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VIRULENCE FACTORS AND SUSCEPTIBILITY PATTERN OF ISOLATES OF TRIBE PROTEAE FROM CLINICAL SAMPLES IN A TERTIARY CARE HOSPITAL

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ABSTRACT

A total of 172 bacterial isolates with the split up of *Proteus* (128), *Morganella* (27) and *Providencia* (17) were isolated from various clinical specimens. Maximum isolates were obtained from urine 86 (50%) and pus 73 (42.4%). *Proteus* and related genera are 97% sensitive to carbapenem groups of antibiotics, 80 - 90% sensitive to piperacillin /tazobactam, amikacin and gentamycin and only 50% sensitive to all other cephalosporin and ciprofloxacin. Haemagglutination was shown by 122 (71%) strains, Hemolysin production in 140 (81%) strains and serum bactericidal activity by 147 (85%) strains of *Proteus* and related genera. All the three i.e. serum bactericidal activity, haemolysin and haemagglutination were present in 87 (51%) strains, any two were present in 63 (37%) strains and any one was present in 34 (20%) strains.The pathogenic potential of Proteus and related species can be assessed by evaluating multiple virulence factors instead of single parameter.

KEYWORDS: **Virulence, Proteus, Haemagglutination, Hemolysin, Serum bactericidal activity**

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INTRODUCTION

Proteus species, a common cause of urinary tract infection, can lead to serious complications. It is also a common nosocomial isolate, seen in the urine of catheterized patients and those with urinary tract abnormalities [1]. It often infects the upper urinary tract where it can lead to acute pyelonephritis, bladder or renal stones, fever and bacteraemia[2,3]. The Mechanism of pathogenesis of *proteus species* is not well resolved. Several potential virulence factors have been suggested for *Proteus species*. These include urease production[3], cell invasiveness[4, 5], cleavage of IgG and IgA by a proteolytic enzyme[6], outer membrane proteins[7], haemolysin production[8], adhesion to uro- epithelium[9, 10], swarming motility by flagella[11, 12] and resistance to bactericidal effect of normal serum[13, 14,15]. It has been suggested adhesions by fimbriae were involved in the initiation of infection by aiding colonisation of epithelial cells. So heavily fimbriated organisms were better able to initiate the infection[16]. Similarly production of haemolysin has been correlated with cytotoxicity for vero cell and with increased virulence in a mouse model of Urinary tract infection[17]. Therefore the present study was undertaken to analyse some of these virulence factors like bactericidal activity of normal human serum (serum bactericidal activity), haemolysin and haemagglutinins of *proteus* and related genera isolated from various clinical specimens and also whether these virulence factors expression vary in urinary isolates as compared to others were assessed. Drug resistance pattern of the isolates were also analized.

MATERIALS AND METHODS

Clinical isolates

A total of 172 bacterial isolates of *Proteus* 128(74%), *Morganella* 27(16%) and *Providencia* 17(10%) were collected for the present study from various clinical specimens for a period of one year from April 2012 to March 2013. The strains were identified into different species on the basis of standard biochemical reactions [18]. All the strains were stored on nutrient agar slopes at 4 ^{0} C for further testing.

Antibiotic sensitivity test

The antibiotic sensitivity test of each isolate was done on Mueller Hinton agar by Kirby Bauer disc diffusion technique using the antibiotic discs of cefazolin(30µg), cefuroxime(30 µg), cefotaxime (30 µg), cefepime(30 µg), ciprofloxacin(30 µg),norfloxacin(30 µg), amikacin(30 µg), gentamicin(10 µg), piperacillin/ tazobactam(100/10 µg), nitrofurantoin(30 µg), Imipenem(10 µg) and meropenem (10µg) according to CLSI guidelines[19].

Detection of Virulence factors

1) Serum bactericidal assay [14]

The serum bactericidal assay was performed by the method described by Kumar S et al 1997. Pooled normal human serum (PNHS) was taken and inactivated at 56 ^{0} C for 30 minutes. In nutrient broth test strains were grown overnight at 37 ^{0} C and were diluted to 10^4 CFU/ml in 5ml fresh nutrient broth. The diluted suspension were incubated at 37^0 C for 2 hours to give a log phase culture of 10^4 CFU/ml. The cultures were centrifuged (1500g for 5 minutes) and the deposit resuspended in 5ml of phosphate buffered saline. Equal volumes (0.2 ml) of this suspension and PNHS were mixed before incubation in a water bath at 0, 60, 120 and 180 minutes. Viable counts were made on nutrient agar by Miles and Mishra method. All the tests were done in duplicate. If the viable counts dropped to < 1% of the initial value the strains were termed sensitive and >90% of organisms survived after 180 minutes then serum resistant.

2) Haemagglutination Test [16]

Each strain bacterial suspension was tested for haemagglutination with human blood group O erythrocytes and sheep erythrocytes. The bacteria were sub cultured satistically thrice for 48 hours in nutrient broth at 37^{0}c, then harvested by centrifugation. The cell pellets were resuspended in 0.5 ml of phosphate buffered saline (PBS) at pH 7.2.

Haemagglutination was carried out by mixing 0.05ml of bacterial suspension tannic acid treated and tannic acid untreated erythrocytes in presence and in the absence of mannose at 23^0c. Haemagglutination was defined as visible clumping of erythrocytes. Mannose sensitive haemagglutination was demonstrated by agglutination of erythrocyte in absence of 50mM Mannose (MS). Mannose resistant *Klebsiella*- like (MR/K) haemagglutination was shown by agglutination of tannic acid treated erythrocytes but not by untreated erythrocytes; the reaction was not inhibited by 50mM mannose. Mannose resistant *Proteus*- like (MR/P) haemagglutination was demonstrated by agglutination of untreated as well as tannic acid treated erythrocytes even in presence of 50mM mannose.

3) Haemolysin production Test [16]

Haemolysin was performed by the method described by Mobley & Chippendale. The bacteria were grown overnight in brain heart infusion broth at 37^0c. Phosphate buffered saline (PBS) containing 0.1ml of two fold dilution of bacterial suspension were mixed with 0.05ml of 3% sheep RBC suspension in PBS (pH 7.2) and incubated for one hour at 37^0c. The highest dilution in which no visible RBC button was observed at the bottom was defined as haemolytic titre.

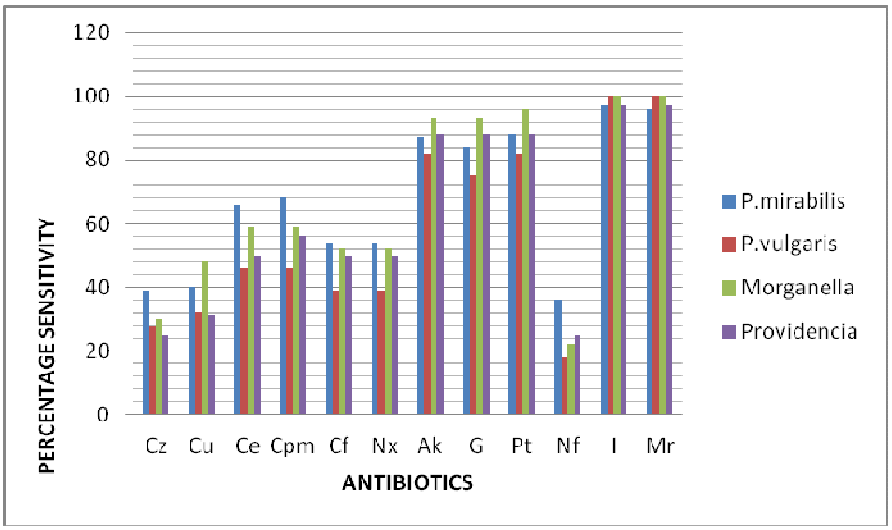
RESULTS

From the total of 172 strains collected, 100(58%) were *Proteus mirabilis*, 28(17%) *Proteus vulgaris*, 27(15%) *Morganella morganii,* 10(6%) *Providencia rettgeri* and 7(4%) *Providencia stuartii*. The distribution of all the isolates from different clinical specimens is shown in table 1. All the isolates of *proteus mirabilis* and *proteus vulgaris* produced urease enzyme. The antibiotic susceptibility patterns of all the isolates are shown in Fig 1. There was no difference of antibiotic susceptibility pattern between the *Proteus species* isolated from urine and from other clinical specimens. Even for *Providencia species* and *Morganella* also no difference in antibiotic susceptibility pattern among various isolates from different clinical sources. The results of virulence factors like heamagglutination, heamolysin and serum resistance (serum bactericidal activity) are shown in table 2. Out of 100 strains of *Proteus mirabilis*, 83 (83%) strains showed haemagglutination. In 28 strains of *Proteus vulgaris*, 16(57%) strains showed haemagglutination. The strains showed mannose sensitive haemagglutination along with other haemagglutinins. Among the 100 *Proteus mirabilis* strains 92(92%) strains were producing haemolysin. All isolates produced measurable haemolytic activity with no significant difference between urinary isolates and isolates from other sources. Serum resistant factor (Serum bactericidal activity) was observed in 91 strains (91%) out of 100 strains of *Proteus mirabilis*. Only 9 strains (9%) were serum sensitive. Correlation of all the three virulence factors is shown in table 3. All the isolates included in this study from urine and other clinical sources possessed either one or more of these three virulence factors.

Table 1
Distribution of Proteus and related isolates from different clinical specimens

Clinical specimen	Proteus mirabilis	Proteus vulgaris	Morganella	Providencia rettgeri	Providencia stuartii	Total Sample no:
Urine	51	11	14	6	4	86
Pus	42	11	13	4	3	73
Sputum	3	1	0	0	0	4
Pleural fluid	2	0	0	0	0	2
Stool	2	3	0	0	0	5
Vaginal swab	0	2	0	0	0	2
Total	100	28	27	10	7	172

Figure 1
Antibiotic sensitivity pattern of the clinical isolates



Cz- cefazolin, Cu- cefuroxime, Ce- cefotaxime, Cpm- cefepime, Cf- ciprofloxacin, Nx- norfloxacin, Ak- amikacin, G- gentamicin, Pt-piperacillin/ tazobactam, Nf- nitrofurantoin, I- Imipenem and Mr- Meropenem

Table 2
Virulence factors of the isolates

Virulence factor		Proteus mirabilis	Proteus vulgaris	Morganella	Providencia rettgeri	Providencia stuartii	Total
Serum Resistant (SR)	**Total**	100	28	27	10	7	172
	Positive	91	21	19	10	6	147
	%	91	75	70	100	86	**85**
Heamolysin (HL)	**Total**	100	28	27	10	7	172
	Positive	92	21	12	8	7	140
	%	92	75	44	80	100	**81**
Haemagglutination (HA)	**Total**	100	28	27	10	7	172
	MR/k	22	3	4	1	0	30
	MR/P	14	2	2	0	0	18
	MR/K&MR/P	47	11	8	5	3	74
	Total	83	16	14	6	3	122
	%	83	57	52	60	43	**71**

Table 3
Correlation of Virulence factors

Virulence factor	Proteus mirabilis (100)	Proteus vulgaris(28)	Morganella(27)	Providencia rettgeri(10)	Providencia stuartii(7)	Total (172)
All 3						
SR+HL+HA	52(52%)	14(50%)	15(56%)	4(40%)	2(29%)	87(51%)
Any 2						
SR+HL	15	2	4	1	2	24
SR+HA	17	2	3	2	1	25
HL+HA	8	2	2	1	1	14
Total	40(40%)	6(21%)	9(33%)	4(40%)	4(57%)	63(37%)
Any 1						
SR	11	2	1	0	0	14
HL	6	4	0	1	0	11
HA	3	2	2	1	1	9
Total	20(20%)	8(29%)	3(11%)	2(20%)	1(14%)	34(20%)

Serum Resistant (SR); Heamolysin (HL); Haemagglutination(HA)

DISCUSSION

In the present study the commonly isolated organism from the urine as well as from the pus is *Proteus mirabilis, Proteus vulgaris* and followed by other isolates of related genera. *Proteus* and related genera is the common causative organism of the hospital acquired infection. In this study all the isolates were collected from the hospitalized patients, hence they are more or less likely to be hospital strains only. In the antibiotic sensitivity profile, there is no difference between the sensitivity pattern among the organism isolated from urine and other clinical sources[21]. In this study *Proteus* and related genera, are 97% sensitive to carbapenem groups of antibiotics (Imipenem and meropenem), 80- 90% sensitive to piperacillin / tazobactam, amikacin and gentamycin. Only 50% sensitive to all other cephalosporin and ciprofloxacin. 3 *Proteus mirabilis* strains and in one *Providencia strain* were carbapenem resistant. Since all the strains were from hospitalised patients, the antibiotic resistance is common among them. In this study three virulence factors like haemagglutination, haemolysin production and serum resistance were analysed. All the isolates showed one or more of these virulence factors productions. Among the virulence factor production also no difference between the isolates from the urine and other clinical specimen. Since all these isolates were from the clinical sources, they are more likely to have the virulence factor in contrast to the environmental ones. For the development of urinary tract infection, adhesion mechanisms have been recognised as a relevant factor [15]. In the present study, all the three different haemagglutination (MS, MR/K, MR/P) were produced by the *proteus* and related genera. Although the distribution of haemagglutinin and fimbriae were more complex among *Proteus, Providencia* and *Morganella,* the role of haemagglutinins of *Proteus* in pathogenesis have been well established [15]. Haemolysin have been considered to be an important virulence factor by some authors [15,4], while others could not observe significant difference in virulence. In the present study, haemolysin was produced by 81% of clinical isolates. Zunino et al 1998 [1] suggested that haemolysin is not essential during early infection but this factor is important at late stages of infection. The resistant to normal serum may be important in the pathogenesis of *Proteus mirabilis* [14]. This virulence factor may contribute to the invasiveness of the bacteria. In the present study 85% exhibited serum resistance property. There was no significant difference in the haemagglutinin, haemolysin production and serum resistant activity of urinary isolates and isolates from other sources together. All the isolates had at least one of the virulence factors, 37% of the isolates exhibited two virulence factors. But 51% of the clinical isolates of *Proteus* and related genera had all the three virulence factors. In the search for potential virulence factors for *Proteus* bacteria, studies have been made on urease activity, pili, growth rate in urine and broth, haemolysin production, hydrophobicity, sensitivity to the bactericidal activity of human serum and cell invasiveness. The multidrug resistance of these strains also contribute to their virulence. Single virulence factor cannot be considered to assess the pathogenic ability of the strain. Various virulence factors may act independently or their actions may be complementary to each other. The presence of more number of virulence in one strain may likely to increase its pathogenic ability. The presence of virulence factors among *Proteus* and related species are same. There is no difference among the urinary isolates and other clinical isolates in exhibiting virulence factors. Always more attention is given to the uropathogenic *proteus* and related species but all hospital acquired strains should receive similar attention.

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