Original article

Hemolysin production, salt tolerance, antibacterial resistance, and prevalence of extended spectrum β-lactamases in *Proteus* bacilli isolated from clinical and environmental sources

Shahla Mansouri[1], Farehnaz Pahlavanzadeh[2]

[1]Department of Microbiology, Kerman University of Medical Sciences, Kerman, Iran
[2]School of Pharmacy, Kerman University of Medical Sciences, Iran

Abstract

Introduction and objective: *Proteus* bacilli are opportunistic members of Enterobacteriaceae and *Proteus mirabilis* is among the most common causes of community or hospital acquired urinary tract infections (UTI) in many countries. In the present study hemolysin production, salt tolerance and resistance to antibacterial agents in environmental and UTIs samples were compared.

Materials and methods: Bacteria were isolated from UTIs (n=80), chicken skin (n=34) and soil (n=10). Resistance to 10 antimicrobial agents was determined by agar dilution method. In addition, β-lactamase and extended spectrum beta-lactamases (ESBLs) were also determined by nitrocefin disks and double disk diffusion methods respectively.

Results: Totally, 62.9% of the isolates showed resistance to 8.5% sodium chloride, and the difference in salt tolerance was significant in respect to species and origin of the samples. However, no significant difference in hemolysin production was detected in respect to species and origin of the samples. Resistance to trimethoprim-sulfamethoxazole (Sxt), amoxicillin (Amx) and chloramphenicol (C) were high (48.4% 41.9%, and 32.26% respectively), while sensitivity to β-lactams except amoxicillin was high (≥93.3%). β-lactamase production was found for 33% of the isolates and the MIC_{90} of Amx resistance isolates was high, but reduced several folds in presence of clavulanic acids, however ESBL phenotype was not observed for any of the isolates.

Conclusion: Hemolysin production was not significantly different in samples isolated from different sources, however isolates from soil were significantly more tolerance to salt (P=0.001). Resistance to some antibacterial agents was significantly higher in the isolates from UTIs and chicken skins in comparison with the soil samples. This is important in the case of Amx and Sxt, which are the important antibacterial agents for the treatment of uncomplicated UTIs.

Keywords: *Proteus*, Enterobacteriaceae, Salt tolerance, antibacterial resistance, ESBL

Introduction

The genus *Proteus* consists of opportunistic gram-negative pathogens, found in

intestinal tract of animal and human, environments, sewage, and manure. *Proteus* especially *P. mirabilis* is among the most common causes of community or hospital acquired Urinary tract infections (UTIs) in many countries [1-3]. UTI with *P. mirabilis* usually starts with colonization of the bladder, causing bacteriuria and cystitis but is rarely involved in severe infections [4]. In the complicated UTI, bacteria can ascend to the kidney, and cause in acute pyelonephritis, chronic inflammation and renal failure [5,6]. Many recurrent causes of bacteriuria and UTI involving *Proteus* have been reported [7]. These infections are usually difficult to treat and persistent [2]. Several virulence factors may be responsible for the pathogenicity of these bacteria. Alternative sigma factors regulate a wide range of physiological processes, which can affect virulence in pathogenic bacteria [8].

Virulence effects can be mediated either through direct virulence gene regulation or indirectly by regulating genes that increase fitness of the bacterium during transmission and infection. For example, the haemolysin secreted by *P. mirabilis* is cytotoxic for cultured urinary tract epithelial cells and these bacteria usually invade the human urothelial cells [2]. Indirect effects of sigma factors on virulence may be more difficult to identify, but alternative sigma factors frequently have roles in virulence by regulating virulence-associated genes that aid in a bacterium's survival during infection. Some sigma factors contribute to environmental survival, and thus transmission of food-borne pathogens in foods and food-processing environments. Others contribute to environmental survival, and have virulence implications, e.g. regulation of biofilm formation [8]. However greater attention is usually paid to the *Proteus* species causing UTIs. Although the strains isolated from other clinical material or environments may also display

the same resistant pattern or virulence markers as the UTI isolates, these harmless colonizers can cause serious other infections especially if the infection is acquired in the hospital [9,10].

Fluoroquinolone and β-lactam antibiotics are most frequently used as the first line treatment of uncomplicated bacterial infections [11]. The continuous trend of empirically treating UTI, results in regional variation in resistant patterns of bacterial isolates. Treating the patients with third generation of cephalosporins, which are the major component of the empiric therapy for UTI results in the emergence of isolates producing extended spectrum beta-lactamases (ESBL). The ESBL isolates are frequently resistant to many other classes of antibacterial agent, which in turn makes in the difficulty of treating infections [12,13]. Plasmid mediated ESBLs, have started spreading in *P. mirabilis* around the world [12]. The incidence of ESBL varies, depending on which area of the world the isolates originate from [3,12]. Since *Proteus* bacilli are not very common in clinical specimens, we did not find any reports on ESBL production either from clinical or environment samples from Iran in the literature.

The aim of this study was to determine the existence of ESBLs and the current prevalence of resistance to antibacterial agents in clinical and non-clinical isolates of *Proteus*. In this study chicken skin was considered as a representative of their fecal flora. The ability to produce hemolysin, and growth in presence of sodium chloride, were tested as the direct and indirect virulence factors respectively. In order to determine a possible correlation between the origin of the bacterial species, virulence factors and antibacterial resistance in the bacterial isolates from chicken skin, environmental samples and UTIs were compared.

Materials and methods

Bacterial isolation

From May 2003 to July 2004, totally 124 isolates of *Proteus* were collected and identified to species level by various biochemical tests [14]. The samples comprised of 80 isolates from urinary tract infections with significant bacteriuria (10^5 CFU/ml), 34 isolates from chicken skin, and 10 environmental samples, isolated from soil. *Proteus* bacilli were very rare in the soil samples; therefore, accidental sampling or nonprobability sampling was used in this preliminarily study.

Hemolysin production

Hemolysin production was determined using blood agar base medium (Merck, Germany) containing 7.5% defibrinated sheep blood, and 3% agar to prevent swarming [6]. The prepared plates were stored at 4°C in sealed plastic bags until use. The plates were dried for one hour at 37°C and then streaked with the test strains which have been grown on trypticase soy broth the previous day. To see both surface and deep hemolytic activity, the bacteria were inoculated on the surface of plates and stabbed inside the agar medium. There is no standard strain for this activity.

Salt tolerance

Growth in presence of salt was tested on nutrient agar medium supplemented with 7.5%, 8.5%, and 10% sodium chloride.

Antibacterial resistance pattern

Sensitivity of the isolates to 10 antibacterial agents, ceftriaxon (Ctx), amoxicillin (Amx), chloroamphenicol (C), azteronam (Azt), gentamicin (Gm), norfloxacin, (Nor), trimethoprim -sulfamethoxazol (Sxt), cephazolin (Cz), ciprofloxacin (Cip), and amoxicillin-clavulanic acid (Amx-Clav) was performed using standard disk diffusion method [15]. Amoxicillin and amoxicillin-clavulanic acid were from Mast

Company, France and other disks were obtained from Hi Med, India. Minimum Inhibitory Concentrations (MICs) against amoxicillin and amoxicillin-clavulanic acid were determined by the standard agar dilution method [15]. ß-lactamase production was tested using nitrocfin disks. ESBL phenotype was determined using double disk diffusion method consisting of cefotaxime-cefotaxime clavulanic acid, ceftazidim-ceftazime clavulanic acid, and cefpodoxime-cefpodoime clavulanic acid, according to the recommended procedure of the manufacturer (nitrocefin and ESBL disks were obtained from Mast Company, France). *P. mirabilis* strain 43071 (ATCC) was used as the quality control organism.

Statistical analysis

The statistical significance of the association between different variables was assessed by chi-square test, using EPI Info., version 6, to perform analysis of the data. P value of <0.05 was considered as significant.

Results

Of 124 bacteria isolated from different sources, the rate of isolation of *P. mirabilis*, *P. vulgaris* and *P. penneri* were found to be 80.6%, 17.7% and 1.6% respectively. All the samples from chicken skin and environment were identified as *P. mirabilis*, 95% of the *P. vulgaris*, and both strains of *P. penneri*, which were identified to be different strains by Dienes phenomenon [2], were isolated from UTIs. All the isolates had the ability to grow on nutrient agar with 7.5% salt, and 78 (62.9%) of the isolates were able to grow in the medium containing 8.5% salt. Higher concentration of salt (10%) inhibited the growth of all isolates. Salt tolerance was more common for *P. vulgaris* isolates (81.8%) in comparison with *P. mirabilis* (59%), (P=0.04), and samples from UTIs and chicken were more

salt tolerant than the environmental isolates
(P=0.003) (Table 1).

Table 1: Hemolysin production and salt tolerance for 124 *Proteus* bacilli isolated from different sources

Sample /Species (No. of the isolates)	Hemolytic activity No (%)	Sodium chloride No (%)
Urinary tract infections (80)	51 (63.75)	55 (68.8)
Chicken skin (34)	21 (61.76)	22 (64.7)
Environmental (10)	5 (50)	1 (9.9)
Proteus mirabilis (100)	56 (56)	59 (59)
Proteus vulgaris (22)	16 (72.7)	18 (81.8)
Proteus penneri (2)	2 (100)	0 (0)

Hemolytic activity on the agar surface and depth of the agar was observed for 74% of the isolates. Both strains of *P. penneri* were hemolytic, while 72.7% of *P. vulgaris* and 56% of *P. mirabilis* isolates showed hemolytic activity on solid medium. No significant difference was noticed between different samples or between *P. vulgaris* and *P. mirabilis* with respect to hemolytic activity.

Antibacterial sensitivity pattern
The isolates were found to be highly sensitive to Ctx, Cz, Amx-clav and Gm (Table 2). Sensitivity to Cip, Nor and Azt was high for UTIs and environmental isolates, while the isolates from chicken were more resistant (P=0.0005). Environmental isolates were significantly more sensitive to Sxt, compared to the other isolates (P=0.005), while chloramphenicol resistance was more common in the isolates recovered in the environment (P=0.0000). *P. penneri* isolates were sensitive to all antibacterial agents except Sxt. *P. vulgaris* isolates were found to be more sensitive to antibacterial agents in comparison with *P. mirabilis* isolates, and the difference in case of C (95.4% for *P. vulgaris* and 63.3% for *P. mirabilis*) and Sxt (77.2% for *P. vulgaris* and 37.6% for *P. mirabilis*) was significant (P=0.008, and P= 0.002 respectively).

Table 2: Sensitivity pattern and β-lactamase production for 124 *Proteus* bacilli isolated from different origins

Sample (No.)		UTIs (80)	Chicken (34)	Environment (10)	Total (124)	P value
Sensitivity to antibacterial agents, No (%)	Amx	49 (61.25)	20 (58.8)	3 (30)	72 (58.1)	>0.05
	Amx/Cla	75 (93.75)	34 (100)	10 (100)	122 (98.4)	>0.05
	Cef	75 (93.75)	34 (100)	10 (100)	122 (98.4)	>0.05
	Ctx	79 (98.75)	34 (100)	10 (100)	123 (99.2)	>0.05
	Ao	78 (97.5)	26 (76.5)	10 (100)	116 (93.5)	0.0005
	C	58 (72.5)	24 (70.6)	2 (10.5)	84 (67.74)	0.0000
	Gm	80 (100)	31 (91.2)	10 (100)	121 (97.6)	>0.05
	Cip	80 (100)	26 (76.5)	10 (100)	116 (93.5)	0.0000
	Nor	80 (100)	26 (76.5)	10 (100)	116 (93.5)	0.0000
	Sxt	37 (46.3)	17 (50)	10 (100)	64 (51.6)	0.005
β-lactamse No (%)		21 (26.25)	9 (26.5)	3 (30)s	33 (26.6)	0.000000

From 72 isolates that were resistant to Amx, 33 isolates (26.6%) produced β- lactamase by nitrocefin test. The MICs for Amx resistance isolates was in the range of 64 to ≥2048 µg/ml, and in presence of clavulanic acid, the MICs level were reduced several folds (Table 3). All Amx resistance β-lactamase negative isolates were sensitive to other β-lactam antibiotics tested, except two isolates which were resistant to Azt, and Cz by disk diffusion method. The Amx resistant β-lactamase positive isolates were sensitive to other β-lactam antibiotics tested, except two isolates which were resistant to Azt, and Cz by disk diffusion method.

Table 3: MIC for Amx and Amx-Cla in Amx-resistant isolates of *Proteus* bacilli isolated from different origins

| Samples | MIC µg/ml | | | | | |
| | Amoxicillin | | | Amoxicillin/Clavulanic acid | | |
	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range
UTIs	512	2048	64-≥2048	32	32	≤8-64
Chicken	256	2048	128-≥2048	≤8	16	≤8-32
Environmental	1024	2048	256-≥2048	32	≥64	32-≥64
β-lactamase +	1024	≥2048	128-≥2048	≥32	≥32	32-64
β-lactamase -	512	1024	64-≥2048	≤8	16	32

Discussion

In the present study, the major isolated species of *Proteus* from UTIs and other samples were *P. mirabilis*. Other investigators also found *P. mirabilis* as the major isolated species of *Proteus* from different samples [16,17]. Since the carriage rate of isolation of *P. mirabilis* is high in human and animal intestine (25%), therefore it is highly distributed in nature [18].

Hemolysin production is an important virulence factor for many bacterial species including *Proteus* [6,18]. Due to the swarming motility of these bacteria on the agar medium, detection of hemolysin on ordinary blood agar is difficult to observe, therefore different methodology with conflicting results are reported [2,6,18,19]. In the present study hemolytic activity both around the colony and inside the agar medium, which seen only after long incubation period (about 48h), was considered as hemolysin production; however there was no significant difference between bacterial isolates with respect to hemolysin production.

Cell surface is an important virulence factor for many bacteria [20], and the ability of a bacterium to tolerate any particular set of ionic conditions could be due to the outermost permeability barrier of that organism [21]. In this study we report the high tolerance of *Proteus* bacilli to salt (7.5%) and higher rate of salt tolerance (8.5%) was found in the UTI and chicken samples compared to the environmental isolates (P=0.0000005). The ability to reproduce, or simply survive, under a wide variety of environmental conditions contributes to a microbial pathogen's potential for transmission by various routes.

Survival under the extreme and rapidly changing conditions requires timely and appropriate alterations in gene expression and protein activity that occur in a bacterial cell in response to stimuli signaling [8]. Virulence factor in commensal bacteria like *Proteus* is basically a multifunctional phenomenon and the significant difference

in samples with respect to some phenotypic characteristics may correspond to the opportunistic nature of these bacteria. However, further work especially in the molecular level is required to see if the salt tolerance may have any effect in this type of signaling (quorum sensing) in these bacteria.

The results obtained by disk diffusion methods showed high sensitivity of isolates to cephalosporins (>98%), gentamicin (97.6%) quinolones and azteronam (93.5%). Resistance to Amx was high and 58% of the isolates showed resistance to this agent. The genus *Proteus* is reported to be the most inherently susceptible of all enterobacteria to β-lactams [22]. Amoxicillin resistance is recently being increased in these bacteria and Chanal *et al*. [3] reported that 48.5% of clinical samples of *Proteus* in a French hospital were Amx resistant [3].

Ampicillin and other β-lactams are widely used in human and veterinary medicine to treat human and animal infections, and the widespread use of these agents could be associated with the emergence of Amx resistant isolates. In this study amoxicillin /clavulanic acid was several folds more active than amoxicillin. Although 19 amoxicillin resistant isolates were not detected as β-lactamase producer by nitrocefin disks, combination of clavulanic acid with amoxicillin has significantly reduced the MIC of these isolates compared to amoxicillin (Table 3).

No ESBL was detected in the isolates resistant to cephalosporins. As far as we know, this is the first report from southeast of Iran on the detection of ESBLs in the enteric bacteria especially the *Proteus* bacilli. However continuous monitoring of clinical isolates for the emergence of ESBLs in any genera of enteric or non-enteric Gram-negative bacteria is recommended. Further work is also necessary to determine the other mechanism of resistance to third generation of cephalosoprins in the absence of ESBLsin these bacteria, such as penicillinase and inhibitor resistance β-lactamase.

Soil and human isolates were 100% sensitive to quinolones, while chicken isolates were significantly less susceptible. Higher flouroquinolone resistant isolates in the chicken could be due to the use of these compounds for growth promotion of the chickens [23,24]. It has been reported that flouroquinolone resistant bacteria has been reported in poultry even in the countries in which these drugs are not used as a growth promotion [23,24]. There is no report that the resistance isolates of a given bacterial species is more virulent than the non-resistance isolates. However the disease caused by the resistance strains is more difficult to treat. Therefore the sensitivity pattern of these isolates was determined and compared because the resistance strains in animals can be transferred to human and their presence is a treat for human infections [10,16,17, 23,24].

In conclusion the most frequent isolate was *P. mirabilis* which isolated from all samples, while *P. vulgaris* and *P. penneri* was only isolated from UTI samples. Hemolysin production and salt tolerance were not significantly different in regard to bacterial species. In this preliminary study, we found a higher level of salt tolerance in the environmental samples compared to chicken and UTIs samples, while hemolysin production was not significantly different in the bacterial isolates from different sources. In order to find a correlation between hemplysin and salt tolerance and the virulence of *proteus* bacilli, more work with a higher number of isolates for comparison is necessary.

Work on other virulence factors such as cell surface hydrophobicity is also recommended. Based on these results, it is recommended that prescription of antibacterial agents be restricted to known bacterial infections. Since animals can be a

source of antibacterial resistance in the community, care should be taken to prevent the spread of resistant bacteria in the chicken hatchery by avoiding the use of antibacterial agents such as the food additives.

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Address for correspondence:
Shahla Mansouri, Department of Microbiology, Kerman University of Medical Sciences, Kerman, Iran
Tel: +98341 3221665; Fax: +98341 3221671,
Email: SMansouri@kmu.ac.ir;
shmansouri_1000@yahoo.com