Virulence of urinary and faecal *Escherichia coli* in relation to serotype, haemolysis and haemagglutination

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SUMMARY

The virulence of faecal and urinary *Escherichia coli* strains was studied in relation to serotype, haemolysin production and haemagglutination pattern. By means of an experimental mouse model *E. coli* strains can be divided into avirulent (I), mouse nephropathogenic (II), and generally virulent (III) strains. Virulent group II and group III strains were more often haemolytic and haemagglutinating than avirulent group I strains. Presence of K antigen could not be associated with virulence. Discriminant analysis for qualitative variables revealed that no combination of the investigated properties contributed more to a strain's virulence level than did one single property. It is concluded that other virulence factors, apart from haemolysin production in group II strains and haemagglutinins in group III strains, must be involved in the determination of a strain's virulence level.

All O2, O6 and O18ac strains tested were virulent, and by far the most O75 strains were avirulent, whereas other O groups were more variable with regard to virulence. Pyelonephritis strains were more often mannose-resistant haemagglutinating than faecal and other urinary isolates, indicating that mannose-resistant adhesins may be important in the pathogenesis of pyelonephritis.

INTRODUCTION

Over the years there has been a dispute about the nature of the infecting *Escherichia coli* strains in urinary tract infections. The *prevalence theory* holds that the *E. coli* strains causing infection are those predominant in the faeces (Turck, Petersdorf & Fournier, 1962; Grüneburg, Leigh & Brumfitt, 1968). On the other

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hand, many investigators have suggested that the infecting *E. coli* strains are a select group with properties which especially enable them to infect the urinary tract: *special pathogenicity theory*. The properties that have been related to nephropathogenicity include O antigen (Grüneberg, Leigh & Brumfitt, 1968; Mabeck, Ørskov & Ørskov, 1971; Dootson, MacLaren & Titcombe, 1973), K antigen (Glynn, Brumfitt & Howard, 1971; Kaijser, 1973; Kalmanson *et al.* 1975; Brooks *et al.* 1980, 1981), haemolysin production (Vahlne, 1945; Cooke & Ewins, 1975; Minshew *et al.* 1978; Brooks *et al.* 1980, 1981) and adhesive capacity (Svanborg Edén, 1978; Varian & Cooke, 1980; Hagberg *et al.* 1981).

In a previous study we found differences in virulence among different *E. coli* strains in a mouse model (van den Bosch, de Graaff & MacLaren, 1979). By following the kinetics of the viable count in the mouse kidney for eight hours after intravenous injection, together with the kinetics in other organs and with the measurement of LD₅₀ values and killing times for the mice, we were able to divide *E. coli* strains into three main groups with different virulence levels: (i) avirulent group I strains show low counts throughout the experiment; (ii) mouse nephropathogenic group II strains show high counts in the kidney after an initial decline; (iii) highly virulent group III strains show high counts in the kidney and other organs immediately after injection. A small group with a pattern intermediate between groups II and III was named group IV. Strains isolated from acute pyelonephritis were more often virulent in the mouse model than strains from acute cystitis and from asymptomatic bacteriuria (ABU) (van den Bosch *et al.* 1980*a*), whereas cystitis strains were not more often virulent than normal faeces strains (van den Bosch *et al.* 1981*a*). Therefore we have suggested that *E. coli* strains reach the bladder in general in proportion to their frequency in the faecal flora and subsequently may invade the kidney and cause serious infection in relation to their virulence.

In the present study we have compared the virulence levels of 119 faecal and urinary *E. coli* strains, from different origins, in relation to serotype, haemolysin production and haemagglutination (HA) pattern of the strains. This is in order to gain a better insight into the possible role of various bacterial properties in urinary virulence.

MATERIALS AND METHODS

Bacterial strains

The 119 *E. coli* strains used in this study all belonged to one or another of the four virulence groups as determined in the mouse model (van den Bosch *et al.* 1979), and were isolated from cystitis in general practices in Amsterdam, The Netherlands, and in Manchester, England (van den Bosch *et al.* 1979); from pyelonephritis, cystitis and ABU in a hospital in Amsterdam (van den Bosch *et al.* 1980*a*); and from cystitis and normal faeces in general practices in Zoetermeer, The Netherlands (van den Bosch *et al.* 1981*a*). The strains were kept lyophilized, and subcultured on nutrient agar not more than three times. Serotyping of O and K antigens was performed as described (van den Bosch *et al.* 1980*a*). H antigens were determined by standard methods according to Edwards & Ewing (1972).

Haemolysin production

Haemolysis was tested on washed-blood agar plates. Defibrinated sheep blood (Gibco, Glasgow, Scotland) was washed three times in phosphate-buffered saline (PBS, pH 7.0) and added to Blood Agar Base No. 2 (Oxoid, Basingstoke, England) to a final concentration of 5%. Haemolysis was read after overnight incubation at 37 °C.

HA pattern determination and electron microscopy

To test the haemagglutination (HA) patterns, bacteria were grown overnight in nutrient broth with very gentle agitation and on washed-blood agar plates at 37 °C, and resuspended in PBS (pH 7.0) to a concentration of 10^9 bacteria/ml by means of optical density. Guinea-pig blood (Albino random bred) and human group A blood were freshly collected 1:1 in Alsever's solution. Blood cells were washed three times in PBS and suspended to a final concentration of 0.75%. Bacterial suspensions (100 μ l) and erythrocytes were placed together into wells of a Multiwell Dispo-tray with a well capacity of 1 ml (Linbro Division, Flow Laboratories, Hamden, Conn.). For testing the mannose sensitivity of HA, assays in the presence of 0.5% D-mannose were included. Agglutination was read after incubation for 2 h at 0–4 °C, after agitation. Electron microscopy was performed as described previously (van den Bosch *et al.* 1980*b*).

RESULTS

Virulence levels of the strains and origin

In Table 1 the distribution into the four groups with different virulence levels is given for strains originating from faeces and from various forms of urinary tract infection (origin). Since only five strains belonged to virulence group IV, this small group will not be included in later analyses. The cystitis strains were collected at three different places, but the distribution into the virulence groups is not found to be different for these three groups of strains from different geographical locations (χ^2 test on 3×3 table, $P = 0.11$). A difference can be shown in the distribution of the virulence groups between the strains from different origins (Table 1; χ^2 test on 4×3 table, $P = 0.0012$). Further analysis by partitioning the χ^2 -statistic shows that none of the origin groups has a similar distribution over the virulence levels. It is seen that faeces strains mainly belong to virulence groups I and III, ABU strains mainly to groups I and II, cystitis strains mainly to groups I and III, and pyelonephritis strains mainly to groups II and III.

Serotypes of the strains

In Table 2 the O serogroups with at least four representatives present are shown, together with the numbers of strains within each serogroup belonging to the various virulence groups, and with the numbers of strains exhibiting the tested properties (K antigen, haemolysis, HA pattern). It is striking that O2, O6 and O18ac strains were invariably associated with virulence, whereas only two out of twelve O75 strains were virulent. Furthermore, it was found that the four O6:K2

Table 1. *Distribution of E. coli strains from various origins into the virulence groups; percentages of strains (the numbers of strains in parentheses)*

Origin	Virulence group*			
	I (51)	II (21)	III (42)	IV (5)
Faeces (30)	50 (15)	0 (0)	47 (14)	3 (1)
ABU† (13)	61 (8)	31 (4)	0 (0)	8 (1)
Cystitis (64)	42 (27)	19 (12)	37 (24)	2 (1)
Pyelonephritis (12)	8 (1)	42 (5)	33 (4)	17 (2)

* Virulence groups determined in mice: I, avirulent; II, mouse nephro-pathogenic; III, virulent; IV, virulence intermediate to groups II and III.

† ABU, asymptomatic bacteriuria.

Table 2. *Properties of strains belonging to common O serogroups with at least four representatives present; numbers of strains*

O antigen	N*	Virulence group†				Properties of strains‡				
		I	II	III	IV	K	Hly	MRHA	MSHA	non-HA
O1	7	4	1	1	1	7	0	2	1	5
O2	5	0	1	4	0	5	1	2	5	0
O6	17	0	5	12	0	16	16	3	16	0
O8	6	3	2	1	0	5	1	1	4	2
O18ac	4	0	0	4	0	4	3	3	2	0
O21	5	1	2	2	0	4	1	1	4	1
O25	5	2	1	1	1	3	2	3	5	0
O75	12	10	2	0	0	11	6	1	7	4
O77	5	1	2	2	0	4	1	0	4	1
O101	4	3	1	0	0	4	0	0	4	0
Other	37	20	4	10	3	25	5	5	21	13
Nontyp.	12	7	0	5	0	?	4	0	8	4

* N, total number of strains.

† See footnote * to Table 1.

‡ K, K antigen; Hly, haemolysis; MRHA, mannose-resistant haemagglutination; MSHA, mannose-sensitive haemagglutination; non-HA, no haemagglutination.

strains all belonged to virulence group II, and the ten O6:K23 strains in the present collection all belonged to virulence group III. However, K2 antigen in combination with O3 and O15 was not found to be associated with virulence, and one O25:K23 strain was also avirulent. Presence of H antigen or presence of certain H antigens did not seem to be associated with virulence, but was related more to the O antigen present. It should be stressed that other, less frequently isolated O groups may be virulent as well.

Presence of K antigen could not be associated with virulence (Table 3; χ^2 test on 3×2 table, $P = 0.69$), or with origin of the strains (Table 4; χ^2 test on 4×2 table, $P = 0.19$).

Table 3. Percentages (numbers) of strains within the virulence groups with *K* antigen (K), haemolysin production (Hly), haemagglutinins (HA), and mannose-resistant haemagglutinins (MRHA) present

Virulence group*	Properties of strains			
	K	Hly	HA	MRHA
I (51)	84 (38)	16 (8)	55 (28)	14 (7)
II (21)	76 (16)	57 (12)	86 (18)	24 (5)
III (42)	84 (31)	45 (19)	93 (39)	17 (7)

* See footnote to Table 1.

Table 4. Percentages (numbers) of strains within the groups of strains from various origins with *K* antigen (K), haemolysin production (Hly), haemagglutinins (HA), and mannose-resistant haemagglutinins (MRHA) present

Origin	Properties of strains			
	K	Hly	HA	MRHA
Faeces (30)	72 (18)	20 (6)	60 (18)	7 (2)
ABU* (13)	100 (10)	23 (3)	69 (9)	15 (2)
Cystitis (64)	85 (52)	39 (25)	81 (52)	17 (11)
Pyelonephritis (12)	75 (9)	50 (6)	83 (10)	50 (6)

* ABU, asymptomatic bacteriuria.

Haemolysin production

From Table 2 it is seen that haemolysin production was observed with all but one of the O6 strains, and was also relatively frequently observed with O18ac, O25 and O75 strains. With regard to the O75 strains, four out of five O75:K95 strains were haemolytic, but none of five O75:K100 strains. The virulent group II and group III strains were more often haemolytic than the avirulent group I strains (Table 3; χ^2 test on 3×2 table, $P < 0.001$). From Table 4 it is seen that the percentages of haemolytic strains increased from faeces to pyelonephritis isolates, but these differences were not significant (χ^2 test on 4×2 table, $P = 0.14$).

HA patterns

Very weak, hardly visible HA was considered negative, since this occasionally observed very weak HA could not be associated with the presence of pili, as judged by electron microscopy. The vast majority of the strains showed mannose-sensitive HA (MSHA) of guinea-pig erythrocytes, especially when bacteria were grown in broth, indicating the presence of type I pili (Table 2). MSHA of human erythrocytes was regularly observed with broth cultures of strains also showing MSHA of guinea-pig erythrocytes, probably also caused by type I pili (Evans, Evans & DuPont, 1979). A minority of the strains showed mannose-resistant HA (MRHA) of human erythrocytes, mostly with plate as well as with broth cultures, indicating the presence of non-type I pili. Some strains showed MRHA of human erythrocytes with broth cultures only (strains with serotypes O8:K-, O21:K-,

O25:K?, O57:K?, and O128:(K67)), perhaps due to still other fimbrial haemagglutinins. As revealed by electron microscopy of strains showing HA with broth cultures only, broth cultures were piliated and plate cultures were not. Two strains showed MRHA of both human and guinea-pig erythrocytes (strains with serotypes O23:K? and O57:K?), a pattern also occasionally found by Evans *et al.* (1980*b*).

Most of the strains that did not show any HA were avirulent group I strains (Table 3). All but a few of the virulent group II and III strains had one or more haemagglutinins. Virulent group II and group III strains were more often HA-positive than avirulent group I strains (χ^2 test on 3×2 table, $P < 0.0001$). No association could be shown between presence of haemagglutinins and origin of the strains (Table 4; χ^2 test on 4×2 table, $P = 0.13$).

Strains showing MRHA belonged to O groups 1, 2, 4, 6, 8, 18ac, 23, 25, 57, 75 and O128. MRHA with plate cultures was relatively frequently observed in the serotypes O6:K2 and O18ac:K?. MRHA by the strains could not be associated with virulence level (Table 3; χ^2 test on 3×2 table, $P = 0.58$), however, strains isolated from pyelonephritis showed more often MRHA than strains from faeces, ABU and cystitis (Table 4; χ^2 test on 4×2 table, $P = 0.011$).

Combination of properties

If one or more of the properties K antigen (K), haemolysin production (Hly) and haemagglutination (HA) are determinative for a strain's virulence level, one should be able to predict the virulence level of a strain from the test results for K, Hly and HA. We have investigated this for each of the four groups of strains from the various origins separately, by use of discriminant analysis for qualitative variables (Gilbert, 1968). If only the origin of a strain is known, the *a priori* probability for a correct classification of the virulence level can be estimated from Table 1, when allocating the strain to the virulence level with the highest probability. This classification implies that the probability of misclassification (PM) is minimized. When the test results for K, Hly and/or HA are also known, the *a posteriori* probability for a correct classification can be calculated. For this we first estimated from Table 5 the probabilities (Pr), such as Pr[virulence = II, K = +, Hly = +, HA = -], by the best fit log-linear model (Goodman, 1970). Using the theorem of Bayes (Lindley, 1975), we can now calculate for each combination of test results the *a posteriori* probability for a correct classification of the virulence level. Thus we derive for each combination of test results a classification with a minimal probability of misclassification (PM). Regarding the relative frequencies of the various combinations of test results, the total PM of this classification procedure can be calculated. By comparing the PMs we get an impression of the influence of one or more test results on the predictability of the virulence level, a low PM indicating a high predictability.

We estimated from the data in Table 1 and Table 5 the lowest probability of misclassification (PM) of a strain within a certain origin group, into a virulence level. This is done first without, and secondly with the various properties taken into account.

(i) The PM of faeces strains was 48 %, which decreased to 30 % when the three properties were taken into account; this decrease was due to HA only.

Table 5. *Numbers of strains* with various combinations of the properties K antigen (K), haemolysin production (Hly), and haemagglutinins (HA) present (+) or not (-), within the three major virulence groups*

Properties			Virulence group†		
K	Hly	HA	I	II	III
+	+	+	6	7	16
+	+	-	1	1	0
+	-	+	12	6	14
+	-	-	19	2	1
-	+	+	0	4	0
-	+	-	0	0	0
-	-	+	6	1	5
-	-	-	1	0	1

* Strains of which it is uncertain whether they may have a K antigen or not were omitted.

† See footnote * to Table 1.

Table 6. *The a posteriori probabilities of correct classification into virulence groups, when taking the properties into account that give rise to the lowest probability of misclassification (see text)*

Origin	Properties of strains*		Probabilities of classification into virulence group†		
	Hly	HA	I	II	III
Faeces	...	+	0.38	0	0.62
	...	-	0.90	0	0.10
ABU‡	+	...	0.40	0.60	0
	-	...	0.80	0.20	0
Cystitis	...	+	0.30	0.22	0.48
	...	-	0.80	0.11	0.09

* Hly, haemolysin production; HA, haemagglutination.

† See footnote * to Table 1.

‡ ABU, asymptomatic bacteriuria.

(ii) The PM of ABU strains was 33 %, which decreased to 24 % when the three properties were taken into account; this decrease was due to Hly only.

(iii) The PM of cystitis strains was 57 %, decreasing to 44 % taking the three properties into account; this decrease was due to HA only.

(iv) The PM of pyelonephritis strains was 50 %, decreasing to 45 % taking the three properties into account; this decrease was due to a combination of Hly and HA.

Thus a considerable decrease in PM was observed for classification of faeces, ABU and cystitis strains into a virulence level, taking into account only a single property of the strains. However, these PMs were still rather high. For pyelonephritis strains the decrease in PM was small. The resulting classification scheme is given in Table 6, in which the pyelonephritis strains were omitted. In conclusion, a combination of the properties Hly and HA did not seem to contribute more to a strain's virulence than a single property; presence of K antigen did not seem to

be important at all; other bacterial properties must be involved as well in the determination of a strain's virulence, because the PMs remained rather high; only in a few cases was the *a posteriori* probability of correct classification into virulence levels satisfactory (Table 6: faeces, HA⁻; ABU, Hly⁻; cystitis, HA⁻).

DISCUSSION

In our experimental mouse model virulent *E. coli* strains behave differently: group II strains are considered mouse nephropathogenic because of their high preference for kidney tissue in particular; group III strains are considered more generally virulent because of their high occurrence throughout the mouse body after intravenous injection (van den Bosch, de Graaff & MacLaren, 1979). Group II strains were frequently found among pyelonephritis and ABU isolates, less among cystitis and not among faecal isolates (Table 1), suggesting that group II strains may be especially pathogenic for human kidney tissue as well. It appears that different mechanisms of pathogenicity must be involved in group II and group III virulence.

Haemolysin production and presence of haemagglutinins could be associated with virulence, whereas presence of K antigen could not. These associations were more pronounced when regarding the different virulence levels (Table 3), than when regarding the different origin groups (Table 4). By means of the discriminant analysis in the present study, we were able to confirm previous results on a small part of the present collection, that haemolysis seems especially important in group II strains and HA in group III strains (Table 6). Previously we showed that in contradistinction to group III strains elimination of haemolysin production changed most group II strains into avirulent group I strains (van den Bosch *et al.* 1981*b*). Presence of haemagglutinins can in general be related to the ability of a strain to adhere to uroepithelial cells, and previously we showed that group III strains adhered better to uroepithelial cells than did avirulent group I strains, whereas adherence of group II strains was more variable (van den Bosch *et al.* 1980*a*).

Several recent studies, comparing properties of isolates from various forms of urinary tract infection and normal faeces, have suggested that combinations of certain bacterial properties are important in nephropathogenicity of *E. coli* (Brooks *et al.* 1981; Green & Thomas, 1981). We feel, however, that caution is necessary in comparing bacterial properties of various groups of clinical isolates without virulence testing, because of the different virulence levels found and the suggested differences in underlying mechanisms of pathogenicity. From the present study it is indicated that no combination of the investigated properties (K antigen, haemolysis and HA) contributes more to a strain's virulence level than does a single property. Thus it is not yet possible to explain fully the difference in virulence between group II and group III strains. We may conclude that apart from haemolysis in group II strains and adhesive capacity in group III strains other, still unknown, factors must be involved in the determination of a strain's virulence level. This can be underlined by the finding that six out of 45 investigated

avirulent group I strains possessed all of the three investigated properties (Table 5).

Another conclusion from the present results is that the group of strains with serotypes commonly found in urinary infections is not homogeneous in relation to virulence. All our O2, O6 and O18ac strains were virulent whereas by far the majority of our O75 strains were avirulent. Strains belonging to other common urinary O groups were variable with regard to virulence (Table 2). For O6 strains these results are in agreement with several studies suggesting their nephropathogenicity (Grüneberg, Leigh & Brumfitt, 1968; Mabeck, Ørskov & Ørskov, 1971; Dootson, MacLaren & Titcombe, 1973). It is not certain whether the special pathogenicity of the O2, O6 and O18ac strains is caused by the antigenic structures itself, or by virulence factors associated with these O antigens. It should be stressed that all four O6:K2 strains belonged to virulence group II and all ten O6:K23 strains to group III, that the vast majority of the O6 and O18ac strains was haemolytic, and that MRHA was frequently observed with O6:K2 and O18ac strains.

The frequent association of haemolysis with certain serotypes (e.g. O6 and O18ac) was shown many years ago (Vahlne, 1945; Brooks *et al.* 1980). Cooke & Ewins (1975) also found O75 strains commonly haemolytic, but we found that haemolysis is only associated with O75:K95 strains and not with O75:K100 strains.

Besides the frequent association of MRHA with serotypes O6:K2 and O18ac, MRHA has been observed with other O groups as well. This is in agreement with observations made by others (Cravioto *et al.* 1979; Duguid, Clegg & Wilson, 1979; Green & Thomas, 1981). Ørskov *et al.* (1980*a, b*) have suggested that MR adherence mediated by MRHA pili, and not MS adherence mediated by type I pili, is of special importance in the pathogenesis of urinary tract infection. This does not seem to be in agreement with our present results. The majority of the strains showed MSHA and only a minority MRHA. Although, as in several other studies (Duguid *et al.* 1979; Varian & Cooke, 1980), no association has been found between origin of the strains and presence of haemagglutinins (Table 4), virulent strains showed HA more often than avirulent strains (Table 3), but strains showing MRHA were equally uncommon in all the three virulence groups. On the other hand, MRHA strains were disproportionately frequent in pyelonephritis strains (Table 4). Several others have found MRHA more frequently present in urinary and other extra intestinal isolates than in normal faecal isolates (Minshew *et al.* 1978; Evans *et al.* 1980; Brooks *et al.* 1981; Green & Thomas, 1981). Additionally, Hagberg *et al.* (1981) have recently shown that pyelonephritis isolates were more often MRHA than normal faecal *E. coli*, whereas MRHA strains adhered in higher numbers to uroepithelial cells than MSHA strains. It is possible that MRHA pili indeed are of some special importance in the initiation of renal infections, representing or associated with virulence factors that are not of importance in our mouse model. However, the number of pyelonephritis strains is still small: more strains should be tested to explain this seeming contradiction between the mouse model and human infection. Evans *et al.* (1980*a*) have shown that MRHA strains are more

virulent in essentially the same mouse model, compared with MSHA and non-HA strains. Furthermore, different pilus types may be involved in MRHA, which are not all virulence associated.

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