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Plasmid mediated virulence factors of some *Proteus* isolates

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ABSTRACT

Various virulence factors including invasion, adhesion, cytotoxicity, protease, lipase, elastase, urease production and swarming migration were determined for 24 *Proteus* isolates recovered from clinical specimens. The results showed that the distribution of virulence factors was different among the test isolates and strain specific in most cases. All *Proteus* isolates showed invasion and adhesion capabilities with different extents. In addition, they were able to produce elastase, urease and exhibit swarming activity. Protease and lipase activities were not detected in any of the isolates. High cytotoxicity was demonstrated in all isolates. A parallel correlation between invasion and cytotoxicity was demonstrated in all isolates. Five isolates of high virulence factors productivities were selected and identified by Analytical Profile Index as *Proteus mirabilis* (Pr2, Pr12 and Pr24) and *Proteus penneri* (Pr6 and Pr20). Plasmid curing by acridine orange resulted in the loss of invasiveness and adhesion capabilities of the five isolates, while other virulence factors levels showed no significant difference changes. The results give a clear evidence that both invasion and adhesion of the tested *Proteus* isolates are plasmid rather than chromosomally encoded.

Key words: *Proteus*, virulence, invasion, adhesion, urease, plasmid and curing

INTRODUCTION

Proteus species, members of the family *Enterobacteriaceae* (Penner, 1984) are motile Gram negative enteric bacteria, they are important pathogens of the urinary tract and are the primary infectious agent in patients with indwelling urinary catheters (Warren *et al*., 1982). Individuals suffering from urinary tract infections caused by *Proteus mirabilis* often develop bacteriuria, cystitis, kidney and bladder stones, catheter obstruction due to stone encrustation, acute pyelonephritis, and fever (Mobley and Warren, 1987; Johnson *et al*., 1993 and Burall *et al*., 2004). In addition strains of *Proteus penneri* can also cause urinary tract infection (Krajden *et al*., 1984).

Several potential virulence factors of *Proteus* had been studied in relation to its virulence and pathogenicity of urinary tract, including hydrolysis of urea by urease, cell invasiveness, cytotoxicity induced by hemolysins, cleavage of IgA and IgG by proteolytic enzyme and adherence to the uroepithelium mediated by fimbriae (Coker *et al*., 2000).

There were many proposed mechanisms and influencing factors for the invasive properties of *P. mirabilis* (Korn *et al*., 1995). Microbial invasion could be facilitated by virulence factors, microbial adherence, and resistance to antimicrobials. Virulence factors assisted pathogens in invasion and resistance of host defences. Bacterial proteins with enzymatic activity (e.g. protease, hyaluronidase, neuraminidase, elastase, collagenase) facilitated local

tissue spread. Microbial adherence to surfaces helps microorganisms establish a base to penetrate tissues. The adhesive properties in the *Enterobacteriaceae* were generally mediated by different types of pili (Ofek and Doyle, 1994).

Urease, which is responsible for the formation of bladder and kidney stones at later stages of infection, could facilitate the colonization of the urinary tract in a mouse model (Jones *et al.*, 1990). Haemolysin, which is cytotoxic for cultured urinary tract epithelial cells (Mobley *et al*., 1991), had been shown to be correlated with the ability of bacteria to invade cells (Peerbooms *et al.*, 1984). The ability of *P. mirabilis* to express virulence factors, including urease and haemolysin, and to invade human urothelial cells, is coordinately regulated with swarming differentiation (Allison *et al.*, 1992; Liaw *et al.*, 2000, 2001 and 2004).

Swarming cell differentiation is thought to be important for the virulence of *P. mirabilis* during urinary tract infections (UTIs) since several virulence factors, including flagellin, urease, the hemolysin HmpA, and the IgA metalloprotease ZapA, are upregulated in the differentiated swarmer cell compared to swimmer cells (Allison *et al*., 1992 and Fraser *et al*., 2002).

A tight relationship between bacterial cells harbouring plasmids and their drug resistance profile was known (Maltezou, 2009). Extended-spectrum β-lactamases (ESBLs) are enzymes that compromise the efficacy of all β-lactams by hydrolysis of the β-lactam ring (Coque *et al*., 2008). The genes encoding ESBLs were usually located on plasmids that were highly mobile and can harbour resistance genes to several other unrelated classes of antimicrobials (Canton and Coque, 2006).

Virulence factors of pathogenic bacteria (adhesins, toxins, invasins, protein secretion systems, iron uptake systems, and others) may be encoded on chromosomal DNA, bacteriophage DNA, plasmids, or transposons (Johnson *et al*., 2003) in particular regions of the prokaryotic genome termed pathogenicity islands (PAIs). Pathogenicity islands were first described in human pathogens of the species *Escherichia coli,* but have recently been found in the genomes of various pathogens of humans, animals, and plants (Haker and Kaper, 2000 & Perna, *et al*., 2001)

Understanding of the common themes in microbial pathogenicity is essential to recognize the microbial virulence in order to develop novel vaccines and other therapeutic agents for the treatment and prevention of infectious diseases. Accordingly, the aim of this work was to quantify some potential virulence factors produced by some *Proteus* spp. and study whether they were chromosomal or plasmid coded.

MATERIALS AND METHODS

Specimens and bacterial isolation

Fifty clinical specimens were collected from El-Demerdash hospital and Ain Shams Special hospital. The collected specimens included urine and swabs from wounds and abscesses. In addition, one isolate was supplied from hospital microbiological labs as grown culture which was recovered from prostatic secretions. Clinical specimens were collected according to the methods described by Cheesbrough (2000).

Maintenance of clinical isolates

All bacterial clinical isolates were grown routinely on nutrient agar slants, stored at 4°C and subcultured monthly. For long term storage,

bacterial isolates were grown in nutrient broth medium at 37°C to exponential phase and spinned down at 10,000 *x*g for 10 min. The pellet was resuspended in 10% (v/v) sterile glycerol in Protect Vials (Technical Service Consultants Ltd., UK) and stored at –20°C.

Vero Cell Line

Vero cell line used in this study was purchased from tissue culture department of VACSERA, Egypt. Vero cell line (ATCC No.CCL-81) is a Kidney epithelial cells derived from African green monkey (*Ceropithecus aethiops*). Preparation of Vero cell monolayer in microtiter plates was carried out according James and John (1998).

Identification of clinical isolates

Identification of clinical isolates for preliminary screening was carried out according to Bergey's Manual of Systematic Bacteriology (Holt and Williams, 1989). The identification of selected isolates was confirmed by Analytical Profile Index (API NE and API 20E (for Enterobacteriaceae) from Biomerex France).

Quantitative assay of virulence factors

Preparation of bacterial inoculum

A twenty four hours tryptic soy broth cultures of tested microorganisms grown in shaking incubator were centrifuged, washed twice with PBS and then standardized to 10^8 CFU ml^{-1} using nutrient broth medium (for protease, elastase, lipase and urease assays) while in case of assay of invasion, adherence and cytotoxcity, Basal tissue culture medium (BTC) was used to standardized the inoculum to 10^8 CFU ml^{-1}.

Growth conditions for production of virulence factors by the tested isolates

A set of 250 ml flasks containing 50 ml tryptic soya broth was separately inoculated by the tested isolated (100 µl of 10^8 CFU ml^{-1}). The flasks were incubated in shaking incubator for 24 h at 37°C. The growth obtained was centrifuges at 10000 rpm for 10 min. and the culture supernatants obtained were used for assaying extracellular virulence factors (protease, lipase and elastase). While for urease assay the pellets were used after sonication.

Quantitative assay of adherence and invasion

Adherence and invasion assay was carried out as described by Plotkowaski *et al.* (1994). Monolayers were grown in 96-well plates and inoculated. After incubation, monolayers were infected by addition of 100 µl of each clinical isolate suspended in Basal tissue culture medium (BTC). After incubation for 2 h at 37ºC in CO_2 incubator, medium were discarded and the wells were rinsed three times with phosphate buffer saline (PBS).

To assess adherence, monolayers were lysed with lyses solution for 10 min at room temperature. Following lyses, bacteria were enumerated by plating ten fold serial dilution onto nutrient agar medium to calculate the total count of adhered and up taken bacterial cells.

Quantitative assay of invasion

Invasion was determined as described by Plotkowaski *et al.* (1994), using a gentamycin survival assay. The same procedure was constructed as previously mentioned. After incubation at 37ºC for 2 h, infected monolayers were rinsed three times with PBS. Monolayers were then incubated for 2 h with fresh tissue culture medium containing 100 µl gentamicin (500 µg ml^{-1}) for each well to kill extracellular bacteria and incubated for 1 h. Following incubation, monolayers were rinsed, lysed, and bacteria were enumerated as for the adherence assay. By subtracting the number of invading cells from the total count of adhered and uptaken

cells, the number of adhering cells was obtained. Eight replicates wells were used for each isolate and all tests were performed at least twice.

Quantitative assay of cytotoxicity

This assay was carried out according to Kueng *et al*. (1986). Crystal violet assay were used to measure the total count of mammalian cells which well reflect the cytotoxic effects of the different selected clinical isolates by using Vero cell line in 96 well plates. The cell culture type (Vero) was subcultured in microtiter plate and each well was inoculated with 20 µl of each clinical isolates (suspended in BTC medium). The plates were incubated for 24 h in CO_2 incubator at 37ºC. After incubation time, the medium was discarded and the wells were washing three times with sterile saline solution, 100 µl of gluteraldehyde of 1% was added for about 30 minutes. The excess gluteraldehyed solution was removed then washed with tap water. Crystal violet solution (0.1%), 100 µl was added for about 30 minutes, dispensed and washed with very slow rate of tap water. The plates were dried then the stained wells were eluted with 100 µl of 10% acetic acid solution. The color intensity in each well was measured using microplate reader at 290 nm. Eight replicates were used for each isolate, and all tests were performed at least twice.

The absorption reading of clinical isolates (average reading of 8 wells) divided by the control reading (average reading of 8 wells containing 20 µl of sterile BTC medium instead of bacterial suspension) multiplied by 10^4 (the total number. of Vero cell per well). This provides the number of viable Vero cells. Subsequently, the previous number was subtracted from 10^4 to obtain the number of dead Vero cells.

Quantitative assay of protease activity

Azo-albumin test was performed as described by Ayora *et al.* (1994) with slight modification, 0.3 ml of bacterial culture supernatant was added to a test tube, 1.7 ml of distilled water, and then 0.1 ml of azo albumin suspension (5.0 mg ml^{-1} in 0.1 M Tris buffer pH 7.5). Blank containing 2.0 ml of distilled water and 0.1 ml of azo albumin suspension. Test tubes were incubated for one hour in a water bath at 30°C then 2.0 ml of 8% TCA were added to stop the reaction. The mixture then poured into a polypropylene centrifuge tube and centrifuged at 10,000 *x*g which was sufficient to precipitate the protein. To a clean tube 2.0 ml of clear supernatant was transferred and mixed with 2.0 ml of 0.5 M NaOH to intensify the color. The absorption was measured at 400 nm. The optical density was used for determination of the amount of protease enzyme U ml^{-1} using a standard curve of protease.

Quantitative assay of lipase activity

Determination of liberated free fatty acid (FFA), as a result of lipase activity was measured by the modified method of Kwon and Rhee (1986). Olive oil was used as a substrate. The reaction mixture 1.0 ml of bacterial free supernatant, 2.5 ml emulsion of (50 ml olive oil in 50ml PBS) and 0.02 ml of 20 mM $CaCl_2.2H_2O$ was incubated in shaking incubator at 200 rpm for 30 min, at 37°C. Then the reaction was stopped by adding 1.0 ml 6 N HCl and then 5.0 ml of iso-octane. The upper layer (4ml) was pipette out into a test tube and 1.0 ml of cupric acetate pyridine was added. The free fatty acid dissolved in iso-octane was determined by measuring the absorbance of iso-octane solution at

715 nm. Lipase activity was determined by measuring the amount of FFA from the standard curve of oleic acid.

Quantitative assay of elastase activity

Elastase activity in bacterial suspension was measured as described by Furuya *et al*. (1993). Bacterial suspension supernatant 0.1 ml was diluted three-fold with 10 mM Tris (pH 7.5) and added to 2 ml of 2 mg of elastin congo red (Sigma Chemical Co., St. Louis, Mo.) suspended in 20 ml of PBS as a substrate. The mixture (2.4 ml) was incubated at 37°C for 1 h, with vigorous shaking. The undissolved substrate was removed by centrifugation at 3000 *xg* for 10 min. absorbance was measured at OD 495. Values of the supernatants were then compared with a standard curve prepared by treating the substrate with purified *P. aeruginosa* elastase (Elastin Products Co., Inc., Owensville, MS, USA) for the same period at enzyme concentrations of 0.75-100 µg l^{-1}.

Quantitative assay of urease activity

Urease activity of bacterial cell extracts was determined by measuring the amount of ammonia released from urea in the phenol-hypochlorite urease assay (Weatherburn, 1967). Bacterial pellets (produced by centrifugation of 2 ml of bacterial suspension) were sonicated in 1 ml of urease buffer (50 mM HEPES pH 7.5 plus 25 mM urea) then incubated at 37°C for 20 min. The reaction was stopped by removal of 1 ml aliquots to cuvettes containing 1.5 ml of solution A (10 g l^{-1} of phenol and 50 mg of sodium nitroprusside) and 1.5 ml of solution B (NaOH 5 mg ml^{-1}-NaClO 0.044%, v/v). The contents were mixed thoroughly. Following incubation at 37°C for 30 min, the absorbance was measured at 625 nm. A standard curve was prepared using ammonium chloride concentrations (0.1-1 µg ml^{-1}).

Assay of swarming migration behavior

The swarming migration distance assay was performed as described by (Gygi *et al*., 1995 and Liaw *et al*., 2001). Briefly, an overnight bacterial culture (5 µl) was inoculated centrally onto the surface of dry LB agar medium (with 2% agar, w/v) plates. After incubation at 37°C, swarming migration for the bacterial cells was detected every 30 min. till 18 h.

Plasmid DNA extraction

Plasmid DNA was extracted and purified from a 5 ml overnight cultures of the selected isolates grown in LB broth medium using a QIA prep Spin Miniprep kit (Qiagen) according to the manufacturer's instructions.

Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to size. Agarose (GibcoBRL) was mixed with 1 x TAE to a final concentration of 1% (w/v) and boiled to dissolve. Ethidium bromide was added to the gel at a final concentration of 0.25 µg ml^{-1} before casting in standard horizontal gel equipment, 1 kb DNA ladder (Fermentas) was used as molecular markers to size DNA fragment. DNA samples (15 µl) were mixed with one fifth volume of loading buffer. Electrophoresis was carried out at 80-100 V for 1-2 h in TAE buffer. After electrophoresis, gels were visualized using a short wave UV trasilluminator (Spectroline, USA) and fluorescent images were captured.

Plasmid curing

Plasmid curing by SDS

The curing was carried out according to Tolmasky *et al.,* (1993). One ml of CA-YE broth medium containing 10% SDS was inoculated with 10 µl of an overnight culture

then incubated at 37°C for 24 h. Two fold dilutions were made, after that, 10 µl was streaked over CA-YE-2 agar medium plates and incubated at 37°C for 24 h. The separate colonies (mutants) were resubcultured on CA-YE-2 plates to ensure their purity. These isolates were tested for their antibiotic sensitivity and presence of plasmids.

Plasmid curing by acridine orange

LB broth tubes containing (different concentrations of acridine orange to a final concentration of 10, 15, 20, 25, 30, 32.5 and 35 µg ml^{-1}) were inoculated with an overnight culture of clinical isolates. The tubes were incubated at 37°C for 24 h. The highest concentration of acridine orange that showed turbidity (0.7-0.8 at OD 660 nm and the isolate still viable) was selected. The selected tubes were plated on LB agar medium and incubated at 37°C for 24 h. The cured isolates were tested for their antibiotic sensitivity and presence of plasmids.

Antibiotic sensitivity test

Antibiotic sensitivity test was carried to study the multi-drug resistant patterns of *Proteus* isolates bacteria. Different antibiotics namely Ciprofor, Garamycin, Cefotax, Isoptophenicol, Terramycin, Amikin, Cefteriaxone, Sulperazone, Cefobid, Leeflox, Meronam, Fortum, Tetracid, Tienam, Tazocin, Augmenten, Unacyine and Amoxile were tested. Nutrient agar plates were surface inoculated with 20 µl of 24 h bacterial inoculum prepared as mentioned in section 2.2 and then discs of different antibiotics were placed uniformly on the surface of the plate. Plates were incubated at 37°C for 24 h. The sensitivity and resistance of the isolates were determined according to the Performance Standards for Antimicrobial Disk Susceptibility Tests (Gram negative antibiotic susceptibility guide GlaxoWellcome).

Statistical analysis

All statistical analysis in this study was carried out using Microsoft Excel 2000, Analysis Toolpack (Microsoft Corporation). All data were calculated from at least 3 replicates and the standard error for each datum was plotted on the graph.

RESULTS

Preliminary identification of clinical isolates

Twenty four isolates of *Proteus* spp. were recovered from different clinical isolates and preliminary identified by different biochemical tests according to the methods recommended by (Holt and Williams, 1989 & cheesbrough, 2000) Table 1.

Table (1): Biochemical tests for identification of *Proteus* spp. the clinical isolates.

Lactose fermentation	Glucose fermentation	Sucrose fermentation	Oxidase test	Citrate test	Motility test	Indol test	Urease test	Hemolytic activity	Nitrate reductase test
-	+	+	-	+	+	-	+	+	+

+ : positive result - : negative results

Quantitative assay of virulence factors

Proteus isolates were predominant in urine samples about 18 isolates (75%), followed by 5 isolates from wound swab (21%), and only 1 isolate from prostatic secretion (4%), hence, *Proteus* spp. considered as a major pathogen of urinary tract infection (Table 2).

All *Proteus* isolates demonstrated high invasion capability on vero cells with a maximum invasiveness recorded with Pr12 and Pr2 38 x 10^4 and 41 x 10^4 CFU ml^{-1} respectively. Adherence capability of *Proteus* spp. showed that Pr12 had maximum adherence (45x 10^4 CFU ml^{-1}) compared with other isolates. A variable cytotoxicity of *Proteus* spp. towards vero cells ranged from 3774

to 6702 dead cells was recorded (Table 2).

Table (2): Virulence factors of *Proteus* spp. isolated from different clinical samples. The highlighted rows represent the selected spp. for further investigations.

Isolate code	Total count of invasion CFU ml^{-1} (x10^6)	Total count of adherence CFU ml^{-1} (x10^6)	Cytotoxicity (no of dead cells)	Elastase activity U ml^{-1}	Urease activity U ml^{-1}	Swarming activity	Source of sample
Pr 1	20	7	5542	0	99	+	Urine
Pr 2	41	36	5560	5	133	+	Urine
Pr 3	22	7	5232	5	59	+	Prostatic pus
Pr 4	25	5	5232	4	89	+	Urine
Pr 5	26	4	5534	0	79	+	Urine
Pr 6	30	29	6074	6	146	+	Urine
Pr 7	18	3	5068	0	92	+	Urine
Pr 8	20	8	5480	4	88	+	Urine
Pr 9	27	4	5263	0	86	+	Urine
Pr 10	29	6	4396	0	79	+	Wound pus
Pr 11	19	17	4935	0	73	+	Urine
Pr 12	38	45	6702	7	145	+	Urine
Pr 13	19	5	5535	4	70	+	Urine
Pr 14	17	2	4245	5	102	+	Urine
Pr 15	20	3	4130	0	88	+	Abscess swab
Pr 16	28	5	5263	0	91	+	Urine
Pr 17	20	4	4706	0	84	+	Urine
Pr 18	23	5	5220	0	90	+	Wound swab
Pr 19	27	3	3774	2	87	+	Urine
Pr 20	28	19	6471	7	154	+	urine
Pr 21	24	4	4706	0	85	+	Wound pus
Pr 22	23	3	5135	0	97	+	Urine
Pr 23	16	5	5466	0	80	+	Wound swab
Pr 24	32	6	6625	5	129	+	Urine

For enzymatic activities, *Proteus* isolates did not show any protease or lipase activities. However, only 10 isolates were able to produce very low concentration of elastase enzyme ranged between 2 and 7 U ml^{-1} (Table 2). For urease activity, all *Proteus* isolates showed urease production with activity ranged from 59 to 154 U ml^{-1}. Maximum urease activity was recorded with Pr20 (Table 2). All *Proteus* isolates showed swarming ability on swarming agar medium.

Depending on the previous results, 5 *Proteus* spp. Pr2, Pr6, Pr12, Pr20, and Pr24 showed maximum production of several virulence factors (Table 2), were selected for further investigations.

Identification of selected clinical isolates

The selected isolates were identified by Analytical Profile Index (API). Pr2, Pr12 and Pr24 were identified as *Proteus mirabilis* while Pr6 and Pr20 were identified as *Proteus penneri* (Table 3).

Table (3): Identification of the selected clinical isolates by Analytical Profile Index (API).

Clinical isolates	*Identification*
Pr2	*Proteus mirabilis*
Pr6	*Proteus penneri*
Pr12	*Proteus mirabilis*
Pr20	*Proteus penneri*
Pr24	*Proteus mirabilis*

Antibiotic sensitivity test

Sensitivity of the identified clinical isolates towards 18 standard antibiotics (representing 8 classes) was carried out to confirm the multi-drug resistance of these isolates (Table 4).

Table (4): Diameter of inhibition zones of different antibiotics (cm) against the selected clinical isolates.

Bacterial isolates	Diameter of inhibition zone (cm)																	
	CIP 5µg	GN 10 µg	FOX 30 µg	C 30 µg	TOB 10 µg	Ak 30 µg	CTX 30 µg	SCF 30 µg	CFP 30 µg	LEV 5 µg	MEM 10 µg	CAZ 30 µg	TE 30 µg	IPM 10 µg	TZP 110 µg	AMC 30 µg	SAM 20 µg	AMP 25 µg
Pr2	09	11	0	0	13	0	0	0	0	0	0	1	0	09	22	3	1	0
Pr6	21	15	0	0	12	0	0	0	0	0	0	0	0	24	2	3.2	0	0
Pr12	18	19	0	0	09	0	0	0	0	0	0	0	0	09	23	24	0	0
Pr20	22	23	0	0	15	13	0	0	0	0	0	09	0	12	21	0	0	23
Pr24	19	24	0	0	0	0	0	0	0	0	0	0	0	11	19	22	11	0

CIP: Ciprofor, GN: Garamycin, FOX: Cifteriaxone, C: Isoptophenicol, TOB: Terramycin, Ak: Amikin, CTX: Cefotax, SCF: Sulperazon, CFP: Cefobid, LEV: Leeflox, MEM: Meronam, CAZ: Fortam, TE: Tetracid, IPM: Tienam, TZP: Tazocin, AMC: Augmentin, SAM: Unasyin and AMP: Amoxile.

The sensitivity and resistance of the isolates were determined according to the Performance Standards for

Antimicrobial Disk Susceptibility Tests (Gram negative antibiotic susceptibility guide GlaxoWellcome). Cifteriaxone, Cefotax and Sulperazon antibiotics did not show any inhibitory activity against any of the tested isolates. *Proteus* spp. showed susceptibility towards different antibiotics. Tazocin showed activity against the 5 *Proteus* clinical isolates. Pr20 was the most sensitive among the others (Table 4).

Plasmid pattern of selected clinical isolates

To assess the relation between plasmids and virulence factors, plasmid extraction from the selected isolates was carried out and analyzed by electrophoresis on 1% agarose gel. All isolates were harbouring plasmids, however, the same pattern was observed with plasmid isolated from Pr2, Pr6 and Pr12 (Fig. 1).

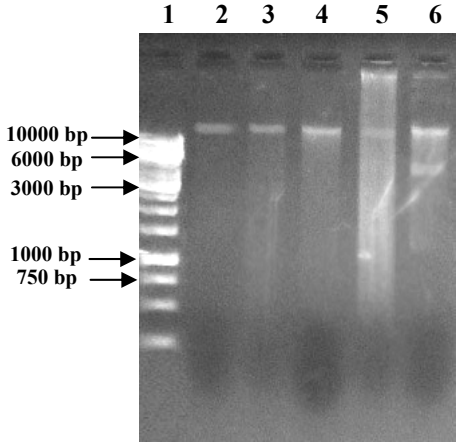

Fig. 1: Electrophoresis of plasmid DNA isolated from the *Proteus* spp. Lane 1 is 1 kb DNA ladder1-; lanes, 2, 3, 4, 5 and 6 are plasmids isolated from Pr2, Pr6, Pr12, Pr20 and Pr24 respectively

Plasmid curing

To demonstrate whether the virulence factors of the selected clinical isolates were plasmid or chromosomal origin, plasmid curing was carried out. Two methods were employed for plasmid curing, the first one by treatment with 10% SDS.

This method showed partial curing i.e. did not remove plasmids completely.

The second method was treatment with different concentrations of acridine orange. The best concentration of acridine orange used to cure plasmids was 32.5 µg ml^{-1} at which the OD at 660 nm ranged from 0.7 to 0.8. Complete plasmid curing was confirmed by plasmid extraction and sensitivity of the cured isolates towards different antibiotics which were resistant to them before.

Effect of plasmid curing on virulence factors

Virulence factors were measured in the selected isolates before and after curing the cells from plasmid to assess the relation between virulence factors and presence of plasmids.

All isolates were found to loose their invasive capability upon plasmid curing (Fig. 2). In addition, the ability to adhere and colonize to the vero cells was lost after plasmid curing. Plasmid free isolates showed no significant difference in cytotoxicity compared with the wild type isolates (non-cured) (Fig. 2). The same pattern was obtained with the other virulence factors elastase and urease activities which showed no significant difference before and after plasmid curing (Fig. 2). Moreover, measuring swarming after growing the wild types and the mutants for 7 h showed no significant difference in swarming migration diameter (Fig. 2).

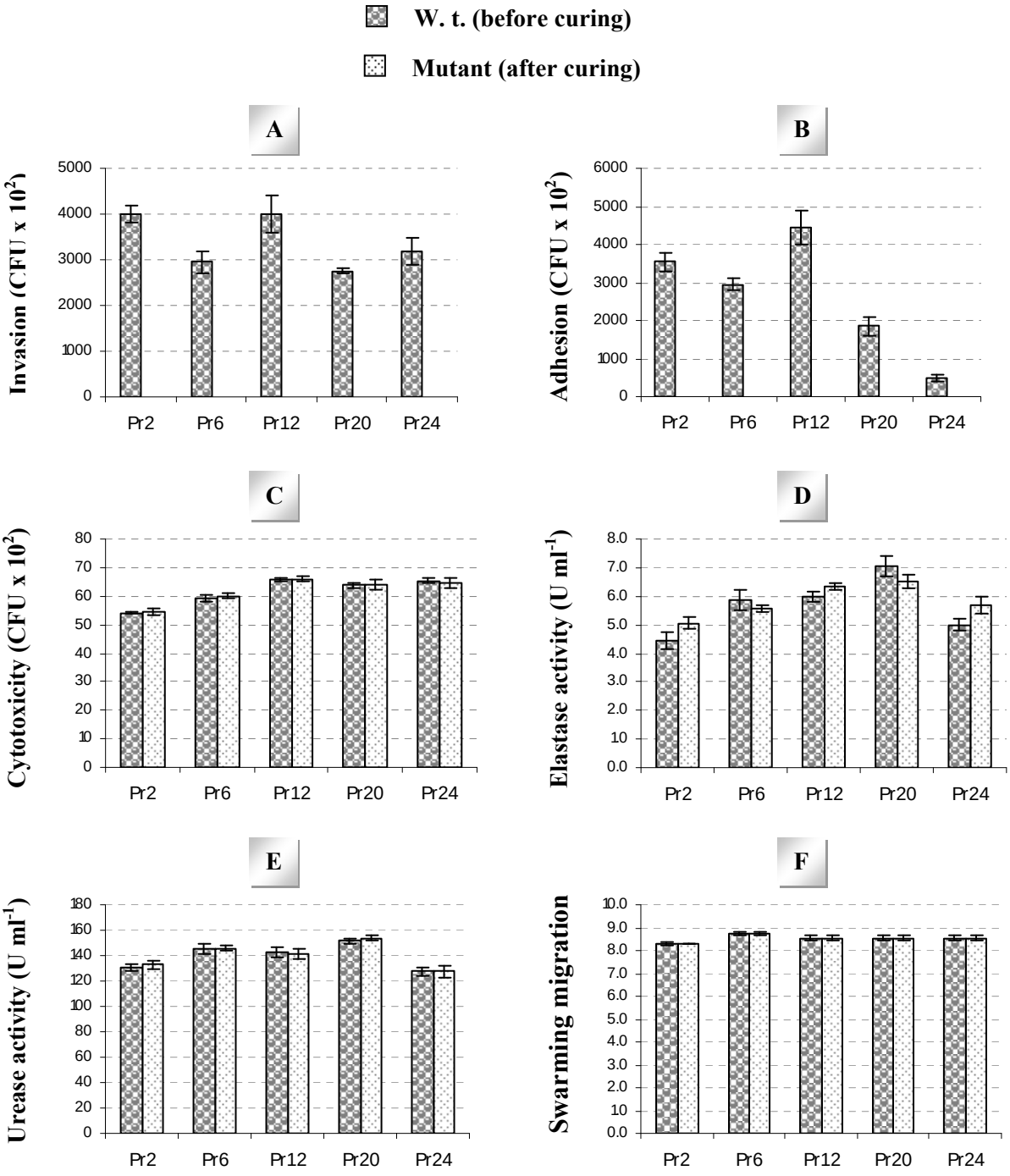

Fig. 2: Effect of plasmid curing on different virulence factors of the selected *Proteus* clinical isolates. A, invasion; B, adhesion; C, cytotoxicity; D, elastase activity; E, urease activity and F, swarming migration after 7 h. W. t., wild type.

DISCUSSION

Recently, significant evidence indicated that markedly different microbial pathogens use common strategies to cause infection and disease (Tang *et al*., 1995). Many diverse bacterial pathogens share common mechanisms in terms of their abilities to adhere, invade, and cause damage to host cells and tissues, (Finlay and Falkow, 1997) as well as to survive host defenses and establish infection. Many of these infections appeared to be related to the acquisition of large blocks of virulence genes from a common microbial ancestor, which can be disseminated to other bacteria via horizontal transfer (Hentzer and Givskov, 2003).

In the present study, a total of 24 clinical bacterial isolates were isolated from different collected clinical specimen from patient suffering from urinary tract infection, wound, burn and abscesses infections. The ability of all clinical isolates to produce different virulence factors including invasion, adherence, cytotoxicity, production of protease, lipase, elastase, urease as well as swarming migration were investigated.

All tested isolates showed variable production of different virulence factors, however all of them sharing certain virulence factors.

The isolates could be classified as either invasive according to gentamycin survival assay or cytotoxic according to crystal violet dye exclusion assays. The results showed that all *Proteus* spp. isolates exhibited both high invasion capability and epithelial cell cytotoxicity. This was reported by Jacobsen *et al.* (2008) who reviewed invasion, cytotoxicity and biofilm formation of *Proteus mirabilis* and *Escherichia coli*.

The parallel relation between the invasion and cytotoxicity in this study was not demonstrated in other microorganisms. Fleiszig *et al*. (1995) reported that *P. aeruginosa* isolates recovered from human infections showed a significant inverse correlation between the ability to induce cytotoxicity and epithelial cell invasion. Although, Gupta *et al*. (1994) demonstrated that disabled cytotoxicity by mutation of a transcriptional activator called ExsA, encoded by *exsA*, showing ability to detect invasion at later time points. This is because cytotoxic bacteria would enter the epithelial cells causing death of these cells consequently, allowing penetration of gentamycin antibiotic into the cells, rendering gentamycin survival assays incapable of detecting the presence of intracellular bacteria accurately. This would also explain why there was an inverse correlation among clinical isolates between their ability to invade cells as measured by gentamicin assays and their cytotoxic capacity (Fleiszig *et al*., 1998). On the other hand, Fleiszig *et al*. (1996) and (1997) suggested that *P. aeruginosa* cytotoxicity may be invasion dependent. Moreover, Evans *et al*. (1998) and Fleiszig *et al*. (1998) showed that cytotoxic strains of *P. aeruginosa* are inherently capable of invasion. Evans *et al*. (1998) reported that invasion and cytotoxicity are independent events, therefore, the therapeutic approaches aimed at preventing invasion were not able to block cytotoxicity. The significance of these results indicated that there is more than one pathway for disease caused by pathogenic isolates, consequently, different therapeutic strategies might be necessary to manage *Proteus* infection.

Adhesion capability of all *Proteus* isolates was demonstrated

with different extents in this study. Adherence to mammalian cells required in the early stage of colonization and biofilm formation and may be mediated by a specific protein, toxin and unique appendage (O'Toole and Roberto, 1998). However, the difference in adhesion capabilities for isolates may be due to strain dependent (Raivio, 2005). Gram-negative uropathogens produce an assortment of adhesins including those attached to the tip of hair-like projections, known as fimbriae or pili, as well as adhesins anchored directly within bacterial cell membranes, known as nonfimbrial adhesins (Jacobsen *et al*., 2008). There was tight relation between invasion and adherence, once initial attachment and permanent adherence commenced on the surface of uroepithelial cells, the establishment uropathogenic *E. coli* infection occurs through the colonization of the bladder by the invasion of host cells and the subsequent formation of biofilms (Mulvey, 2002).

Concerning the production of urease enzymes, all *Proteus* spp. showed high production of urease enzymes. This was similar to previous reports by Gendlina *et al.* (2002) who demonstrated that *Proteus* spp. produced high amount of urease compared with other bacteria. Urease enzyme was known to hydrolyzes urea into ammonia and ultimately carbon dioxide. As a result of ammonia production, an increase in local pH causes precipitation of normally soluble calcium and magnesium ions. These salt crystals can grow to remarkable size to produce bladder and kidney stones (Li *et al*., 2002) which are a hallmark of infections with *Proteus* spp.

Proteus spp. collected in this study did not show any protease or lipase activities. This was contradictory to the work done by Stankowska *et al.* (2008) who demonstrated the proteolytic activities of 12 *Proteus mirabilis*, however they proved that the virulence factors in *P. mirabilis*, were strain-specific. This was also demonstrated in this study that only 42% of the collected samples were able to produce elastase activities. Swarming migration was known to be an important virulence factor, the present work showed that all *Proteus* isolates exhibited swarming migration.

In general, many of infections caused by the pathogenic strains were due to the acquisition of large blocks of virulence genes from a common microbial ancestor, which can be disseminated to other bacteria via horizontal gene transfer (Hentzer and Givskov, 2003). The widespread use of antibiotic since the middle of the last century has created a powerful selection force driving the evolution of drug resistant bacterial pathogens (Levy, 1992 and Virve *et al*., 2004). This was clearly demonstrated in this study. The antibiotic susceptibility pattern of the 5 clinical isolates showed a slight susceptibility to Ciprofor, Garamycin, Augmentin and Tazocin which are members of β-lactams antibiotics. The resistance could be explained on the basis of observation by Bush *et al.* (1995) who showed that members of the family enterobacteriaceae commonly express plasmid encoded β-lactamases (e.g., TEM-1, TEM-2, and SHV-1). which confer resistance to penicillins (Knothe *et al*., 1983). Resistance generally arises due to mutations in penicillin binding proteins, production of metallo-β-lactamases, or resistance to diffusion across the bacterial outer membrane (Piddock *et al*., 1997 and George *et al*., 2005). The most common

mechanism of resistance in Gram negative bacteria causing clinically significant infection remains the production of β-lactamases, including chromosome and plasmid encoded enzymes (Bush *et al*., 1995**).**

Plasmids isolated from the selected 5 clinical isolates (exhibited high virulence factors) showed the same pattern in 3 isolates which was different from the other 2 isolates. However a diverse pattern was detected in *Proteus mirabilis* (Pr2, Pr12 and Pr24) as well as *Proteus penneri* (Pr6 and Pr20) isolates. This explains the difference in sensitivity of the isolated towards the different antibiotics. According to previous studies, it is well known that most Gram negative bacteria are harbouring plasmids (Helling*, et al*., 1981, Parkhill*, et al.,* 2001 and Virve, *et al*., 2004) that are responsible for antibiotic resistance and some virulence factors.

As a result of plasmid curing for the 5 clinical isolates, bacteria lost their resistance towards different antibiotics and became sensitive to different previously exhibited resistance towards them. This demonstrated that the antibiotic resistant genes are plasmid encoded rather than chromosomal. This was confirmed previously the relation between plasmids and antibiotic resistance (Laraki *et al*., 1999 and Lauretti *et al.*, 1999).

In addition, only two virulence factors were lost upon plasmid curing (invasion and adherence capabilities), however no effect was detected with the other virulence factors tested. This assessed the relation between plasmids and virulence factors. Moreover, it demonstrated that the tested virulence factors were chromosomal coded rather than plasmid coded (Stankowska *et al*., 2008) except for invasion and adherence capabilities.

Previous reports correlated plasmids with virulence and curing plasmids resulted in loss of virulence. Rotger and Casadesus (1999) reported that virulence factors responsible for pathogenicity in enteric bacteria are often encoded by plasmids, as in *E. coli*, *Yersinia* spp. and *Shigella* spp. Sekizaki *et al.* (1989) found that a reduction in virulence of *E. coli* against chickens was correlated with loss of a large plasmid with molecular size of 95 kilobases (kb). Jackson *et al*. 1999 reported that cured strains of phytopathogen *Pseudomonas syringae* pv. phaseolicola (*Pph*) form the 154-kb plasmid lost virulence toward bean. Paetzold *et al.* (2007) reported that invasion of *Shigella flexneri* was due to the *Shigella* entry region (SER), carried in a large virulence plasmid.

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ARABIC SUMMARY

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تم تعيين بعض عوامل الضراوة وهى الإختراقية ،الالتصاق ،السمية لخلايا العائل ،انتاج بعض الإنزيمات المحللة البروتيز،اللايبيز،الإلستيز ،اليوريز والقدرة على السريان لـ٢٤عزله لنوع البروتيس معزولة من عينات طبية. وقد أظهرت الدراسة ان معدل فعوامل الضراوة مختلفة و ومتخصصة السلالة فيما بين العزلات.

أظهرت كل عزلات بروتيس قابلية الإختراق والالتصاق بمعدلات مختلفة بالاضافة الى قدرتهم على انتاج بعض إنزيمات الإلستيز و اليوريز. لم تظهر اى من العزلات القدرة على انتاج إنزيمات البروتيز و اللايبيز. كما أظهرت كل العزلات السمية العالية لخلايا العائل بالاضافة الى وجود علاقة بين قدرة الإختراق و السمية فى كل العزلات.

وقد تم اختيار ٥ عزلات من جنس بروتيس أظهرت أعلى انتاجية لعوامل الضراوة وتعريفهم بواسطة مؤشر التحليل الشكلى الى بروتيس ميرابيلس (Pr2, Pr12 and Pr24) و بروتيس بينيرى (Pr6 and Pr20) .

وقد أظهرت إزالة البلازميد بواسطة الاكريدين البرتقالى من العزلات الخمسة الى فقدهم القدرة على الإختراق و الالتصاق فى حين لم يلاحظ اى اختلاف على بقية عوامل الضراوة الاخرى مبينة بذلك أن الإختراق و الالتصاق مشفرة بلازميديا وليس كروموزوميا.