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ORIGINAL ARTICLE

Virulence Factors Associated with Quinolone Resistance in *Proteus* Species Isolated from Patients with Urinary Tract Infection

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Proteus, quinolones, virulence, urinary tract infections

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ABSTRACT

Background: *Proteus is an important causative organism of urinary system infections. The invasive nature of Proteus is supported by expression of multiple virulence factors; the infection outcome gets worse when those virulent isolates acquire antibiotic resistant determinants.* ***Objectives:*** *The present study was aiming at isolation of Proteus from urine of patients with urinary tract infections (UTIs) and to assess the relation between virulence factors expression and presence of quinolones resistance genes in those isolates.* ***Methodology:*** *Quinolone resistant Proteus isolates were chosen for detection of quinolone resistance genes, also they were tested for presence of different virulence factors.* ***Results:*** *Sixty eight quinolone resistant Proteus isolates were determined. aac(6')- Ib-cr was the most frequently detected quinolone resistance gene. Haemagglutination, haemolytic activity, protease production and biofilm formation were documented in 79.4%, 76.5%, 70.6% and 83.8% of the isolates respectively.* ***Conclusion:*** *Proteus isolated from urine displayed many virulence factors and harbored a variety of quinolone resistance genes.*

INTRODUCTION

Proteus bacteria are extensively disseminated as reasons of infections in human, particularly the immune compromised patients [1]. The genus *Proteus* is usually recorded as an opportunistic microorganism associated with serious invasiveness [2]; most of these infections are caused by *Proteus mirabilis (P. mirabilis*) and it was observed as one of the main causes of urinary tract infections (UTIs), wound infections, respiratory tract infections, otitis media and burn infections [3].

The contagious nature of *Proteus* at different human infection sites is usually related to their antibiotic insensitivity that supports their continued existence in different healthcare centers, the great ability of *Proteus* to invade different host tissue is linked to many tools of virulence, like cellular adhesion and diffusion all over the body [4] which is supported by different types of motility, twitching movement helps isolates to move among two hard surfaces, swimming movement facilitates the bacterial progress in liquid environment, however swarming mobility is the strongest type as the bacteria intend to increase the number of their flagella serving their progress on solid exteriors [5]. *Proteus* also display other important virulence factors like Lipopolysaccharides, protease, hemolysine, urease and iron gaining structures [6], additional serious virulence character exhibited by *Proteus* is their ability of biofilm organization which assist their continued existence and antimicrobial resistance [7].

Urinary system infections are common reasons of hospital admission all over the world. Progression to complicated urinary tract infections is frequent, affecting either upper or lower part of the urinary system [8].

Simple forms of UTIs are usually managed by cephalexin, cotrimoxazole, ceftriaxone and nitrofurantoin [9]. Fluoroquinolones are usually suggested by medical guiding principles as an experiential therapy for complicated urinary tract infections [10], though they had been widely used together with beta-lactams in management of UTIs in patients without any underlying complications [9].

Resistance to fluoroquinolones among patients is increasing, in several communities; it may go beyond 50%[11]. Resistance to quinolones may be chromosomally mediated due to mutation in the coding areas controlling topoisomerase IV and DNA gyrase programming genes [12]. Plasmide controlled resistance to quinolones was also declared including, aminoglycoside acetyltransferase gene (*aac* (6')-*Ib-cr)*, efflux pumps coordinating genes (*qepA* and *oqxAB*) and genes that suppress the quinolone inhibitory action on topoisomerase IV and DNA gyrase (qnrS, qnrD, qnrC, qnrB, qnrA) [13].

Proteus bacteria are considered to be one of the most important organisms which are listed as causes of UTIs, unfortunately these types of infection are insistent and hard to be eliminated [14]. *P. mirabilis* wild isolates are generally sensitive to fluoroquinolones and even to the antimicrobials belongs to the beta-lactam families, however, the medical isolates of *Proteus* which are

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more virulent exhibited an increasing rate of resistance to the cephalosporins of broad spectrum and also to fluoroquinolones [15] which are the corner stone in treatment of UTIs due to their neglected side effect, easy administration and the poor possibility of resistance development [16].

To the maximum extent of our ability to know, the relationship between quinolones resistance and virulence factors expression in *Proteus* bacteria has not been previously evaluated. The aims of the present study were to isolate *Proteus* bacteria from urine samples of patients suffering from UTIs and to assess the relation between different virulence factors expression by those isolates and the presence of quinolones resistance genes.

METHODOLOGY

Design of the study:

The present research was carried out during the time between March 2019 and July 2020. The study was a descriptive cross sectional and it was performed on 215 isolates of *Proteus* bacteria detected from urine samples of patients admitted at different inpatient wards of Mansoura University Hospitals (MUHs) who showed symptoms and signs suspecting UTIs and did not receive any antibiotics. Quinolone resistant *Proteus* isolates were determined both phenotypically and genotypically then they were tested for presence of different virulence factors.

The Medical Research Ethics Committee of the Faculty of Medicine in Mansoura University approved the study protocol with code number: R.20.10.1041.

Pathological specimens collection and processing:

Urine specimens were collected from targeted patients either catheterized or non catheterized using the standard aseptic precautions [17], then they were subjeced to full processing in the Microbiology Diagnostic and Infection Control Unit (MDICU) of the Medical Microbiology and Immunology department at the Faculty of Medicine, Mansoura University.

Identification of *Proteus* bacteria isolates:

Proteus isolates which were found in a significant number suggestive of bacteruria ($\geq 10^5$ CFU/ml) were identified after cultivation of urine samples on CLED agar media**.** Conventional scheme of identification was used [18] including colonial morphology, Gram stained films and biochemical reactions. Full identification to the species level was performed using the API 20 E analytical profile index *(Bio-merieux SA, Montalieu Vercica and France).*

Quinolone antibiotics susceptibility testing:

All of the isolated *Proteus* were examined for quinolone resistance following the principles recommended by the CLSI [19]. Isolates found to be resistant to the examined fluoroquinolnes and showed intermediate sensitivity or resistance to nalidixic acid were interpreted as high level quinolone resistant, whereas isolates exhibited sensitivity to fluroquinolones and resistance or intermediate sensitivity to nalidixic acid were recorded as low level quinolone resistant [20].

PCR detection of quinolone resistance genes:

The quinolone resistant *Proteus* isolates were subjected to PCR assay for detection of quinolone resistance genes including *aac(6')- Ib-cr*, *qepA* , *oqxAB*, *qnrA*, *qnrB*, *qnrC*, *qnrD* and *qnrS* according to the previously described protocol [21] with primers listed in table (1).

Table 1: Primers used for PCR detection of quinolone resistant genes

Gene	Primer	Product Size, bp	Melting temperature (^{0}C)	Reference
aac(6')- Ib-cr	aac-F TTGCGATGCTCTATGAGTGGCTA aac-R CTCGAATGCCTGGCGTGTTT	482	57	[22]
qepA	qepA-F CTGCAGGTACTGCGTCATG qepA-R CGTGTTGCTGGAGTTCTTC	403	60	[23]
oqxA	oqxA-F GACAGCGTCGCACAGAATG oqxA-R GGAGACGAGGTTGGTATGGA	339	62	[21]
oqxB	oqxB-F CGAAGAAAGACCTCCCTACCC oqxB-R CGCCGCCAATGAGATACA	240	62	[21]
qnrA	qnrA-F AGAGGATTTCTCACGCCAGG qnrA-R GCAGCACTATKACTCCCAAGG	619	57	[21]
qnrB	qnrB-F GGMATHGAAATTCGCCACTG qnrB-R TTTGCYGYYCGCCAGTCGAA	264	57	[24]
qnrC	qnrC-F GGGTTGTACATTTATTGAATC qnrC-R TCCACTTTACGAGGTTCT	447	57	[25]
qnrD	qnrD-F CGAGATCAATTTACGGGGAATA qnrD-R AACAAGCTGAAGCGCCTG	582	57	[26]
qnrS	qnrS-F GCAAGTTCATTGAACAGGCT qnrS-R TCTAAACCGTCGAGTTCGGCG	428	57	[24]

Note: bp, base pair

Detection of virulence factors in quinolone resistant *Proteus* isolates:

Testing for different types of motility*[5]*:

This test was performed to asses three different types of motility; swimming, swarming and twitching motility using a variety of agar content suitable for each type of motility in Luria-Bertani (LB) medium. For swimming motility, 0.3% agar contained medium was used on which the bacteria were puncture inoculated. For swarming motility, 0.7% agar contained medium was used on which picked colonies from the swimming agar plates were inoculated. Twitching motility was detected using media with 1% agar on which bacterial isolates (10 µl) were inoculated under the agar layer using micropipette.

Haemagglutination assay*[27]*:

This assay tested the capacity of fimbria to adhere erythrocytes causing haemagglutination. Briefly, 250 µl of bacterial suspension was cultured in 5 ml of Brain Heart Infusion Broth (BHIB). Cell pellets harvested by centrifugation were resuspended in 0.5 ml of phosphate buffer saline (PBS). Testing for haemagglutination was performed in microtitration plate using 0.1 ml of erythrocytes suspension mixed with 0.05 ml from the prepared suspension of the bacteria at 23^{0}C.

Haemolysin assay*[27]*:

This test depends on cytotoxic activity of haemolysin produced by *Proteus* causing erythrocyte haemolysis. The test was performed on bacteria cultivated in BHIB at 37^{0}C overnight (1x10^5 CFU/ml) in microtitraton plates. 3% sheep RBCs (0.05 ml) was added to 0.1 ml of bacterial suspension (two fold dilution in PBS, pH 7.2) and incubated for 1 hour at 37^{0}C. The highest dilution at which no RBCs button was seen at the bottom was considered haemolytic titer.

Protease assay*[28]*:

The examined isolates were inoculated by streaking on skimmed milk agar surfaces, and then they were incubated for 24 hours at 37^{0}C. Visible milk clearance around the colonies indicated positive results for protease production.

Formation of biofilm*[29]*:

According to the previously described method, the examined isolates were cultured on brain heart infusion broth with 4% glucose in microtitration plates then they were incubated for 24 hours at 37^{0}C. Washing step was done three times for the microtitration plates' wells. The plates were left to be air dried. Crystal violet (0.1%) was used to stain the adhered biofilms. Quantitative assessment of biofilms was done at 595 nm OD, every strain was assessed in triplicate.

Statistical Analysis:

Data entry and statistical analysis were done using Statistical Package of Social Science (SPSS), software version 21. Qualitative data descriptions were performed using numbers and percentages. Association between categorical variables was performed using the Chi square test. Statistically significant *P* value was less than 0.05.

RESULTS

During the study period, 215 *Proteus* isolates were detected in the examined urine speciemens of patients with suspected UTIs, out of them 143 (66.5%) were *P. mirabilis* and 72 (33.5%) were *P. valgaris*. Female patients showed higher prevalence of *Proteus* isolates (133, 61.9%) than male patients (82, 38.1%). The range of the patients age was 6-73 years (mean age was 39.4).

The isolated *Proteus* showed different distribution among various hospital departments, medical wards were found to be the the most prevalent place from which *Proteus* bacteria had been isolated (121, 56.3%) followed by surgical wards (62, 28.8%), intensive care units (21, 9.8%) and pediatric wards (11, 5.1%). *Proteus* were mostly isolated from catheterized patients, 134 (62.3%) versus 81 (37.7%) isolated from noncatheterized patients.

Testing for quinolones susceptibilty (table 2) revealed that 68 isolates showed insensitivity to nalidixic acid (20 were nalidixic acid intermediate and 48 were resistant), of those isolates, 39 (57.4%) were also resistant to fluroquinolones and recorded as high level of quinolone resistant, the remainig 29 nalidixic acid non sensitive isolates showed sensitivity to the examined fluroquinolones and they were documented as low level of quinolone resistnat.

PCR testing of the 68 quinolone resistant *Proteus* isolates showed that *aac(6')- Ib-cr* was the most common quinolone resistance gene detected in those isolates (57, 83.8%), *oqxB* and *oqxA* genes were also found to be present with a substantial rate (43, 63.2% and 26, 38.2% respectively). *qepA* gene was found to be harbored by 3 (4.4%) of the tested isolates. Among the *qnr* genes, *qnrA* and *qnrD* were the only types detected in the examined isolates (2, 2.9% and 1, 1.5% respectively), non of the examined quinolone resistant *Proteus* isolates were found to be positive for either *qnrB*, *qnrC* or *qnrS* genes.

All the examined isolates exhibited at least one type of motility, most of them (61, 89.7%) were found to be positive for swimming, sawrming and twitching motility, 5 (7.4%) showed both swimming and twitching motility but they didn't swarm, the remaining two isoaltes (2.9%) demonstrated only swimming type of motility. Testing for presence of other virulence factors in the quinolone resistant *Proteus* isolates revealed that, 54 (79.4%) were found to be positive for haemagglutination. Haemolytic activity was documented in 52 (76.5%) of the examined isolates with variable haemoltic titre (table 3). Protease production was recorded in 48 (70.6%) of the tested isolates.

Regarding biofilm formation assay, (57, 83.8%) of *Proteus* isolates displayed the ability of biofilm formation. The correlation between quinolone resistance genes and presence of virulence factors in the the examined isolates was described in table 4, a significant association was observed between both haemagglutination and biofilm formation as virulence factors and presence of *aac(6′)- Ib-cr* and *oqxB* genes. Isolates exihibited presence of *oqxA* gene was found to be significantly postive for Haemolysin.

Table 2. Susceptibility pattern of the isolated *Proteus* for tested quinolones

Antibiotics	The examined *Proteus* isolates n= 215					
	Sensitive		Intermediate		Resistant	
	No	%	No	%	No	%
Nalidixic acid	147	68.4	20	9.3	48	22.3
Ciprofloxacin	140	65.1	43	20	32	14.9
Norfloxacin	166	77.2	19	8.8	30	14
Levofloxacin	152	70.7	34	15.8	29	13.5
Ofloxacin	156	72.6	28	13	31	14.4

Table 3. Haemolytic titre of quinolone resistant *Proteus* isolates

Haemolytic titre	Haemolysin positive quinolone resistant *Proteus* isolates n= 52	
	No	%
2	2	3.8
4	6	11.5
8	13	25
16	11	21.2
32	10	19.2
64	4	7.7
128	5	9.6
256	1	1.9

Table 4. Association between quinolone resistance genes and presence of virulence factors in the the examined quinolone resistant *Proteus* isolates.

Quinolone resistance genes		Virulence factors			
		Haemagglutination Positive *Proteus* isolates No=54 n(%)	**Haemolysin Positive *Proteus* isolates** No=52 n(%)	**Protease Positive *Proteus* isolates** No=48 n(%)	**Biofilm Positive *Proteus* isolates** No=57 n(%)
aac(6')- Ib-cr	***aac(6')- Ib-cr* positive isoltes No= 57**	49(90.7%)	46(88.5%)	39(81.2)	51(89.5%)
	***aac(6')- Ib-cr* negative isoltes No=11**	5(9.3%)	6(11.5%)	9(18.8%)	6(10.5%)
P value		**0.008***	0.13	0.59	**0.014***
oqxB	***oqxB* positive isolates No=43**	38(70.4%)	32(61.5%)	27(56.2%)	41(71.9%)
	***oqxB* negative isolates No=25**	16(29.6%)	20(38.5%)	21(43.8%)	16(28.1%)
P value		**0.037***	0.82	0.11	**0.002***
oqxA	***oqxA* positive isolates No=26**	19(35.2%)	24(46.2%)	19(39.6%)	24(42.1%)
	***oqxA* negative isolates No=42**	35(64.8%)	28(53.8%)	29(60.4%)	33(57.9%)
P value		0.47	**0.033***	0.93	0.24
qepA	***qepA* positive isolates No=3**	2(3.7%)	3(5.8%)	1(2.1%)	3(5.3%)
	***qepA* negative isolates No=65**	52(96.3%)	49(94.2%)	47(97.9%)	54(94.7%)
P value		0.86	0.77	0.42	0.98
qnrA	***qnrA* positive isoaltes No=2**	2(3.7%)	0(0%)	2(4.2%)	1(1.8%)
	***qnrA* negative isolates No=66**	52(96.3%)	52(100%)	46(95.8%)	56(98.2%)
P value		0.87	0.081	0.88	0.73
qnrD	***qnrD* positive isolates No=1**	1(1.9%)	0(0%)	0(0%)	1(1.8%)
	***qnrD* negative isolates No=67**	53(98.1%)	52(100%)	48(100%)	56(98.2%)
P value		0.46	0.52	0.64	0.35

***** Significant *P* value

DISCUSSION

Quinolone resistance among *Proteus* bacteria is a real trouble facing clinicians, particularly in patients suffering from UTIs as those isolates are suspected to be more virulent, thus adding difficulty in their management [15], in the present study it was proposed that virulence factors and quinolone resistance are expected determinants in *Proteus* isolated from patients with UTIs.

In the current study, *Proteus* species were mostly isolated from patients with UTIs admitted at medical wards (56.3%) supporting the previous finding that recorded isolation of *Entrobacteriaceae* including *Proteus* mostly from medical wards, specially among patient with UTIs [30], this is logically accepted as those patients seems to be more liable to increased incidence of infections due to their prolonged hospital stay and presence of underlying chronic diseases. *Proteus* isolation rate was also higher in patients with urinary catheters compared to those who were none catheterized, as the catheter usually provides the appropriate media for uropathogens including *Proteus* and other Gram negative bacteria [4].

Resistance to ciprofloxacin was observed in 14.9% of the examined isolates approximating the previously recorded results from near localities (Sudan, 13.3%) [31]. The percentage of ciprofloxacin resistance among *Proteus* isolates in Egypt seems to be increasing that it was about 11% as reported by El-Sokkary et al. [32] reaching 14.9% in the present study which raise the attention to the problem of quinolone resistance, the hazards of difficult management of patients with UTIs and the drawbacks of excessive and unnecessary antibiotic prescriptions.

In the present study, 39 (18.1%) of the isolated *Proteus* recorded as high level quinolone resistant isolates, higher percentage was observed in a previous study conducted in 2014 [33] as 38.3% of examined *Proteus* species were classified as highly resistant to quinolone, that was explained by presence of mutation associated with quinolone resistance in the genes encoding topoisomerase II in those isolates, These types of mutations could be linked with presence of plasmid mediated quinolone resistance genes [34].

Testing for quinolone resistance genes by PCR revealed that *aac(6′)- Ib-cr* gene was the most frequent one responsible for quinolone resistance in the examined isolates, 83.8% of the isolated *Proteus* were found to be positive for that gene, these results match that previously reported by Azargun et al.[30] who found that 66.7% of the examined *Proteus* isolates were positive for *aac(6′)- Ib-cr* gene, the authors also recorded it as the most prevalent quinolone resistance gene among the *Enterobacteriaceae* isolates. The presence of *aac(6′)- Ib-cr* as the most prevalent plasmid-mediated quinolone resistance gene in the examined extraintestinal *E.coli* isolates was also approved by Chen et al. [21].

oqxA and *oqxB* were also found to be common as quinolone resistance genes occupying the second position after *aac(6′)- Ib-cr* [21, 30], this was in accordance with the present results (the prevalence of *oqxB* and *oqxA* was 63.2% and 38.2% respectively). The present study revealed that *qnrD* was the least frequent quinolone resistance gene in the examined *Proteus* isolates (1.5%); however, it was the unique plasmid mediated gene of quinolone resistance (prevalence rate was 3.1% among *Proteus* species) in an American study published in 2014 [33]. *qepA* gene was found to be harbored by 4.4% of the tested isolates approximating the percentage that was previously recorded by extraintestinal *E.coli* isolates causing human infections (4.2%)[21], however this gene couldn't be detected in *Proteus* that were previously examined in other studies[30].

Regarding the results of virulence factors expression in the examined quinolone resistant *Proteus* isolates, it was found that motility is the most prevalent factor of virulence exhibited by those isolates (89.7% were found to be positive for all types of motility), this had been already established in pervious study (96.1% of the isolates showed all types of movement) [4]. Swarming motility hadn't been expressed in seven isolates, however 100% of the examined *Proteus* isolates in previous studies showed swarming [35], lacking of swarming in some isolates may be explained by deficiency in their peritrichious flagella.

Assessment of other virulence factors was very essential to determine the pathogenic potential of the quinolone resistant *Proteus* isolates. Testing for haemagglutination in the present study was very important as adhesion is an essential step in establishment of urinary system infection, 79.4% of the examined isolates showed haemagglutination activity which approximates the previously recorded results by Mishra et al. [27] who stated that 61.49% of the examined *Proteus* were positive for haemagglutination assay, however 100% of *Proteus* isolates could agglutinate human red blood cells in a subsequently performed study [36].

Haemolysin as a virulence factor produced by *Proteus* is very essential for enhancement of bacterial spread to the kidneys by forming pores in the target cells [37]. Haemolytic activity was assessed in the present study for all quinolone resistant *Proteus* isolates and it was positive in 76.5% approximating the previously recorded results [35], even though it was produced by all of the examined *Proteus* isolates in other studies [38].

Proteus isolates produce protease enzyme which is important for degradation of host protein and IgA implicated in mucosal immune response [39], protease

activity was explored in 70.6% of *Proteus* species subjected to examination in the current study, however lower percentage was found to be positive for protease in other studies (45.8%) [40].

In the present study biofilm formation was investigated as an important virulence factor in urinary *Proteus* isolates. Most of the examined *Proteus* (83.8%) showed the ability of biofilm construction matching the previously recorded results [40].

The current study highlighted an evident association between quinolone resistance and presence of virulence factors particularly biofilm formation, haemagglutination and Haemolysin production, meaning that virulent *Proteus* species were more liable to acquire resistance to various antibiotics and this may aggravate the outcome of infection caused by those isolates later on.

Further studies are recommended to detect more resistance determinants in *Proteus* species isolated from other infection sites particularly those expressing a variety of virulence factors.

CONCLUSION

Proteus bacteria being an important cause of UTIs were found to be highly virulent with expression of different types of motility. They were found to harbor multiple quinolone resistance genes with significant association between *aac(6′)- Ib-cr* and *oqxB* genes presence and both haemagglutination and biofilm formation as virulence factors.

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- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

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