade 5 or 6 responses to serum were antibiotic resistant and colicinogenic, there was no clear indication that these characteristics were associated with serum resistance. Of the 17 serum-resistant strains, 9 were antibiotic resistant (55%), compared with 35 of 74 (47%) strains which showed complete or intermediate serum sensitivity; 4/17 (24%) and 16/74 (22%), respectively, possessed self-transmissible antibiotic resistance determinants. The differences between the mean serum responses of antibiotic-resistant and antibiotic-sensitive strains (Table 2) were not significant at the 5% level, nor were those between strains carrying and lacking transmissible antibiotic resistance determinants.

A prerequisite for the increase in serum resistance mediated by the F<sub>II</sub> plasmids R1 and R100 seems to be the presence of LPS O side chains which determine the delayed sensitive response typical of grades 3 and 4 (25). No difference in serum sensitivity between antibiotic-resistant and antibiotic-sensitive strains was observed, however, even when those strains of high serum sensitivity (grades 1 and 2) were omitted from statistical analyses (data not shown).

There was also no obvious correlation between serum resistance and colicinogeny (Table 2). Of the 17 serum-resistant strains, none (<6%) produced colicin(s) of group A (A, E, D, etc.), and 4 (24%) produced colicins of group B (V, B, I, etc.). Of the latter, two (12%) were found to be Cva<sup>+</sup>. This compares with 20 strains producing group A colicins (27%) and 18 produc-

TABLE 1. Serum sensitivity in relation to O type

Type	n <sup>a</sup>	UTI (%) <sup>b</sup>	No. of isolates with following serum sensitivity grade:			
			1-2	3-4	5-6	Mean
O1	12	7	8	3	1	2.2
O2	8	2	6	2	0	2.0
O4	13	5	10	1	2	2.4
O6	14	10	6	4	4	3.4
O7	8	3	6	2	0	3.0
O9	8	2	3	3	2	3.4
O18	16	5	3	6	7	4.0
O75	12	9	6	5	1	2.9

<sup>a</sup> n, Number of isolates examined.

<sup>b</sup> Percentage of urinary tract infection (UTI) caused by these O types (detected at Charing Cross Hospital, London [24]).

TABLE 2. Mean serum response of hemolytic, antibiotic-resistant, and colicinogenic isolates

Type	<i>n</i> <sup>b</sup>	Mean grade of serum response <sup>a</sup>									
		Antibiotic resistance <sup>c</sup>		Antibiotic resistance <sup>d</sup> ( <i>tra</i> )		Colicin <sup>e</sup>		Colicin V <sup>f</sup>		Hemolysin	
		+	−	+	−	+	−	+	−	+	−
O1	12	2.6	1.9	2.6	1.9	1.9	2.6	1.8	2.4		
O2	8	2.3	1.8								
O4	13	1.7	3.0	1.8	2.7	1.3	2.7				
O6	14	3.7	3.1	3.7	3.3	2.2	4.0			3.7	2.0
O7	8	3.2	2.8								
O9	8	2.8	4.0			3.8	3.0	3.8	3.0		
O18	16	4.3	3.6	4.8	3.8	4.0	4.0	3.0	4.1	4.9	2.9 <sup>g</sup>
O75	12	2.8	2.3							3.8	1.7 <sup>g</sup>
Total	91	3.1	2.8	3.0	2.9	2.7	3.1	2.7	3.0	3.5	2.4 <sup>g</sup>

<sup>a</sup> See the text and Fig. 1.  
<sup>b</sup> *n*, Number of isolates examined.  
<sup>c</sup> +, Antibiotic resistant; -, antibiotic sensitive; approximately equal numbers of each.  
<sup>d</sup> +, Strains carrying transmissible antibiotic resistant determinants; -, remaining strains including those bearing nontransmissible markers.  
<sup>e</sup> +, Strains producing one or more colicins, including colicin V; -, noncolicinogenic strains.  
<sup>f</sup> +, Strains producing colicin V; -, remaining strains including those producing colicins other than colicin V.  
<sup>g</sup> Significant differences (*P* < 0.01) in mean serum response of hemolytic and nonhemolytic strains.

ing group B colicins (24%) (13 of which were Cva<sup>+</sup> [24%]) among the remaining 74 of prompt and intermediate serum sensitivity. Only in serotype O9, where all five colicinogenic strains were Cva<sup>+</sup>, did these strains exhibit a higher mean degree of serum resistance than those which were not colicinogenic. Again, the difference was not significant at the 5% level.

(iii) **Relation to hemolysin production.** Possession of hemolysin determinants was strongly associated with serum resistance. Of the 17 serum-resistant isolates, 13 (77%) were hemolytic, compared with 27 (37%) of the remaining 74, and this difference was also apparent within individual O types, i.e., independent of qualitative differences in O antigen. This is demonstrated most clearly in types O18 and O75 (Fig. 2), in which about 50% of the strains were hemolytic. The mean serum resistance grades obtained for Hly<sup>+</sup> and Hly<sup>-</sup> strains differed to a large extent when the number and distribution of the Hly<sup>+</sup> character allowed comparison, i.e., in serotypes O6 (Hly<sup>+</sup> mean grade of serum resistance, 3.6; Hly<sup>-</sup>, 2.0), O18 (Hly<sup>+</sup>, 4.9; Hly<sup>-</sup>, 2.9), O75 (Hly<sup>+</sup>, 3.8; Hly<sup>-</sup>, 1.7), and in the total population (Hly<sup>+</sup>, 3.5; Hly<sup>-</sup>, 2.4). In the last three cases, the differences were significant (*P* < 0.01).

DISCUSSION

It has been demonstrated that plasmid-borne genes linked to antibiotic resistance (19, 31) and colicin (1) determinants can increase to a marked degree the serum resistance of individ-

ual strains of *E. coli*. Our data show, however, that those markers are not associated to a significant extent with serum resistance among *E. coli* causing urinary tract infections, indicating that the carriage of ColV and R plasmids, although

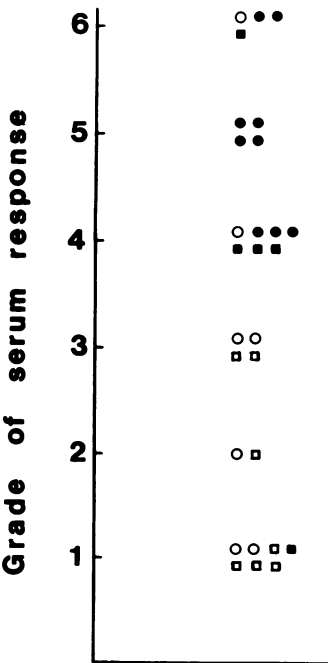


FIG. 2. Serum response of hemolytic (closed symbols) and nonhemolytic (open symbols) isolates of *E. coli* types O18 (circles) and O75 (squares).

widespread throughout these organisms (5, 23), is not a major factor in determining their serum resistance. This does not exclude the possibility that these plasmids are decisive in determining the serum response of either individual strains colonizing the urinary tract or of *E. coli* causing other infections. Although the data do not allow for the lower occurrence of *tra* factors in antibiotic-sensitive strains, they do nevertheless indicate that serum resistance is a property not ubiquitously encoded by R plasmids and may be, as recent experiments suggest (34), limited to the  $F_{II}$  plasmids such as R1, R100, and R6-5. It is also possible that R factor modifications of serum response such as that due to the *traT* product require a genetic background limiting their influence to a minority of strains.

In contrast to antibiotic resistance and colicinogeny, the production of hemolysin, found to occur in 44% of the isolates examined, was clearly associated with high levels of serum resistance. Within the total population and also within the three O types where its incidence allowed comparison, the Hly<sup>+</sup> character was correlated with the higher grades of serum response. This was particularly evident among the strains of types O18 and O75, which were about 50% Hly<sup>+</sup>, thus allowing comparison independent of O antigen. The mean serum resistance of Hly<sup>+</sup> strains among these O types was significantly higher than that of their Hly<sup>-</sup> counterparts. However, isolates of the generally non-hemolytic types O7 and O9 were relatively serum resistant, whereas strains of O4, all of which were hemolytic, were rather serum sensitive. These data doubtless reflect, as do those indicating the lack of overall association between serum resistance and R plasmid carriage, the multifactorial basis of the phenomenon. The *hly* determinant or other associated factor evidently increases serum resistance but only with certain O types. This could be explained, for example, by a variation in the association of *hly* and a serum resistance factor throughout the O types or, if the *hly* determinant itself is responsible, by induced alterations in the cell envelope only affecting serum response when superimposed on a particular O antigen background.

The possibility that the *hly* determinant itself causes a decisive change in envelope topology is attractive in view of both the demonstration that the  $F_{II}$  R plasmid-induced serum resistance is specifically due to the action of a *traT*-encoded outer membrane protein (19) and the knowledge that the plasmid-borne *hly* determinant, comprising at least three cistrons, encodes for protein(s) responsible for transport of the activated hemolysin through the outer membrane (20). Preliminary experiments have suggested, however, that this is probably not the case, as the

introduction of well-characterized Hly plasmids of different incompatibility groups (6) into non-hemolytic O18 isolates of intermediate or low serum resistance has not led to significant changes in serum response. Nevertheless, recent studies have shown that the large majority of hemolytic strains causing urinary tract infection, in contrast to the hemolytic fecal strains originally examined (11, 20), carry their *hly* determinants on the chromosome (Hughes, Müller, and Hacker, unpublished data), and although these share extensive homology with those which are plasmid borne, they are not necessarily identical (Müller et al., Proc. Soc. Gen. Microbiol. 8:102, 1981) and will presumably be associated with genes not normally carried on plasmids. Clarification of the relationship between hemolysin production and other factors, such as serum resistance, associated with urinary tract infection therefore awaits further investigation of the chromosomal *hly* determinant.

Our data on the relationship between O type and serum response confirm in most respects those reported previously from this hospital (28), which indicated that serum-resistant urinary and fecal strains were not randomly distributed throughout the O types. The present data, however, suggest that such differences among urinary isolates are not solely a reflection of the qualitative differences in O antigen. The high resistance to serum observed among urinary strains of O types O6, O18, and O75 appears to be largely associated with the carriage of *hly* determinants; most nonhemolytic strains of these serotypes are as serum sensitive as strains of the sensitive O types, e.g., O1 and O2.

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