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Research Article

Phenotypic Screenings of AmpC and MBL Mediated Resistance in Proteus mirabilis & Proteus vulgaris, An Emerging Threat to Clinical Therapeutics.

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Abstract: ***Background:*** β-lactamases such as AmpC β-lactamases and Metallo-β-Lactamases (MBL) caused resistance to various β -lactam drugs which mostly create serious therapeutic problems. Among clinical isolates of Proteus mirabilis and Proteus vulgaris secreted hemolysins were extremely common and hemolytic activity was either cell associated or cell free. The true rate of occurrence of AmpC beta-lactamases and MBL along with hemolysin production in P. mirabilis and P. vulgaris are rapidly increasing. ***Objectives:*** To find the prevalence of P. mirabilis and P. vulgaris with antibiogram pattern against different antibiotics. To perform different phenotypic screenings e.g. AmpC resistance and Metallo Beta- lactamases (MBL) screenings with the virulence factor by phenotypic identification of hemolysin production. ***Study design:*** Retrospective Cross-sectional study ***Place and duration of study:*** This Retrospective Cross-sectional study was designed from three major hospitals of Peshawar i.e. Hayatabad Medical Complex (HMC), Northwest Hospital (NH) and Khyber Teaching Hospital (KTH), Peshawar. In current study three hundred and fifty samples were collected from July 2017 to December 2017. ***Methodology:*** In this study a total of 350 patients with different age groups of both gender were selected. These samples will be collected from different sources such as surgical site, pus abscess, urine and blood stream. Samples were collected with sterile swabs and then cultured on different media i.e. Nutrient agar, Cystein lactose electrolyte deficient (CLED), Sheep Blood agar, and MacConkey media, Trypton Soya Broth (TSB) and Molar Hinton Agar (MHA). The target microorganisms were identified by colony morphology, Gram staining, Analytical profile Index (API) System and standard biochemical tests i.e. Urease, Triple sugar iron (TSI), Indole test. Antibiotic sensitivity was conducted by "disc-diffusion" method on Mueller Hinton Agar using CLSI guidelines. A three-dimensional extract test and Double Disk Synergy Test (DDST) were applied for phenotypic detection that harbor AmpC β -lactamase and Metallo β – lactamase. ***Results*** In the current study, a total of 350 consecutive, nonrepeating isolates of Proteus spp. 311(88.8%) were found positive, among positive isolates P. mirabilis and P. vulgaris were 174 (51.7%) and 137 (48.2%) respectively. The prevalence of male and female patients were 140 (45%) and 171 (54.9%) respectively. P. mirabilis showed highest resistance to Nalidixic acid (63.97%), Colistin –SO4 (63.35%) and Minocycline (62.73%), whereas it showed higher sensitivity to Moxifloxacin (78.8%), Cefipime (78.26%), Ampicillin and Meropenem (73.29%) and Levofloxacin (72.04%). Similarly, P. vulgaris was highly resistant to Colistin –SO4 (63.33%), Nitrofurantoin (58.00%) and Nalidixic acid (57.33%), whereas it shows higher sensitivity to Cefipime (88.66%), Moxifloxacin (88.00%), Cefixime (76.66%), Meropenem (73.33%), Levofloxacin and Piperacillin/ Tazobactum (70.00%). Out of total positive cases, maximum number of MBL positive cases of P. mirabilis were 75 (24.15%) and P. vulgaris were 55 (17.6%), and the ratio of AmpC β -lactamases in P. mirabilis and P. vulgaris were 180 (57.87%) and 85 (47.22%) respectively. Similarly, hemolysis activity was observed in 168 (54.01%) isolates of Proteus spp. Among 168 (54.01%) hemolytic isolates the frequency of P. mirabilis and P. vulgaris were 94 (55.9%) and 74 (44.04) respectively. ***Conclusion:*** It is concluded from the study that emergence of MBL and AmpC - β lactamases in clinical isolates leads to failure of anti- microbial therapies worldwide and these MDR organisms are mostly capable to produce hemolytic toxins.

Keywords: (MBL) Metallo β – lactamase, (DDST) Double Disk Synergy Test, (MDR) Multi drug resistance, Hly+ = hemolytic, Hly-= non- hemolytic.

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INTRODUCTION

Antimicrobial resistance (AMR) is a major challenge to the management of bacterial infections worldwide. Resistance to commonly used antibacterial agents can limit treatment options, increase the risk of therapeutic failure, and complicate infection prevention and control. The World Health Organization's global surveillance program emphasizes the importance of reliable microbiological testing, standardized reporting, and continuous surveillance to characterize the changing burden of AMR [1,2].

The genus Proteus comprises Gram-negative members of the order Enterobacterales that are associated with human, animal, and environmental conditions. Among clinically important species, P. mirabilis is a recognized cause of urinary tract infections, particularly complicated infections associated with urinary catheters and structural abnormalities. P. vulgaris can also cause clinically significant infections, although its distribution and susceptibility characteristics may differ from those of P. mirabilis [3]. Both organisms exhibit biological characteristics that can facilitate persistence in susceptible hosts and complicate the management of infection.

The pathogenic potential of Proteus species is associated with several characteristics, including motility, urease activity, biofilm formation, and other virulence-associated factors. In urinary infections, urease activity may contribute to urinary stone formation and persistent infection. Hemolysins and other bacterial factors have also been investigated for their possible roles in host-cell injury and disease progression. Nevertheless, the presence of a particular virulence-associated phenotype does not necessarily establish the severity of an individual infection or prove a direct relationship with antimicrobial resistance [3,4].

As Proteus consists of five important species i.e. P. vulgaris, P. mirabilis, P. penneri, P. myxofaciens, and P. hauseri but among these P. mirabilis and P. vulgaris are clinically very important. Swarming nature is the important characteristic of Proteus species. [5]

β-Lactam antibiotics are widely used to treat infections caused by susceptible Gram-negative bacteria. Their activity can be compromised by β-lactamases, which hydrolyze the β-lactam ring and render susceptible antibiotics ineffective. AmpC β-lactamases can affect several penicillins and cephalosporins, while carbapenemases may compromise the activity of carbapenems. Metallo-β-lactamases are a group of carbapenemases that depend on metal ions for enzymatic activity and can inactivate many β-lactam antibiotics. Their clinical significance depends on the enzyme present, the bacterial species, and the susceptibility profile of the isolate. MDR isolates leads to increasing prevalence of infections that has been reported worldwide. Recent genomic research has also demonstrated the diversity of resistance determinants among clinical P. mirabilis isolates, including plasmid-associated mechanisms and changing resistance patterns over time [6, 7].

In Gram negative Bacilli the most common cause of resistance to the β- lactam antibiotics is the production of ESBL, which is the main cause for the resistance to the 3rd generation cephalosporins. The antimicrobial activity is destroyed by β-lactamase which catalyze the hydrolysis of the β-lactam ring of antibiotic. In many different genera of Enterobacteriaceae and P. aeruginosa ESBLs have been reported worldwide. ESBL producing organisms are also resistant to several other classes of antibiotics. [8]

The emergence of resistant strains is a natural process that occur when inaccurate replication of microorganisms takes place and when they exchange resistant traits among their species. By the misuse of antimicrobial drugs, through improper infection control practices in medical fields, improper sanitary system and poor handling of food products the spread of antimicrobial resistant infection were accelerated. [9].

MATERIALS AND METHODS

Study design and Sample collection

The original study was described as a retrospective cross-sectional analysis. Specimens were collected between July and December 2017. A total of 350 samples were collected from three major hospitals of Peshawar i.e. Hayatabad Medical Complex (HMC), Northwest Hospital (NH) and Khyber Teaching Hospital (KTH), Peshawar. These samples were collected from different sources such as surgical site, pus abscess, urine and blood stream [10].

Culturing media

All the samples were cultured on different media i.e. Nutrient agar, Cystein Lactose Electrolyte Deficient (CLED), Sheep Blood agar, and MacConkey media, Trypton Soya Broth (TSB) and Muller Hinton Agar (MHA). The target microorganisms were identified by colony morphology, Gram staining, Analytical profile Index (API) System and standard biochemical tests i.e. Urease, Triple sugar iron (TSI), Indole test [11]. By colouring cells red or violet, Gram negative and Gram positive groups were differentiated.

Antibiotic sensitivity test

Antibiotic sensitivity was carried out by applying different antibiotics discs on MHA culture plates according to modern CLSI guidelines.

Combine disk test

The test CDT was used to carry out phenotypic detection of MBL that was reported by [12].

AmpC screenings and hemolysin production

For non-susceptible organisms, following the NCCLS criteria, for beta-lactamase testing isolates having zone diameters less than 18 mm were selected i.e. for AmpC screenings. E. coli was used as a control strain, extended zone of inhibition was considered an evidence for the presence of AmpC beta- hemolysin production to detect hemolysis and virulence factors, all isolates of P. mirabilis and P. vulgaris were investigated by tube assay method, as reported by [19].

RESULTS

Out of 350 samples, 311(88.8%) were found positive, while 39 (11.14%) were negative, among positive isolates P. mirabilis and P. vulgaris were 174 (51.7%) and 137 (48.2%) respectively, **(Table 4.1)**. Among the positive blood samples, the prevalence of male and female patients were 140 (45%) and 171 (54.9%) respectively, **(Table 4.2).**

Table 4.1: Distribution of specimens and identified Proteus species

Total Samples	Total Positive Samples	Percentage (%) of Positive Samples	Total Negative Samples	Percentage (%) of Negative Samples
350	311	88.8	39	11.14
Total Positive Samples	P. mirabilis	(%)	P. vulgaris	(%)
311	174	51.7	137	48.2

Among collected samples, 140(45.01%) were collected from male patients and among these the frequency distribution of P. mirabilis and P. vulgaris were 78 (55.7%) and 62 (44.2%), respectively. Similarly, 171(54.9%) isolates were collected from female patients the frequency distribution of P. mirabilis and P. vulgaris were 96 (56.1%) and 75 (43.8%) respectively. The collected samples were categorized into different age groups, i.e.NB-19, 20-49 and 50-80 years. **(Table 4.3).**

Table 4.2: Distribution of species according to patient gender.

Gender	Positive Isolates	P. mirabilis	(%)	P. vulgaris	(%)
Male	140(45.01%)	78	55.7	62	44.2
Female	171(54.9%)	96	56.1	75	43.8

Table 4.3: Age and gender wise distribution of P. mirabilis and P. vulgaris.

	Uropathogens							
	P. mirabilis (174)				P. vulgaris (137)			
	M (78)	(%)	F (96)	(%)	M (62)	(%)	F (75)	(%)
NB-19 years	13	16.6	21	21.8	11	17.7	15	20
20–49 years	23	29.4	25	26.0	18	29.0	22	29.3
50–80 years	42	53.8	50	52.0	33	53.2	38	50.6

All identified isolates of P. mirabilis and P. vulgaris were screened for antibiotics susceptibility test against different antibiotics. P. mirabilis showed resistance to Nalidixic acid (23.60%), Co-trimxazole (26.08%), Colistin- SO4 (26.70%), Minocycline (28.57%) and nitrofurantoin (32.91%). Similarly, it showed more sensitivity towards 3rd and 4th generation flouroquinolones like Levofloxacin (72.04), Moxifloxacin (78.88%) and towards 3rd and 4th Cephalosporins like Cefixime (60.86%), Cefipime (78.26%) and also show sensitivity towards broad spectrum Penicillins like Ampicillin (73.29%) and Carbapenems like Meropenem (73.29%). The interpretation of zone of inhibition was carried out according to CLSI

guidelines. **(Figure 4.6)** P. vulgaris showed resistance to Colistin- SO4 (16.66%), Nalidixic acid (25.33%), Minocycline (30.00%), Co-trimxazole (32.00%), and nitrofurantoin (30.00%). Similarly it showed sensitivity towards 3rd and 4th generation flouroquinolones like Levofloxacin (70.00%), Moxifloxacin (88.00) and towards 3rd and 4th Cephalosporins like Cefixime (76.66%), Cefipime (88.66%) and also show sensitivity towards broad spectrum Penicillins like Piperacillin/Tazobactam (70.00%) and Carbapenems like Meropenem (73.33%) respectively **(Figure 4.7).**

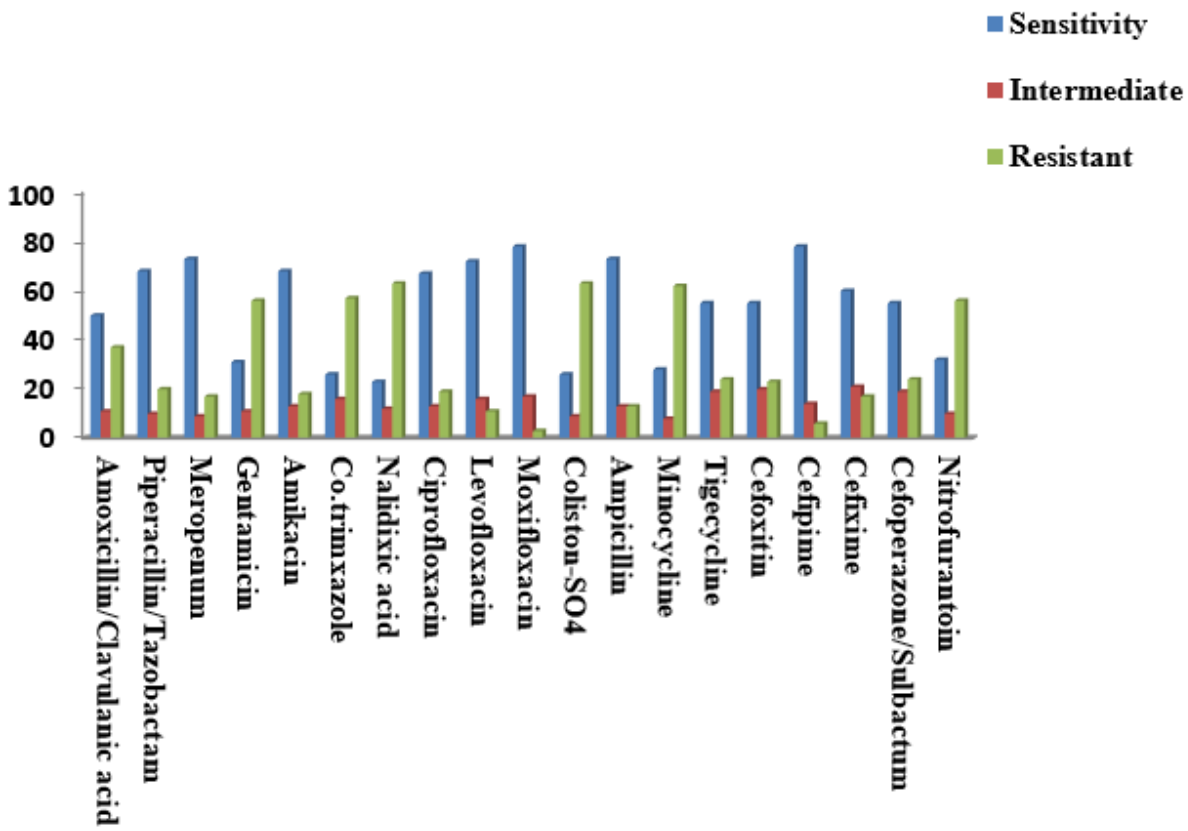


Figure 4.6: Antibiotic sensitivity pattern of P.mirabilis.

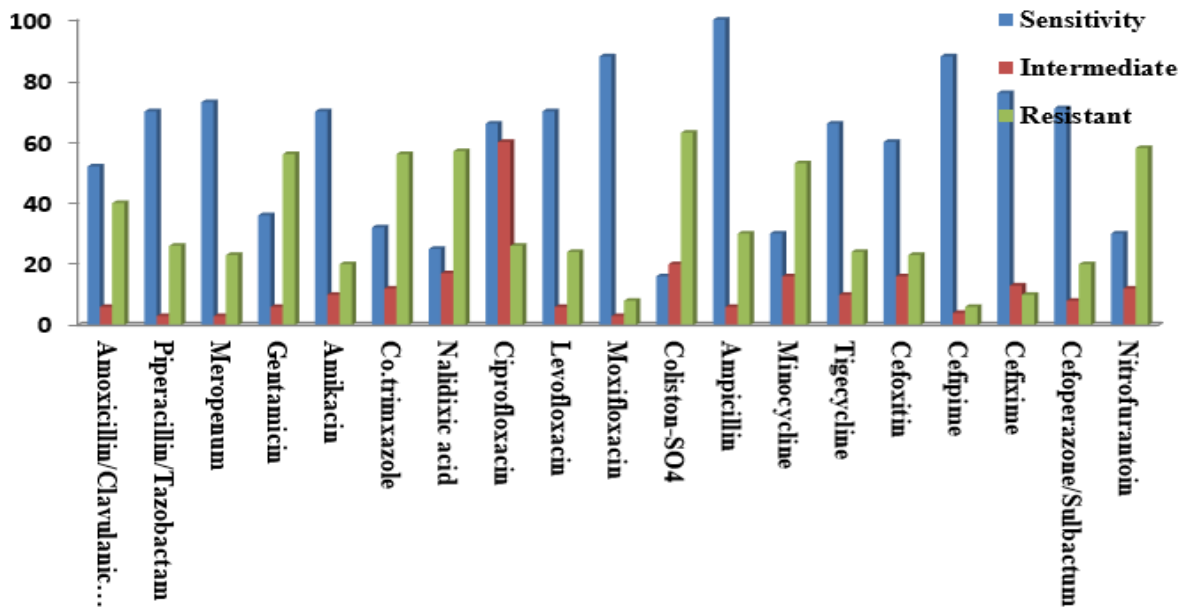


Figure 4.7: Antibiotic sensitivity pattern of P.vulgaris.

P. mirabilis and P. vulgaris both were checked for the production of Metallo β – lactamase (MBL). All identified isolates of P. mirabilis and P. vulgaris were screened for the sensitivity of EDTA-IPM and IPM. The results showed that maximum number of P. mirabilis MBL producer were present in urine (32) and pus (18) samples as compare to blood (14) and surgical samples (11)

Table 4.4: Frequency distribution of positive MBL samples on the basis of age groups between male and females.

Age groups	Uropathogens (MBL)							
	P. mirabilis (75)				P. vulgaris (55)			
	M (31)	(%)	F (44)	(%)	M (21)	(%)	F (34)	(%)
NB-19 years	6	19.3	9	20.4	4	19.0	7	20.5
20–49 years	11	35.4	16	36.3	7	33.33	11	32.3
50–80 years	14	45.1	19	43.1	10	47.61	16	47.0

From the current study, it was observed that maximum cases of MBL producers were detected in female as compared to male. Similarly, age groups of 50-80 years were more affected by MBL producers as compare to other age groups. **(Table 4.4)**. On the basis of clinical features the prevalence of hemolytic isolates of P. mirabilis was observed higher in patients suffering from UTIs 43 (45.74%), SSIs 32 (34.04) and PIs 19 (20.21) respectively **(Table 4.5).** Similarly the prevalence of hemolytic isolates of P. vulgaris were observed higher in patients suffering from UTIs 38 (51.35%), SSIs 22 (29.72%) and PIs 14 (18.91) respectively **(Table 4.6).**

Table 4.5: Distribution of Hly^+ and Hly^- isolates of P. mirabilis 94 (55.9%).

		Isolates n (%)	Males n(%)	Females n (%)	UTIs n (%)	SSIs n (%)	PIs n(%)
Hemolysi	Strong	21 (22.3)	37 (39.36)	57 (60.63)	43 (45.74)	32 (34.04)	19 (20.21)
	Moderate	42(44.6)					
	Weak	31 (32.97)					
	Total Positive	168(54.01)					
	Hly^-	143(45.98)					

Table 4.6: Distribution of Hly^+ and Hly^- isolates of P. vulgaris 74 (44.04%)

		Isolates n (%)	Males n(%)	Females n (%)	UTIs n (%)	SSIs n (%)	PIs n(%)
Hemolysi	Strong	18 (24.32)	27(36.48)	47(63.51)	38 (51.35)	22 (29.72)	14 (18.91)
	Moderate	30 (40.54)					
	Weak	26 (35.1)					
	Total Positive	168(54.01)					
	Hly^-	143(45.98)					

Occurrence of AmpC-producing organisms:

Among MBL positive samples, of P. mirabilis and P. vulgaris the positive samples were further screened for the production of cephalosporins. Among AmpC positive isolates of P. mirabilis (37) were detected in urine, (24) in pus, (20) in blood stream and (14) in surgical site. Similarly, among AmpC positive isolates of P. vulgaris (33) were detected in urine, (23) in pus, (18) in blood stream and (11) in surgical site in **(Table.4.7)**

Table 4.7: Susceptibilities of AmpC-producing isolates, as total number of P. mirabilis 95 (52.77%) and P. vulgaris cases are 85 (47.22%).

Total positive isolates 180 (57.87%)	AmpC MBL Producers (n)		Sensitivity (%)	
	P. mirabilis	P.vulgaris	P. mirabilis	P.vulgaris
Urine	37	33	38.94	38.82
Pus	24	23	25.26	27.05
Blood stream	20	18	21.05	21.17
Surgical site	14	11	14.73	12.94

From the current study, it was observed that maximum cases of AmpC producers were detected in female as compared to male. Similarly age groups of 50-80 years were more affected by AmpC- MBL producers as compare to other age groups in **(Table.4.8)**

Table 4.8: Frequency distribution of positive AmpC metallo beta-lactamases samples on the basis of age groups between male and females.

Age groups	Uropathogens							
	P. mirabilis 95 (52.77%)				P. vulgaris 85 (47.22)			
	M (37)	(%)	F (58)	(%)	M (32)	(%)	F (53)	(%)
NB-19 years	9	24.32	12	20.68	7	21.87	11	20.75
20–49 years	12	32.43	20	34.48	11	34.37	19	35.84
50–80 years	16	43.24	26	44.82	14	43.75	23	43.39

DISCUSSION

Out of 350 samples, 311(88.8%) were found positive, among these P. mirabilis and P. vulgaris were 174 (51.7%) and 137 (48.2%) respectively. Among the positive blood samples, the prevalence of male and female patients were 140 (45%) and 171 (54.9%) respectively. [13] also carried out similar study isolated different Gram negative bacteria including P. mirabilis and P. vulgaris from different sources i.e. urine, catheter tips, stool, semen, urogenital and pus abscesses.

The isolates included E. coli (278), K. pneumoniae (128), P. mirabilis (89), P. vulgaris (32), P. aeruginosa (83), S. typhi (4), Serratia marcescens (1), Morganella spp. (2) and Enterobacter spp. (16). In this study the percentage of positive samples 633 (100%) were more as compare to our study (88.8%). This difference is because we focused only on Proteus spp. and only in patients were included.[14] conducted a cross-sectional study in the emergency department (ED) of a general hospital. Among collected samples, 212 cases (72.8%) were reported in females (median age). The predominant uropathogens was E. coli (76.6%), followed by P. mirabilis (10.3%) and S. saprophyticus (4.1%). P. mirabilis was the most important uropathogens in males and accounted for 24% of the cases of UTI in males as compared to E.coli, S. saprophyticus and P. vulgaris. The current study included high number of females and maximum number of females and maximum number of bacteria were isolated from old age males and females as compared to other age groups, because they are immune-compromised having diabetes and UTI at the same time, hence they are more susceptible to infections.

All identified isolates of P. mirabilis and P. vulgaris were screened for antibiotic susceptibility test. P. mirabilis showed resistance to Nalidixic acid (23.60%), Co-trimxazole (26.08%), Colistin- SO4 (26.70%), Minocycline (28.57%) and nitrofurantoin (32.91%). Similarly it showed more sensitivity towards 3rd and 4th generation flouroquinolones like Levofloxacin (72.04), Moxifloxacin (78.88%) and towards 3rd and 4th Cephalosporins like Cefixime (60.86%), Cefipime (78.26%) and also show sensitivity towards broad spectrum Penicillins like Ampicillin (73.29%) and Carbapenems like Meropenem (73.29%). Similarly [15], reported that the strains of P. mirabilis were usually more sensitive to antibiotics, as compared to P. penneri, P. hauseri and P. vulgaris.

As P. mirabilis showed resistance to Nitrofurantoin and Tetracycline but generally it was sensitive to these specific Cephalosporins (Cefotaxime, Ceftriaxone, Ceftazidime, Ceftizoxime, Cefoxitin, Cefuroxime and Cefepime), Amino- and Ureido-penicillins (Ampicillin, Amoxicillin, and Piperacillin), Aminoglycosides (Tobramycin Amikacin, and Gentamicin), Trimethoprim-Sulfamethoxazole and Imipenem, Ciprofloxacin). The results obtained are almost in the same line to the current project, but the little difference is due to the misuse of antibiotics in Pakistan. This study also represents that, among all Proteus spp. P. mirabilis shows more sensitivity towards used antibiotics, but due to the irrational use of antibiotics Proteus developed resistance.

P. vulgaris showed resistance to Colistin- SO4 (16.66%), Nalidixic acid (25.33%), Minocycline (30.00%), Co-trimxazole (32.00%), and nitrofurantoin (30.00%). Similarly it showed sensitivity towards 3rd and 4th generation flouroquinolones like Levofloxacin (70.00%), Moxifloxacin (88.00) and towards 3rd and 4th generation of Cephalosporins like Cefixime (76.66%), Cefipime (88.66%) and also show sensitivity towards broad spectrum Penicillins.[16] conducted similar study on 23 Proteus isolates which exhibited 100% resistance towards Ceftazidime, Amikacin, Sulphamethoxazole, Chloramphenicol, Ampicillin and Streptomycin and were marked as MDR organisms. They additionally had 95.6 %(22/23) resistance towards kanamycin, 91 % (21/23) to ceftriaxone, 86.95% (20/23) to Cefepime and the most reduced rate of resistance is of 39.1% for levofloxacin. This study correlates with the current study, regarding sensitivity of levofloxacin, and in this study the resistance percentage of 3rd and 4th generation of Cephalosporins is higher than our results, due to the emergence of MDR cases.

Similarly in Nigeria, 10% MDR P. mirabilis with 100% resistance to Tetracycline and Ampicillin, however sensitive to Ciprofloxacin, Ofloxacin and Gentamicin were isolated from poultry feed samples screened bacteriologically at Calabar, Eastern part of Nigeria, reported by [17].

The high Carbapenems and Cephalosporins resistant isolates were subsequently screened for the production of Metallo beta – lactamase (MBL) and AmpC beta- lactamases. All identified isolates of P. mirabilis and P. vulgaris were screened for the sensitivity of EDTA-IPM and IPM in case of MBL screening, whereas production of AmpC beta- lactamases were identified through FOX sensitivity patterns. The results showed that maximum number of P. mirabilis and P. vulgaris MBL and AmpC producers were present in urine and pus samples as compare to blood and surgical samples. A similar study was also performed by [18], in Egyptian hospital. Among total collected isolates from urine (143), the identified isolates were E. coli (102), K. pneumoniae (30), K. oxytoca (5), P. mirabilis (4), and P. vulgaris (2). Twenty-six (18.2%) isolates were cefoxitin resistant. Among these resistant isolates, E. coli (21), K. pneumoniae (3), K. oxytoca (1), and P. mirabilis (1) were thus considered as putative AmpC markers.

The percentage of isolates showing positive ESBL by phenotypic techniques was 80.7. The study correlates with current research work. A slight difference may be due to source and number of samples. Hemolysis was observed in 168 (54.01%) isolates while 143 (45.98%) were found to be non-hemolytic. Among 168 (54.01%) hemolytic isolates the frequency of P. mirabilis and P. vulgaris were 94 (55.9%) and 74 (44.04) respectively. Among 94 (55.9%) hemolytic isolates of P. mirabilis: 21 (22.3%) were strongly hemolytic, 42 (44.6%) were moderately hemolytic and 31 (32.97%) were weakly hemolytic, whereas in 74 (44.04%) hemolytic isolates of P. vulgaris: 18 (24.32%) were strongly hemolytic, 30 (40.54%) were moderately hemolytic and 26 (35.1%) were weekly hemolytic.

In four hospitals, ninty-five strains of the Proteae tribe from a wide range of clinical specimens were isolated. Among the three species; 94% of P. mirabilis, 84% of P. vulgaris, and 56% of M. morganii strains there was a high incidence of hemolysin production and showed clear hemolysis when they were grown in brain heart infusion broth [19]. In the present study the ratio of hemolytic isolates of P. mirabilis and P. vulgaris was in similar view to our results as secreted hemolysins were extremely common among clinical isolates of Proteus spps.

CONCLUSION

This study was designed for P. vulgaris and P. mirabilis in order to determine the occurrence of MBL and AmpC β – lactamases. In addition, we also carried out a phenotypic screening method for the detection of AmpC and MBL mediated

MDR Proteus species, which is nowadays emerging as a serious clinical threat throughout the world. It is also concluded from the study that emergence of MBL and AmpC - β lactamases in clinical isolates leads to failure of anti- microbial therapies worldwide and these MDR organisms are mostly capable to produce hemolytic toxins.

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