UROPATHOGENIC PROPERTIES OF *PROTEUS MIRABILIS* AND *PROTEUS VULGARIS*

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SUMMARY. A group of faecal isolates of *Proteus vulgaris* and *P. mirabilis* was studied for the presence of possible virulence factors such as growth rates in urine and broth, haemolysin production, hydrophobicity, sensitivity to the bactericidal activity of human serum and cell invasiveness. Differences were found in haemolysin production, cell invasiveness and experimental virulence in a mouse model. These differences might explain why *P. mirabilis* is much more common in human urinary-tract infections than *P. vulgaris*.

INTRODUCTION

Proteus vulgaris and *P. mirabilis* are common inhabitants of the human gut; Krikler (1953) reported prevalences of 17% for *P. mirabilis* and 5% for *P. vulgaris* in the faeces of healthy persons. Urinary pathogens are thought to originate mainly from the gut and it is interesting that *P. mirabilis* is disproportionately more frequently isolated from patients with urinary-tract infections than *P. vulgaris*. Senior (1979), for example, isolated 258 strains of *P. mirabilis* from infected urines against only four strains of *P. vulgaris*, two of which were isolated together with *P. mirabilis*. These epidemiological data suggest that *P. mirabilis* is more likely to cause urinary-tract infections than *P. vulgaris*. As both species are very similar, genetically and biochemically (Brenner *et al.*, 1978), a comparative study of isolates of both species could yield valuable information about bacterial properties important in the pathogenesis of urinary-tract infections. Therefore, we have compared faecal isolates of both species, isolated from the same population, with regard to several properties: experimental virulence in a mouse model, growth rates in urine and broth, haemolysin production, hydrophobicity, sensitivity to the bactericidal activity of human serum, cell invasiveness and serotype.

MATERIALS AND METHODS

Isolation of Proteus strains from faeces. Stool samples were obtained from patients seen in general practice. Most samples had been submitted for examination for occult blood or for suspected parasitic infestation. A suspension of the sample in phosphate-buffered saline (PBS) was inoculated in the centre of a fresh nutrient-agar plate (Oxoid). The plates were incubated for

up to 3 days at 37°C and inspected for swarming growth. From such growth a pure culture was obtained and identification was performed with the API 20E system (API System S.A., France). All isolated strains were lyophilised and also kept in a glycerol-broth mixture (30:70 v:v) at -70°C.

Experimental virulence in a mouse model. The 50% lethal dose (LD50) and the 50% kidney-infecting dose (KID50) were determined after intravenous injection of log-phase bacteria in 6-week-old male mice (Swiss Random, TNO, Zeist, The Netherlands) as previously described (Peerbooms, Verweij and MacLaren, 1982).

Growth rates in urine and broth. Overnight cultures in nutrient broth (Oxoid) were diluted 1 in 50 in sterile pooled urine or nutrient broth in microtitration plates (Microtitre, Dynatech). The growth rates were determined by measuring the absorbances at 570 nm at serial intervals of 30 min with a Dynatech M600 plate reader. The urine had been treated previously to remove alkali-precipitable material (Senior, Bradford and Simpson, 1980).

Haemolysin production by Proteus strains was measured as described by Peerbooms, Verweij and MacLaren (1983). In short, haemolytic titres were recorded as the highest dilution of a late-log-phase culture in Brain Heart Infusion Broth (Difco) that produced complete haemolysis of a standardised suspension of horse erythrocytes.

Hydrophobicity of the bacterial surface was studied in an aqueous two-phase partitioning system composed of dextran T500 (Pharmacia, Sweden) and polyethyleneglycol 600 (PEG; Merck, Germany) as described by Stendahl, Norman and Edebo (1979) with some modifications. PBS was used instead of Tris buffer and the partition of the bacteria between the two phases was determined by viable counts instead of by using radio-active labelled bacteria. About 10⁵ cfu of log-phase bacteria were used.

Sensitivity to the bactericidal activity of human serum. Approximately 10⁶ cfu of log-phase bacteria in PBS were added to pooled human serum to give a final serum concentration of 67% v/v. The mixture was incubated for 90 min at 37°C. Viable counts at the beginning and end of the test were obtained by serial dilution and plating out on Cystine Lactose Electrolyte Deficient (CLED) Agar (Oxoid). The pooled human serum was obtained from healthy volunteers and was stored at -70°C.

Cell invasiveness was studied on monolayers of Vero cells as described by Peerbooms, Verweij and MacLaren (1984). In short, monolayers of Vero cells were incubated for 2 h with a 1 in 500 dilution of an overnight broth culture in modified Hanks's Balanced Salt Solution (HBSS). After this incubation, the monolayer was washed with HBSS and subsequently incubated in tissue culture medium containing kanamycin 250 µg/ml to kill extracellular bacteria (Okamura *et al.*, 1983). In a final step, the cells were lysed and intracellular bacteria were counted by plating on CLED agar plates. Cell invasiveness was expressed as a percentage of the cell invasiveness of reference strain F21.

Serotyping. O and H serotyping was kindly performed by Dr Peter Larsson, Göteborg, Sweden.

All bacterial properties except experimental virulence and serotyping were determined at least twice in independent experiments.

RESULTS

Bacterial strains

We examined 233 stool samples and found 20 strains of *P. mirabilis* (8.6%) and 10 strains of *P. vulgaris* (4.3%). All experimental data are shown in table I for *P. mirabilis* and in table II for *P. vulgaris*.

Experimental virulence in a mouse model

The mean KID50 value and the mean LD50 value were lower for the *P. mirabilis* strains than for the *P. vulgaris* strains. The difference between the KID50 values was

TABLE I
Properties of P. mirabilis strains

Strain	Serotype*	KID50 (10 ⁸ cfu)	LD50	Growth rate† (min)		Hydrophobicity (percentage in dextran)	Haemolytic titre	Invasiveness‡ (%)	SBB§ (%)
				broth	urine				
F15	27	3.1	7.8	43	41	89	32	ND	131
F18	Na	2.3	3.0	29	55	87	128	210	49
F19	17	9.8	9.8	31	30	57	<2	20	28
F21	29	4.2	8.4	32	32	75	128	100	36
F22	Na	0.94	2.1	32	35	87	64	ND	102
F23	28	1.4	2.4	36	36	73	256	303	92
F24	18	2.7	5.4	38	32	76	128	91	98
F25	Na	1.9	2.4	34	32	85	192	153	80
F26	30	4.3	7.7	35	36	68	96	98	56
F36	9	0.93	1.3	33	34	62	128	152	114
F38	Na	5.7	11.4	35	32	78	96	163	5
F39	10	9.2	18.4	31	38	91	48	ND	110
F40	2	16.0	20.5	45	42	99	10	7	160
F41	Na	4.3	10.8	30	55	87	24	42	130
F42	31	0.95	0.95	33	40	3	256	170	21
F45	9	0.98	1.95	30	45	76	128	ND	114
F48	Sa	6.4	6.4	31	44	28	256	119	0
F49	23	1.7	1.9	35	34	71	384	187	149
F50	27	8.2	10.3	36	40	97	48	ND	252
F51	2	...	...	43	40	87	192	159	192
Mean	...	3.1	4.9	35	38	...	130	132	96

ND = Not determined because of kanamycin resistance.

* O antigen; Na = Non-agglutinating; Sa = spontaneously agglutinating.

† Expressed as generation time.

‡ Expressed as a percentage of invasiveness of strain F21.

§ Sensitivity to the bactericidal activity of human serum (SBS): percentage of survivors.

TABLE II
Properties of P. vulgaris strains

Strain	Serotype*	KID50 (10 ⁸ cfu)	LD50	Growth rate† (min)		Hydrophobicity (percentage in dextran)	Haemolytic titre	Invasiveness‡ (%)	SBB§ (%)
				broth	urine				
F16	Na	5.2	10.0	41	44	89	80	10	175
F17	Na	4.3	6.7	37	33	78	80	8	209
F20	Na	...	...	41	48	72	12	4	0
F35	30	> 13.0	> 13.0	36	36	6	40	10	12
F37	Na	2.2	2.6	31	32	60	48	6	121
F44	Sa	4.7	4.7	39	36	0	32	33	168
F46	Na	> 13.0	> 13.0	38	48	2	24	16	104
F48	21	> 21.0	> 21.0	38	41	61	80	27	119
F52	Na	> 16.0	> 16.0	39	32	0.5	48	20	76
F53	Na	2.2	2.2	32	51	58	96	18	79
Mean	...	> 6.8	> 7.7	37	40	...	54	15	106

*, †, ‡, § see footnote to table I.

significant (Student *t*-test, $p < 0.05$) whereas the difference between the LD50 values was on the border of significance by the same test ($p \sim 0.05$).

Growth rates in urine and broth

The mean growth rates in each medium were similar for *P. mirabilis* and *P. vulgaris*.

Haemolysin production

All *P. vulgaris* strains possessed only a low-to-moderate activity whereas the *P. mirabilis* strains, though showing large variations, as a group showed a significantly higher activity (Student *t*-test, $p < 0.05$).

Hydrophobicity

The great majority of the *P. mirabilis* strains showed preference for the dextran phase (18 out of 20 strains) against only 6 out of 10 *P. vulgaris* strains that showed this preference. This difference is on the border of significance (χ^2 -test, $p \sim 0.05$).

Sensitivity to the bactericidal activity of human serum

Neither species was very sensitive to the bactericidal activity of human serum; only two strains of *P. mirabilis* and one of *P. vulgaris* were very sensitive ($< 10\%$ survivors).

Cell invasiveness

Vero cell invasiveness could not be measured for some strains of *P. mirabilis* because of their resistance to kanamycin ($MIC > 50 \mu g/ml$). However, the cell invasiveness of the 15 remaining strains of *P. mirabilis* was much higher than the invasiveness of the 10 *P. vulgaris* strains. This difference is statistically highly significant (Student *t*-test, $p < 0.001$).

DISCUSSION

To evaluate which bacterial properties are important in the pathogenesis of urinary-tract infections, different approaches can be chosen. From epidemiological studies on the prevalence of bacterial species in urinary infections and in the faecal flora, and from studies on the frequency with which defined bacterial properties are found in isolates from these origins, deductions may be drawn about the possible relevance of bacterial properties in the pathogenesis of these infections. Another approach is to study the importance of bacterial properties in experimental infections. We combined both approaches in this study of possible virulence factors of *P. mirabilis* and *P. vulgaris*.

Strains isolated from a non-hospital population were used because in hospital patients colonisation with hospital strains is known to occur readily. The selective pressure of antibiotics in the hospital environment might introduce yet another variable and invalidate the results of an epidemiological study.

Whereas the incidence in faeces of *P. vulgaris* (4.3%) was comparable with the data from other authors, *P. mirabilis* was found to have a lower incidence (8.6%) (Krikler, 1953: 5% and 17%; Rustigian and Stuart, 1945: 3% and 24%). This difference could be explained by geographical variations or by different isolation methods.

No significant differences were found between *P. mirabilis* and *P. vulgaris* in growth rates in urine and broth or in serum sensitivity. Differences in growth rates have been held responsible for differences in prevalence in urinary-tract infections of *Escherichia coli* and other enterobacteria (Anderson *et al.*, 1979) and of *P. mirabilis* and *P. morgani* (Senior, 1983), but cannot account for the difference in prevalence of *P. mirabilis* and *P. vulgaris* in urinary-tract infections.

The lack of difference in the sensitivity to the bactericidal effect of serum is in agreement with the results of other authors, indicating that serum sensitivity does not seem to be an important property in the pathogenesis of urinary-tract infections (Larsson and Olling, 1977; Brooks *et al.*, 1980). No conclusion can be drawn about the prevalence of particular serotypes because of the small number of strains and the diversity of serotypes. However, the study of Larsson (1980) suggests that in *Proteus* spp. there is no relationship between serotype and virulence.

As to their hydrophobicity, *P. mirabilis* strains seemed to be quite homogenous whereas the *P. vulgaris* strains were more heterogenous, though the difference between the species was of borderline significance. Accumulation of bacteria in the dextran phase has been associated with a hydrophobic surface (Stendahl *et al.*, 1979). Although a hydrophobic surface makes bacteria more liable to phagocytosis (Stendahl, Tagesson and Edebo, 1973) it also permits greater association with mucosal cells (Perers *et al.*, 1977), which might be favourable in the pathogenesis of urinary-tract infections.

Significant differences between the two *Proteus* species were found in their experimental virulence, haemolysin production and cell invasiveness. The greater virulence of *P. mirabilis* in experimental infections is in agreement with its greater prevalence in human infections. Although this suggests that data from this model are relevant for the human situation, we must be careful because it is possible that not all bacterial virulence factors, important in the pathogenesis of ascending infection in man, are measured in a haematogenous infection model.

Haemolysin production has been shown to be an important virulence factor in urinary isolates of *E. coli* (Brooks *et al.*, 1980). We reported earlier a correlation between haemolytic activity of *P. mirabilis* strains and their experimental virulence in mice. All *P. vulgaris* strains showed this haemolytic activity but at a significantly lower level than the *P. mirabilis* strains; it is a strongly cell-associated property (Peerbooms *et al.*, 1983). The possible role of haemolysin production in the pathogenesis of urinary-tract infections could be the liberation of iron compounds (Waalwijk, MacLaren and De Graaf, 1983) stimulating bacterial growth *in vivo* (Hart, Kadis and Chapman, 1982), creation of favourable growth conditions by cell destruction or stimulation of cell invasiveness. This latter phenomenon we described earlier for *P. mirabilis* (Peerbooms *et al.*, 1984). Indeed, *P. vulgaris* strains, were much less efficient in cell invasion than *P. mirabilis* strains, as was to be expected from their lower haemolytic activity.

This study showed that there are several differences between *P. mirabilis* and *P. vulgaris*, which could account for the higher prevalence of *P. mirabilis* in urinary-tract infections. None of these differences is so pronounced that it alone could account for this higher prevalence of *P. mirabilis*. Studies such as those of Brooks *et al.* (1980) and of Van den Bosch *et al.* (1980) have already shown that virulence in these commensal pathogens is a multifactorial phenomenon. Judgement of the contribution of each

separate factor to the bacterial virulence is therefore difficult and requires further studies.

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