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Original Research Article

Detection of Macrolides Resistance Gene in *Proteus mirabilis* Strains Isolated from Different Clinical Specimens by Using PCR Based on *mef*(A) gene

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Abstract

Different species of *Proteus mirabilis* may vary in type of infections in both the community and hospital environments. *Proteus mirabilis* can cause a variety illnesses including urinary tract (uncommon in normal hosts), uncomplicated cystitis or pyelonephritis, wound, and blood stream infections (BSI). This study aimed to determine the macrolides resistance gene *mef*(A) in *P. mirabilis* strains isolated from different clinical samples by using PCR.

This a prospective study involving the analyses of different clinical samples (i. e. sputum, wound swabs, urine and ear swabs) were collected from 86 patients suspected of bacterial infections from Al-Sader Medical City and Al-Zahraa hospital for maternity and children were cultivated to isolate *P. mirabilis*. Bacterial isolates diagnosed according to Baily and Scott's Diagnostic Microbiology then further identified by Vitek® 2- system, the sequence of the primer used for detection of *mef*(A) gene was amplified by using Thermal Cycler. Statistical analysis was performed by using SPSS v.20. Eighty-six patients were randomly recruited; they were (65.12%) males and (34.88%) females. *P. mirabilis* occupies the lowest percentage 3.5 % among other microorganisms in this study. Other bacterial genera occupied different percentages there were 33.7%, 31.4%, 22.1% and 5.8 % for *Pseudomonas aeruginosa, E. coli, Proteus vulgaris* and *Klebsiella pneumoniae*. The antimicrobial activity patterns were detected using Kirby- Bauer method. Augmentin (AUG) occupied the highest value of effectiveness (93%) against the bacterial strains in comparison with other antimicrobial agent while the lowest value was (41.9%) for Ceftazidime (CAZ) (P<000). Only one strain carry the 402 mef(A) gene among the three Proteus mirabilis strains previously reported as macrolides resistant (phenotypically).

Key Words: Macrolides resistance, *mef* (A) gene, *Proteus mirabilis*.

الخلاصة

تختلف انواع بكتريا *Proteus mirabilis* في قابليتها على احداث اصابات في كل م ن بيئة المجتمع والمستشفيات . حيث تسبب هذه البكتريا *اصابات مختلفة* تتضمن المسالك البولية والتهاب المثانة غير المعقد ، التهاب حوض الكلية ، التهابات الجروح، وغيرها. هدفت الدراسة الى تحديد جينات مقاومة المايكروليدات في البكتريا اعتمادا" على تحديد جين المقاومة *mef*(A)، باستعمال تقنية تفاعل البلمرة المتسلسل. تضمنت هذه الدراسة جمع 86 عينة سريرية مختلفة مثل البلغم، مسحات الجروح، الادار ، ومسحات الاذن من مرضى يشتبه في اصاباتهم بالعدوى من مدينة الصدر الطبية ومستشفى الزهراء التعليمي للولادة والاطفال في محافظة النجف الاشرف . زرعت العينات على الاوساط الزرعية لعزل جرثومة *P. mirabilis*. تم تشخيص البكتريا المعزولة استنادا " الى مرجع بيلي وسكوت للاحياء الدقيقة ، ثم تم تاكيد التشخيص باستخدام Vitek® 2– system .استعملت تقنية تفاعل البلمرة المتسلسل لتضخيم البادئ باستعمال *جهاز* Thermal Cycler. كما استعمل نظام الحزمة الاحصائية SPSS اصدار رقم ٢٠ لتحليل البيانات . تم اختيار ٨٦ مريضا بصورة عشوائية منها (٦٥.١%) ذكور و(٣٤.٨%) اناث. احتلت بكتريا *P. mirabilis* اقل نسبة من نسب العزل (٣.٥%) بينما سجلت بقية الانواع البكتيرية نسبا" مختلفة للعزل لكل من *P. aeruginosa*،*E. coli*،*P. vulgaris* ، و *K. pneumonia*. أظهر مضاد اوكمنتين أعلى نسبة للفاعلية (٩٣%) بطريقة الانتشار من القرص بينما كانت اقل نسبة (٤١.٩%) لمضاد للسيفتازيديم (P<000). أظهرت نتائج الدراسة ايضا ان هناك عزلة واحدة تحمل الجين ٤٠٢ *mef (A)* من بين العزلات الثلاث التي حددت قابليتها لمقاومة المضادات (مظهريا").

الكلمات المفتاحية: الجين mef (A)، ماكروليدات، *Proteus mirabilis*،

Introduction

The bacteria of the genera *Proteus*, *Morganella*, and *Providencia* belong to the tribe Proteeae in the family Enterobacteriaceae. They have one biochemical characteristic that distinguishes them from all other members of the Enterobacteriaceae, with the exception of the rare genera *Tatumella* and *Rahnella*: the ability to deaminate oxidatively certain amino acids to the corresponding keto acid and ammonia. Tests for phenylalanine or tryptophan deaminase are the ones commonly made and a rapid method for this reaction has been devised [1]. *Proteus* strains occur in the soil, polled water and in the intestine of human and a wide variety of animals. They form part of normal flora and can be opportunistic human pathogens [2].

These strains gave a characteristic spreading growth ('swarming') on appropriate solid media, oxidatively deaminate amino acids, hydrolyze urea but fail to acidify mannose. The G+C content of the DNA is 38–40% mol. The genus, named after the Greek deity *Proteus* who had the ability to assume different shapes. Presently has five named species: *Proteus mirabilis* and *Proteus vulgaris* (formerly known together as *Proteus hauseri*), *Proteus myxofaciens* described by Cosenza and Podgwaite [3], *Proteus penneri* (formerly *P. vulgaris* biotype 1) described by Hickman *et al*. [4], and *Proteus hauseri* (formerly *P. vulgaris* bio group 3) described by O'Hara *et al*. [5].

Proteus mirabilis can cause a variety of community or hospital acquired illnesses including urinary tract infections (uncommon in normal hosts), uncomplicated cystitis or pyelonephritis, wound, and bloodstream infections (BSI). However, it is one of the most important pathogens associated with complicated urinary tract infections (acute pyelonephritis, bladder infections, and kidney stones) and bacteremia, affecting patients with anatomical abnormalities, immuno-deficiency, and long-term urinary catheterization [6,7].

There are many mechanisms were by bacteria confer resistance to the drugs including intrinsic impermeability and acquired resistance as plasmids, transposons and mutations [8]. Yah reported in a study on a wide spread of plasmids resistance genes among *Proteus* species that 44 % of antibiotic resistance were plasmid mediated, 32 % by chromosome, while 24 % of the resistance pattern to antibiotics could not be ascertained [9].

The use of macrolide and related antibiotics (ketolides, oxazolidinones, streptogramins, and lincosamide) have increased dramatically over the last 15 years. A number of different mechanisms of macrolide resistance have been reported for gram-negative bacteria. These mechanisms include two esterase genes [ere (A) and ere (B)] found in *E. coli* and *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Proteus* species and more recently, in *Providencia stuartii*, *Pseudomonas* species, and *Vibrio cholerae* [10- 12].

Agents within the tetracycline, aminoglycoside and macrolide lincosamide-streptogramin (MLS) classes of antibiotics target the ribosome to inhibit protein translation. Tetracyclines and aminoglycosides interact with 16S rRNA (rrs) and the macrolide-lincosamide-streptogramin (MLS) family binds to 23 S rRNA (rrl). In most bacteria, multiple rrs and rrl operons are present and susceptibility mediated by any one of these targets can be dominant, making resistance difficult to attain without a mutation in all or a majority of the other operons [13]. Macrolide resistance is generally mediated by two mechanisms: 23 S rRNA methylation encoded by the *erm* (B) gene or macrolide efflux via the *mef* (A) gene [14, 15].The aim of the present study is to determine the macrolides resistance gene mef(A) in *P. mirabilis* strains isolated from different clinical samples by using PCR.

Materials and Methods

Specimens' collection

Different clinical samples i.e. sputum, wound swabs, urine for culture, ear swabs, were collected from 86 patients suspected of bacterial infection from Al-Sadder Medical City and Al-Zahraa hospital for maternity and children. Clinical samples were cultivated on the laboratory media to isolate the *Proteus mirabilis*. Prior to sample collection demographic data of the patients were recorded i.e. age, gender and smoking.

Identification approach of the isolated bacteria

Bacterial isolates firstly diagnosed according to Baily and Scott's Diagnostic microbiology then the suspected colonies further identified by Vitek®2-System (Biomérux, France).

Antimicrobial susceptibility patterns

An antimicrobial sensitivity patterns of *Proteusmirabilis* isolates were studied for only nine different antibiotics (Table-1) according to disk diffusion technique [28] i.e. the inoculum were prepared by growing on separate agar plates and colonies from the plate were transferred with an inoculating loop into 3 ml of sterile normal saline in a test tube. The density of these suspensions adjusted to 0.5 McFarland standards. The surface of Muller-Hinton agar (Oxoid Cambridge, UK) plate evenly inoculated with the organisms using a sterile swab. The swab dipped into the suspension and pressed against the side of the test tube to remove excess fluid. The wet swabs used to inoculate the Muller-Hinton agar by evenly streaking across the surface. By means of Disc Dispenser (Oxoid Cambridge, UK), the antibiotic discs applied to the surface of the inoculated agar and the plates incubated overnight at 37˚C. The diameter of zone of growth-inhibition observed measured and compared to the chart provided by Clinical and Laboratory Standards Institute (CLSI).

Table 1 : The antibiotic used, their symbols and concentration.

ID.	Antimicrobial agent	Symbol	Disc Conc.
1	Ampicillin	AMP	10 µg/disc
2	Augmentin®	AUG	30 µg/disc
3	Gentamicin	GM	10 µg/disc
4	Cephalothin	KF	30 µg/disc
5	Amikacin	AK	30 µg/disc
6	Ceftazidime	CAZ	30 µg/disc
7	Ciprofloxacin	CIP	5 µg/disc
8	Erythromycin	ERY	15 µg/disc
9	Azithromycin	AZT	15 µg/disc

PCR primer

The sequences of the primer used for PCR was as follows: *mef*(A) (402 bp): 5'-CTGTATGGAGCTACCTGTCTGG-3', 5'-CCCAGCTTAGGTATACGTAC-3 [16].

Preparation of bacterial pellet

One milliliter of an overnight culture was added to a 1.5 ml micro-centrifuge tube. Centrifuge at 13,000–16,000 ×g for 2 minutes to pellet the cells. Remove the supernatant and use the pellet.

DNA extraction protocol

Promega Wizard® Genomic DNA Purification Kit was used to extract the DNA from the bacterial isolates.

Amplification reaction mixture

Twenty-five microliters of Master Mix (Go Taq® Green Master Mix, 2X, Promega) were poured in 100 µl PCR tube, followed by 5 µl DNA extract, 2.5 µl of 16 pica mole/µl upstream primer specific solution as mentioned above, 2.5 µl of 16 pmol/µl downstream primer specific solution and 15

µl nuclease free water for a final volume 50µl.

PCR condition and gel electrophoresis

The reactions consisted of initial denaturation at 94°C for 5 minutes, followed by cycling of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and elongation at 72°C for 2 min for 35 cycles and then the final extension of 72°C for 5 minutes. The PCR products were separated on a 1.5% agarose gel with 0.5 TBE running buffer [16].

Statistical Analysis

Statistical analysis was performed by using SPSS V. 20.

Results

Eighty six patients were randomly recruited; they were 56 (65. 12%) males and 30 (34.88%) females from Al-Najaf province, (p <0.005).The age of the patients ranged from 10to 53 years (Mean: 30.24, Std. Deviation: 11.392). Three isolates (75%) of *P. mirabilis* were isolated from non-smoker patients (75%). There are high discrepancy (p<0.00) in the rates of isolation of bacterial genera from various clinical specimens under study. *P. aeruginosa* occupies the highest percentages 33.7% followed by 31.4% for *E. coli*, while the lowest percentage was 3.5% for both undiagnosed microorganisms and *Proteus mirabilis* respectively. Other bacterial genera occupied different ratios there were 22.1%, for *P. vulgaris* and 5.8% for *K. pneumoniae* (Table 2).

Table 2: The percentage of the isolated bacterial types.

Isolated bacteria Spp.	No.	%
E. coli	27	31.4
Klebsiella pneumoniae	5	5.8
Pseudomonas aeruginosa	29	33.7
Proteus vulgaris	19	22.1
Proteus mirabilis	3	3.5
Other microorganisms	3	3.5
Total	**86**	**100.0**

The results in figure one demonstrated that all *P. mirabilis* were isolated from urine samples in this study, in comparison with other infections.

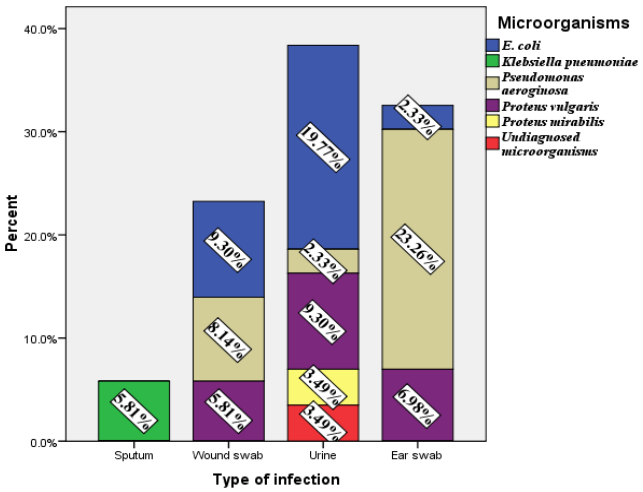

Figure 1: Distribution of the isolated microorganisms according to type of infections.

There were clear differences among the antimicrobial activity, Augmentin (AUG) occupied the highest value of effectiveness 80 (93%) against the bacterial strains under test in comparison with other antimicrobial agents while the lowest value was 36 (41.9%) for Ceftazidime (CAZ) ($p< 0.000$). Other antimicrobial agents' takes up different values, there were 63 (73.3%), 56 (65.1%), 50 (58.1%), 44 (51.2%), 44 (51.2%), 39 (45.3%), and 38 (44.2%) for Amikacin (AK), Erythromycin (ERY), Gentamicin (GM), Ampicillin (AMP), Azithromycin (AZT), Cephalexin (KF), and Ciprofloxacin (CIP) respectively. Only three isolates of six *Proteus mirabilis* were resistant to erythromycin (50%), whereas Azithromycin resistant do not detected in this study by the same bacterium(Table 3).

Table 3: Antimicrobial Susceptibility patterns against the bacterial isolates under test (the missing values were 3 (3.5%)

Antimicrobial agent	Sensitive* No. / (%)	Resistant (No. / %)	Intermediate Sensitivity (No. /%)	Total (No. /%)
Ampicillin (AMP)	44/(51.2%)	29/(33.7%)	10/(11.6%)	83/(96.5%)
Augmentin (AUG)	80 /(93%)	0	3/(3.5%)	83/(96.5%)
Gentamicin (GM)	50/(58.1%)	26/(30.2%)	7/(8.1%)	83/(96.5%)
Cephalexin (KF)	39/(45.3%)	37/(43%)	7/(8.1%)	83/(96.5%)
Amikacin (AK)	63/(73.3%)	16/(18.6%)	4/(4.7%)	83/(96.5%)
Ceftazidime (CAZ)	36/(41.9%)	42/(48.8%)	5/(5.8%)	83/(96.5%)
Ciprofloxacin (CIP)	38/(44.2%)	43/(50%)	2/(2.3%)	83/(96.5%)
Azithromycin (AZT)	44/(51.2%)	35/(40.7%)	4/(4.7%)	83/(96.5%)
Erythromycin (ERY)	56/(65.1%)	14/(16.3%)	13/(15.1%)	83/(96.5%)

Genetic results, there is only one strain carry the 402 *mef*A gene (lane 7) among the three *P. mirabilis* genera previously reported, as macrolides resistant (phenotypically) i.e. 3 isolates were erythromycin resistant. On the other hand, all *Proteus mirabilis* isolates (6 isolates) were sensitive to azithromycin (Figure 2).

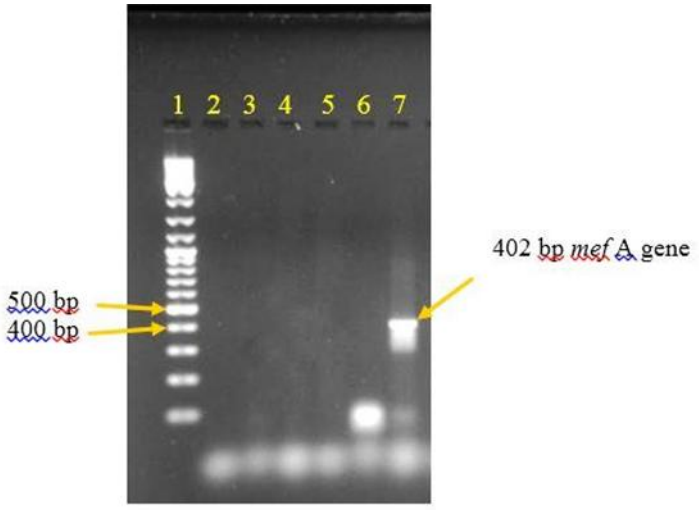

Figure 2: PCR amplification products of *mef* A gene 402-bp. (1: 100-bp ladder marker, 1, Lane 2, 3, 4, 5, 6 negative results, Lane 7 positive results).

Discussion

This study deals with the infections of different bacterial genera (i.e. *E. coli, K. pneumoniae, P. aeruginosa, Proteus vulgaris,* and *Proteus mirabilis*) among patients; identification of them and founding of gene that is responsible for the resistance of bacteria against different antibiotics. The infection by *P. aeruginosa* occupies the highest percentage in comparison with other bacteria i.e. *K. pneumoniae, P. mirabilis,* and other microorganism, also the isolated microorganism from urine samples more than another samples like (ear swabs, wound swabs and sputum) respectively. These results agreed with Kiffer [21] *et al.,* who observed that the role of different microorganism especially (*P. mirabilis*) as an important urinary pathogen in-group of patients and it is evinces the high isolation rates in this group of patients in taken urine sample. *P. mirabilis* has a diverse mode of transmission, and hence can cause infection in different anatomical sites of the body. It is one of the most common gram-negative pathogens encountered in clinical specimens and can cause a variety of community or hospital-acquired illnesses, including urinary tract, wound, and bloodstream infections (BSI) [26, 27].

The difference among male and female groups were clearly observed in this study. The results demonstrate superiority of male in comparison with female, these differences due to different pharmacokinetics and pharmacodynamics, more likely to report adverse events than among of them, or take more medications than another, also may be due to cultural or social variation, and other biological processes [22]. Infection with different bacteria in non-smoker group of patients more than other groups (X- smokers and Smokers) that may be due to the collected samples, conditions of sample collecting and depending upon immunity of these non-smoker patients. This study is disagreement with noticing of Juhi *et. al.,* that confirm the evidence associating to tobacco smoke exposure with bacterial infections, and to introduce interactions between tobacco smoke, bacteria, and the immune system that may help explain the increased susceptibility to infectious disease in smokers.

The result of this study demonstrate that there are relationship between age and the infection with different bacterial genera, patients about (30-39) years have larger average more than (50-59) years may be due to the young individual patients more exposure than another and careless with themselves especially in case of cleaning. This can support the role of these microorganism as an important pathogen in this group of patients, moreover seems to represent an important pathogen in young patients as supported by the high isolation rates in this group of patients observed in this study and by Kiffer *et. al.*. *In vitro* antibacterial sensitivity tests carried out according to Kirby-Bauer technique.

The results showed that the susceptibility of *P. mirabilis* to augmentin® (amoxicillin/clavulanic acid) occupied a highest value [80 (93%)] of effectiveness. Amoxicillin is belong to β-lactam antibiotic, bactericidal by mechanism of cell wall synthesis inhibitor and extended spectrum penicillin [17]. In comparison with other antibiotics (Amikacin, Erythromycin, Gentamicin, Ampicillin, Azithromycin, Cephalexin, Ciprofloxacin, and Ceftazidime), respectively according to effectiveness value. The results of this study is disagreement with observing of [19] that is found susceptibility of *E. coli* to amoxicillin/clavulanate was higher (77.5%). Now microorganisms have become resistant to many antibiotics due to the misusing of drugs, which is decreasing efficiency of conventional medicine [20]. An antibiotic (Ceftazidime) has more resistance than other antibiotics by different microorganisms, this resistance may be due any way of different mechanisms in microorganism production of enzymes that inactivate the drug (e.g. β - lactamase, which inactivates beta lactam antibiotics, or alteration of the drug-binding

site: this occurs with penicillin's, Reduction of drug uptake by the bacterium [23]. So that, antibiotic resistance is a growing problem, which may delay or reduce effective treatment, and high exposure to antibiotics, is considered a major cause of antibiotic resistance [18].

There is only one strain carry the 402 *mef*A gene among the three *P. mirabilis* genera previously reported, as erythromycin resistant (phenotypically). This result may be due to the using of one type of macrolides resistant genes, in which there are more than one type of macrolides resistant mechanisms reported previously include two esterase genes [*ere*(A) and *ere* (B)] found in *E. coli* and *Enterobacter*, *Klebsiella*, *Citrobacter*, and *Proteus* species. Three phosphorylase genes [*mph* (A), *mph* (B), and *mph* (D)] found in *E. coli* and *Pseudomonas*, and one rRNAmethylase gene [*erm*(B)] previously found in *E. coli* and *Actinobacillus*, *Klebsiella*, *Neisseria*, and *Wolinella* species [25].

Conclusion

Clearly few studies have examined erythromycin and azithromycin resistant of *Proteus mirabilis* in Al-Najaf province, Iraq, especially by using PCR technique. Deduce from the above that the resistance gene has been diagnosed for the first time and this is alarming for the development of resistance to macrolides group of antibiotics.

References

1. Giammanco, G., Pignato, S. and Agodi, A.. A simple chromogenic test for rapid screening of Proteus and Providencia bacteria. Microbiologica, 1985;8: 395–397.
2. Pelczar, M.J.JR., Chan, E.C.S., Krieg, N.R. Microbiology. Fifth ed. Tata McGraw Hill, India. 1986.
3. Cosenza, B.J. and Podgwaite, J. D. A new species of Proteus isolated from larvae of the gypsy moth *Porthetria dispar* (L). Antonie van Leeuwenhoek. 1966. 32 (1): 187–191.
4. Hickman, F.W., Steigerwalt, A.G., et al. Identification of *Proteus penneri* sp. nov., Formerly Known As *Proteus vulgaris* Indole Negative or As *Proteus vulgaris* Bio-group 1. J Clin Microbiol. 1982; 15 (6): 1097–1102.
5. O'Hara CM, Brenner FW, Steigerwalt AG, Hill BC, Holmes B, Grimont PA, Hawkey PM, Penner JL, Miller JM, Brenner DJ.. Classification of *Proteus vulgaris* bio-group 3 with recognition of *Proteus hauseri* sp. nov., nom. rev. and unnamed Proteus genomospecies 4, 5 and 6. International J Systematic Evol Microbiol. 2000; 50: 1869–1875.
6. Mobley H.L.T., Belas R.. Swarming and pathogenicity of *Proteus mirabilis* in urinary tract. Trends Microbiology. 1995;3:280-284.
7. O'Hara C.M., Brenner F.W., Miller J.M. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. Clin Microbiol Rev. 2000; 13:534-546.
8. Gutmann L. Cross-resistance to nalidixic acid, trimethoprim and chloramphenicol associated with alteration in the outer membrane of *Klebseilla*, *Enterobacter* and *Serratia*. J Infect Dis., 1985; 154: 501-507.
9. Yah SC, Eghafona NO, Oranusi S, Abouo AM. Widespread plasmid resistance genes among Proteus Species in diabetic wounds of patients in the Ahmadu Bello university teaching hospital (ABUTH) Zaria. Afr J Biotechnol 2007; 6(15):1757-1762.
10. Kim, YH., CJ. Cha, CE. Cerniglia. Purification and characterization of an erythromycin esterase from an erythromycin-resistant *Pseudomonas* sp. FEMS Microbiol. Lett.2002.210:239–244.
11. Putman, M., H. W. van Veen, and W. N. Konings. Molecular properties of bacterial multidrug transporters. Antimicrobial Agents Chemotherapy.2000.64:672– 693.
12. Thungapathra, M., Amita, K. K. Sinha, S. R. Chaudhuri, P. Garg, T. Ramamurthy, G. B. Nair, and A. Ghosh. Occurrence of antibiotic resistance gene cassettes *aac*(*6*)-*Ib*, *dfrA5*, *dfrA12*, and *ereA2* in class I integrons in non-O1, non-O139 *Vibrio cholerae* strains in India. Antimicrob Agents Chemother., 2002.46:2948–2955.

13. Alekshun, MN, Levy SB. Molecular Mechanisms of Antibacterial Multidrug Resistance "Leading Edge Review. Cell. 2007; 128: 1037-1050.
14. Roberts, MC., J. Sutcliffe P. Courvalin LB. Jensen J. Rood, H. Seppala. Nomenclature for macrolide and macrolide lincosamide streptogramin B resistance determinants. Antimicrob Agents Chemother 1999; 43: 2823-2830.
15. Tait-Kamradt A, Clancy J, Cronan M, Dib-Hajj F, Wondrack L, Yuan W, Sutcliffe J. *mef E* is necessary for the erythromycin-resistant M phenotype in *Streptococcus pneumoniae*. Antimicrob Agents Chemother., 199;. 41: 2251–2255.
16. Nagai, K., Shibasaki, Y., Hasegawa, K., Davies, T. A., Jacobs,M. R., Ubukata, K. and Appelbaum, P. C. Evaluation of PCR primers to screen for *Streptococcus pneumoniae* isolates and β-lactam resistance, and to detect common macrolide resistance determinants. Antimicrobial Agents Chemotherapy.2001. 48:915–918.
17. Bertram G., Susan B., Anthony J. Basic and Clinical Pharmacology.11th edition, University of California, San Francisco. 2009. 1139, 661t .pp. 1069-1070.
18. Costelloe C, Metcalfe C, Lovering A, Mant D, Hay AD. Effect of antibiotic prescribing in primary care on antimicrobial resistance in individual patients: Systematic review and meta-analysis. British Med J 2010. 340: c2096.
19. Enrico M, Vittorio G, Loredana D, Antonia L, Roberto M, Paolo R, and Clementina C. Gender and Age-Dependent Etiology of Community Acquired Urinary Tract Infections. The Scientific World Journal, 2012. Article ID349597,
20. Gerner-Smith P, Whichard JM. Foodborne Pathogens Disease trends and reports,2008;5 (2):, 111-4.
21. Kiffer, CR, Mendes, C, Oplustil, C P and Sampaio, J L. Antibiotic resistance and trend of urinary pathogens in general outpatients from a major urban city. International Brazilian Journal of Urology. 2007. 33(1): 42–48.
22. Fishman, J R, Wick, M A, and Koenig B A. The use of “sex” and “gender” to dene and characterize meaningful differences between men and women. Agenda for Research on Women’s Health for the 21st Century. 1999. 1:15–18.
23. Al-Charrakh AH, Yousif SY, Al-Janabi HS. Occurrence and detection of extended-spectrum β-lactamases in *Klebsiella* isolates in Hilla, Iraq. Afr J Biotechnol., 2011; 10 (4): 657-665.
24. Juhi B, Donald R D, and David A S. Tobacco use increases susceptibility to bacterial infection. Published online 2008 Dec 18. doi: 10.1186/1617-9625-4-12;
25. Ojo K K,Ulep, C, Van Kirk N, Luis, H. Bernardo, M.Leitao, J. and Roberts, M. C. The *mef*(A) Gene Predominates among Seven Macrolide Resistance Genes Identified in Gram-Negative Strains Representing 13 Genera, Isolated from Healthy Portuguese Children. Antimicrob agents Chemother 2004; 48 (9): 3451–3456.
26. O’Hara, C M, Brenner F W, and Miller J M. Classification, identification, and clinical significance of *Proteus*, *Providencia*, and *Morganella*. Clin. Microbiol. 2000. 13:534–546.
27. Pandey J K, Narayan A, and Tyagi S. Prevalence of Proteus species in clinical samples, antibiotic sensitivity pattern and ESBL production. Int J Current Microbiol Appl Sci, 2013; 2 (10):253-261.
28. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disk method. American J Clin Pathol, 1966; 45:493-6.