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Salt Aggregation Test for Measuring Cell Surface Hydrophobicity of Urinary *Escherichia coli*

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The recently developed salt aggregation test for measuring relative surface hydrophobicity of bacteria was used to study *Escherichia coli* strains isolated from urinary tract infections. Of the 232 strains tested 166 (71.5 %) aggregated in salt solutions of varying molarity (0.1–1.6 M final concentration). Mannose-resistant haemagglutination of various erythrocyte species and/or mannose-sensitive haemagglutination of guinea-pig erythrocytes was seen in 144 of the 166 salt aggregating strains. Two salt aggregation-negative (hydrophilic) strains exhibited mannose-sensitive haemagglutination. Fimbriae of varying morphology were seen in salt aggregating strains. Strains with type 1 fimbriae only generally showed lower surface hydrophobicity than strains exhibiting mannose-resistant haemagglutination. Growing strains at 18 °C suppressed fimbriation with a concomitant decrease in surface hydrophobicity and haemagglutination. The salt aggregation test proved to be a rapid and reproducible screening test for detecting bacteria with high surface hydrophobicity due to surface protein of fimbrial (haemagglutinating or non-haemagglunating) and non-fimbrial nature.

During the past decade much interest has focused on cell surface antigens of bacteria and their possible role in the colonization of mucosal surfaces (1, 2). Enterotoxigenic *Escherichia coli* isolated from intestinal infections possess different fimbriae – K88, K99, CFA/I and CFA/II – which have different host specificity and exhibit mannose-resistant haemagglutination (MRHA) of erythrocytes of different animal species (3, 4). More recently, fimbrial antigens causing MRHA were described in *Escherichia coli* isolated from urinary tract infections (5, 6, 7). On the other hand, type 1 (common type) fimbriae causing mannose-sensitive haemagglutination (MSHA) of guinea-pig erythrocytes are common in *Escherichia coli* independent of origin (8).

Recent studies in one of our laboratories showed that prototype strains of *Escherichia coli* carrying type 1 fimbriae have a relatively hydrophilic surface character, whereas enterotoxigenic *Escherichia coli* with CFA/I and CFA/II have pronounced surface hydrophobicity as determined by two independent tests, hydrophobic interaction chromatography and bacterial cell aggregation in salt solutions, i.e. the salt aggregation test (SAT) (4, 9). Growing these latter strains in different media to suppress fimbriae production always resulted in a parellel diasappearance of the ability to haemagglutinate and a drastic decline in surface hydrophobicity as measured by both methods. These observations encouraged us to use the simple SAT together with haemagglutination and electron microscopy to investigate whether strains with high surface hydrophobicity always display MRHA and are fimbriated. Furthermore, we wanted to evaluate the SAT as a new test for rapid and simple screening for fimbriated strains and to explore the observation that strains with type 1 fimbriae only can be discriminated by this test since type 1 fimbriae show relatively low surface hydrophobicity despite similar content of nonpolar amino acids (10, 11).

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Materials and Methods

Bacterial Strains. *Escherichia coli* (232 strains) were isolated from either hospitalized patients or outpatients of all age groups (0–98 years) with urinary tract infection ($> 10^6$ bacteria per ml). The urine samples were cultured on Endoagar (Oxoid, London) and blood agar with 5 % horse erythrocytes overnight at 37 °C. The strains were typed to species level by API 20E (Analytab Inc., Plainview, NY, USA). The strains were stored in trypticase soy broth with 20 % glycerol (vol/vol) at −70 °C. Faecal *Escherichia coli* were isolated from 42 individuals (3–61 years) with no known history of urinary tract infection or diarrhoeal disease. Four colonies from each individual were tested. After initial isolation strains were serially transferred in static CFA broth at 37 °C (3). Strains were also grown on blood agar at 18 °C and at 42 °C aerobically and at 37 °C anaerobically in a GasPak jar (BBL Microbiology Systems, Cockeysville, MO, USA).

Salt Aggregation Test (SAT). Bacteria from the primary blood agar isolation plate were suspended in 0.001 M sodium phosphate buffer, pH 6.8, at a concentration of 10^8 bacteria per ml (9). Ten microliters of the bacterial suspension were mixed with an equal volume of ammonium sulphate of varying molarity (3.2, 1.8 and 0.2 M), pH 6.8, on a glass slide and observed for aggregation after 1 min at 20 °C. The SAT value represented the lowest concentration of ammonium sulphate at which aggregation was observed.

Haemagglutination Test. Bacteria were suspended in sodium phosphate buffered saline, pH 7.1, supplemented with 1 % bovine serum albumin at a concentration of 10^9 bacteria per ml. Bacterial suspensions (50 µl) were mixed with equal volumes of washed erythrocytes suspended in sodium phosphate buffered saline to 3 % (vol/vol) in plastic trays with holes of 16 mm diameter (Linbro Sc. Comp., Hamden, CT, USA) and observed for agglutination at 20 °C and at 4 °C after 1 h and 18 h (3). To discriminate between mannose-sensitive and mannose-resistant haemagglutination, 50 µl of 0.5 % D-mannose was added to the bacteria-erythrocyte mixture. All strains were tested against human group A and guinea-pig erythrocytes. Strains that did not haemagglutinate these species were further tested against monkey, horse, ox, sheep and chicken erythrocytes.

Hydrophobic Interaction Chromatography. Forty-two strains were chromatographed on Octyl-Sepharose equilibrated with 1 M ammonium sulphate, pH 6.8, in Pasteur pipettes as previously described (4). The eluate was compared visually with suspensions of bacteria with known concentrations. The test was considered positive when > 90 % of the bacteria were retained on the gel.

Electron Microscopy. Bacteria were suspended in sodium phosphate buffered saline at a final concentration of 10^5 bacteria per ml. One drop of the suspension was placed on a carbon-coated grid, washed with distilled water and stained with 1 % phosphotungstic acid. The grids were investigated in a JEOL 100 C transmission electron microscope at 60 KV. At least 200 cells per grid were observed.

Other Tests. Eight strains exhibiting MRHA of human erythrocytes were serotyped (O: H: K) by Dr. B. Kaijser, Institute of Medical Microbiology, Gothenburg, Sweden.

Chemicals. Trypticase soy broth and agar bases (No. 2) were from Oxoid Ltd, London, UK. Ammonium sulphate and D-mannose were purchased from Merck, Darmstadt, FRG, and Octyl-Sepharose from Pharmacia Fine Chemicals, Uppsala, Sweden.

Results

Salt Aggregation and Haemagglutination Tests

Cell surface hydrophobicity as revealed by the salt aggregation test (SAT) was seen in 166 of the 232 strains (71.5 %). Thirty-nine strains aggregated only at the highest salt concentration (1.6 M final concentration), and 83 strains at 0.9 M final concentration (Table 1). In contrast, 18 (10.7 %) of the 168 faecal *Escherichia coli* strains isolated from healthy invidivuals aggregated in 1.6 M ammonium sulphate, and three strains in 0.9 M salt solution.

Thirty-eight strains out of 42 tested bound to the gel matrix in the standard hydrophobic interaction chromatography (HIC) assay. All these HIC-positive strains also aggregated in the SAT. With one strain 80–90 % of the bacteria were retained on the gel, and this strain aggregated only at the highest salt concentration and showed MSHA. Three strains were negative in both HIC and the SAT (data not shown).

Of all 166 strains that aggregated at 0.1–1.6 M ammonium sulphate, 144 haemagglutinated one or more erythrocyte species upon initial testing (Table 1). Only two strains out of 66 that did not salt-aggregate demonstrated haemagglutination (MSHA). Most of the strains which demonstrated MSHA only had a positive SAT at the highest salt concentration, whereas 88 % of the strains demonstrating MRHA also aggregated at lower salt concentrations.

After serial transfer in CFA broth six times, 11 out of 15 initially non-haemagglutinating

Table 1: Distribution of haemagglutination among strains aggregating in ammonium salt solutions.

Strains exhibiting	Number of strains aggregating in ammonium sulphate (final concentration) 0.1M	0.9M	1.6M	> 1.6M
MR_{human}	6	10	4	0
MR_{human} and $MS_{g\text{-}p}$	14	16	2	0
$MR_{non\text{-}human}$	7	6	3	0
$MR_{non\text{-}human}$ and $MS_{g\text{-}p}$	9	34	5	0
$MS_{g\text{-}p}$	5	8	15	2
No haemagglutination	3	9	10	64
	44	83	39	66

MS = Mannose-sensitive haemagglutination of guinea-pig erythrocytes.
MR = Haemagglutination of monkey, horse, ox, sheep, chicken and human erythrocytes alone or in various combinations in the presence of D-mannose.

strains exhibited MSHA (Table 2). Five of these 11 strains also exhibited MRHA. Six of these strains showed a concomitant increase in hydrophobicity as measured by the SAT, whereas one strain showed a decrease in hydrophobicity. This strain exhibited MSHA after serial transfer and became heavily fimbriated, presumably with type 1 fimbriae.

Electron Microscopy

Out of thirty strains which were positive in SAT and haemagglutination tests, 29 were fimbriated, and of 27 salt-aggregating but initially non-haemagglutinating strains 24 were fimbriated. With two of the four non-haemagglutinating and non-fimbriated strains more

Table 2: Fimbriation in relation to surface hydrophobicity and haemagglutination of initially non-haemagglutinating strains at initial testing and after serial transfer six times in CFA broth.

	Initial testing		After serial transfer in CFA broth		
Strain	SAT	Number of fimbriae/cell	SAT	HA	Number of fimbriae/cell
66	0.9	None. Flagellae	0.1	–	10–20 on 1 %
149	1.6	1–5 on 1 %	0.9	–	1–5 on 5 %
166	1.6	1–5 on 1 %. Flagellae	0.9	–	1–5 on 2 %
177	1.6	30–50 on 20 %	1.6	–	1–5 on 5 %
189	0.1	20–50 on 5 %	0.9	MS	100–200 on 90 %
99	0.9	None. Flagellae	0.9	MS	10–50 on 5 %
102	0.9	10–50 on 10 %	0.9	MS	10–50 on 10 %
148	0.9	5–10 on 5 %	0.1	MR, MS	2–10 on 80 %
204	0.9	10–30 on 1 %	0.1	MR, MS	100–200 on 90 %
50	1.6	10–20 on 10 %. Flagellae	1.6	MS	10–50 on 10 %
111	1.6	1–5 on 1 %	0.1	MR, MS	1–5 on 1 %
119	1.6	10–50 on 5 %. Flagellae	1.6	MS	10–50 on 5 %
167	1.6	100–200 on 50 %. Flagellae	0.9	MR, MS	100–200 on 50 %
176	1.6	1–5 on 1 %	0.9	MS	10–50 on 10 %
178	1.6	100–200 on 5 %	0.1	MR, MS	100–200 on 90 %. Flagellae

SAT = Salt aggregation test.
HA = Haemagglutination test.
MR = Mannose-resistant haemagglutination.
MS = Mannose-sensitive haemagglutination.

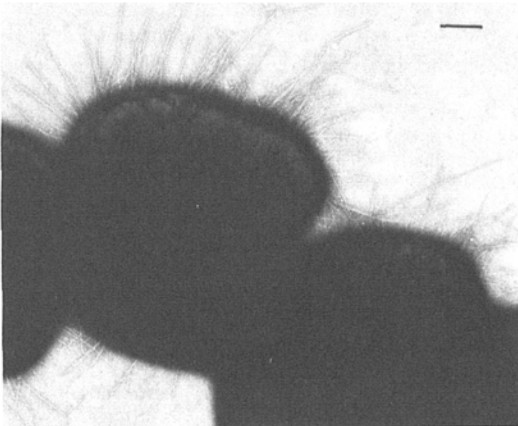

Figure 1: Strain 122 grown in CFA broth at 37 °C which aggregates in 0.1 M ammonium sulphate at pH 6.8 and exhibits marked mannose-resistant haemagglutination of human erythrocytes. Bar 200 nm.

than 95 % of the bacteria carried one or more flagellae. Five strains which were negative in both SAT and haemagglutination tests were also analysed but none of them was fimbriated (data not shown).

Bacteria of salt-aggregating strains seemed to form clusters on the grid. Fimbriae from strains showing MRHA of human erythrocytes tended to aggregate, presumably due to pronounced hydrophobic interactions (Figure 1), while fimbriae from strains showing MSHA only did not. The mean diameter of fimbriae from four strains with MRHA of human erythrocytes was about 5.5–6 nm (Figure 1). Morphologically different fimbriae with mean diameters of 2.5–7.5 nm were observed in strains showing MRHA of non-human erythrocytes or non-haemagglutinating strains. Generally, fimbriae were seen overlying and extending from the cell margin.

Comparison of strains showing MRHA of human erythrocytes and aggregating at 0.1 M or 1.6 M ammonium sulphate showed that strains that aggregated only at the highest salt concentration had fewer fimbriae per cell and fewer bacteria were fimbriated than in strains aggregating at a lower salt concentration.

Strain 122 (non-typable in O and K antisera) aggregated in 0.1 M ammonium sulphate and displayed MRHA of human erythrocytes when grown on blood agar at 37 °C aerobically as well as anaerobically and was heavily fimbriated (Figure 1). However, when grown at 18 °C this strain salt-aggregated in 0.9 M ammonium sulphate and higher concentrations, and showed only weak MRHA of human erythrocytes. Cells were then less fimbriated. After repeated cultures at 42 °C the strain did not display MRHA or salt-aggregate,.and no fimbriae were detected.

In seven of the nine strains which showed increased cell surface hydrophobicity as measured by the SAT after serial transfer, the increase in hydrophobicity was concomitant with an increased number of fimbriated cells and in three strains also an increased number of fimbriae per cell (Table 2).

Discussion

Type 1 fimbriae and purified fimbriae from urinary *Escherichia coli* causing MRHA have about the same content of nonpolar amino acids (2, 11) which are postulated to produce strong hydrophobic interactions between the fimbrial subunits (11). However, recent studies show that *Escherichia coli* isolated from urinary tract infections which possess fimbriae causing MRHA all seem to show pronounced hydrophobic surface properties while strains showing MSHA only, i.e. which probably carry only type 1 fimbriae, have a more hydrophilic surface character (9). The results presented in Table 1 confirm these observations since 15 out of 28 strains showing MSHA aggregated only at a high ammonium sulphate concentration (1.6 M) in the standard SAT and two strains were negative in the SAT. The remaining strains with MSHA only aggregated in 0.1–0.9 M ammonium sulphate which may be explained as follows: (i) these strains also carry non-haemagglutinating fimbriae with pronounced surface hydrophobicity analogous to non-haemagglutinating 987p fimbriae in *Escherichia coli* colonizing the small intestine (12); (ii) these strains possess hydrophobic surface proteins of non-fimbrial nature (13). Since studies on gonococci have revealed up to four different antigenic types of fimbriae on one strain (14), and recent studies on fimbriae of uropathogenic *Escherichia coli* revealed serological heterogeneity, it seems quite probable that these MSHA-only strains possess yet undefined fimbriae (10, 15). Screening of these

hydrophobic strains with MSHA for haemagglutination of erythrocytes of a great number of animal species did not reveal any specific pattern which could indicate new fimbrial haemaggutinins in these strains (unpublished data). Furthermore, unpublished studies on a number of semi-rough and rough *Escherichia coli* and *Salmonella* strains seem to rule out the possibility that an incomplete lipopolysaccharide could explain the pronounced surface hydrophobicity of these strains.

In our study, 62 % of the strains haemagglutinated one or more erythrocytes species which is consistent with other studies (5, 6, 16, 17, 18, 19). The incidence of *Escherichia coli* carrying fimbriae which cause MRHA of human erythrocytes, e.g. the recently defined P- and X-fimbriae, is higher among patients with pyelonephritis than in patients with lower urinary tract infection (5, 6, 20). Strains isolated from older patients less frequently display MSHA and MRHA (21). This is consistent with data from the present study in which 70 % of the isolates that were negative in the SAT and haemagglutination tests originated from patients above 60 years of age. The dominance of SAT-positive strains among urinary strains (71.5 %) compared to normal faecal strains (12.5 %) suggests that the hydrophobic cell surface, on account of fimbriae or non-fimbrial surface proteins, is involved in colonization of the urinary tract or maintenance of urinary tract infection.

Several methods have been described for measuring cell surface hydrophobicity such as (i) hydrophobic interaction chromatography, (ii) two phase partition systems, (iii) binding to hydrophobic probes such as dodecanoic acid, and (iv) binding to hydrocarbon (4, 22, 23, 24). The salt aggregation test provides a simple, rapid and reproducible method for measuring cell surface hydrophobicity well suited for screening of large number of strains. We found that the SAT in combination with haemagglutination and electron microscopy is a valuable tool for investigating strains of *Escherichia coli* and other species isolated from mucosal surfaces for the presence of fimbriae and other surface proteins which may represent specific adhesive antigens (1, 2).

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