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RESEARCH ARTICLE

DETECTION OF PLASMID-MEDIATED QUINOLONE RESISTANCE (PMQR) GENESIN TRIBE *PROTEEAE* ISOLATED FROM BACTERIURIA IN NAJAF HOSPITALS, IRAQ

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ABSTRACT

Tribe *Proteeae* is one of the most common causes of UTI. Plasmid-mediated quinolone resistance (PMQR), which complicates treatment, has been increasingly identified in this tribe worldwide. The purpose of this study was to identify the frequency of PMQR genes among *Proteeae* isolates. Five PMQR genes [*qnrA, qnrB, qnr S, qnrD,* and *aac(6')-Ib-cr*] were screened bymonoplex PCR. Of the 1132 patients with significant bacteriuria, tribe *Proteeae* accounted for 75 (6.6%) of the urinary isolates, 72 were identified as *Proteus mirabilis*, while one as each *P. vulgaris, P. penneri,*and *Morganellamorganii*. Forty-two exhibited decreased susceptibility or resistance to nalidixic acid and/or ciprofloxacin. The antibiotic susceptibility testing revealed a high frequency of the resistance to the majority of antibiotics tested. It was found that 14.3%, 9.5%, and 7.1% of the isolates harbored *aac(6')-Ib-cr*, *qnr D* and *qnrA*, respectively either alone or in combination. This study constitutes the first *qnr D* determinants in Iraq.

INTRODUCTION

Tribe *Proteeae* belongs to the family *Enterobacteriaceae*. It includes three genera, which are *Proteus*, *Morganella*, and *Providencia*. They are opportunistic pathogens causing numerous kinds of infections with the domination of UTIs (Pal *et al*., 2014). In Iraq;*P. mirabilis* constitutes the third most commonly isolated pathogen of UTIs after *E. coli* and *K. pneumoniae* (Fayroz-Ali, 2012; Al-Mayahi, 2013). However, MDR in *Proteeae* isolates including resistance to quinolones is rising, causing a growing public health problem worldwide (Pal *et al*., 2014). Resistance to quinolones among the *Enterobacteriaceae* frequently results from mutation of chromosomal genes encoding topoisomerase IV, DNA gyrase, regulatory efflux pumps, and/or porins-related proteins (Redgrave *et al*., 2014). Three types of PMQR mechanisms have been designated to date: protection of the quinolone targets mediated by proteins encoded by the *qnr* genes; acetylation of ciprofloxacin and norfloxacin by the functional enzyme *aac(6')-Ib-cr*; and efflux pumps that (Rodriguez-Martinez *et al*., 2013; Kim *et al*., 2014).Five classes are currently known (*qnrA, qnrB, qnr C, qnr D*, and *qnr S*) (Kulková *et al*., 2014; Alheib *et al*., 2015).

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The *aac(6')-Ib-cr* (cr for ciprofloxacin resistance) is a variant of *aac(6')-Ib*(responsible for resistance to kanamycin, tobramycin, and amikacin) with two amino acid substitutions compared to the wild-type allowing it to acetylate and subsequently reduce the activity of norfloxacin and ciprofloxacin(Rodriguez-Martinez *et al*., 2016; Yanat *et al*., 2017). In Najaf, there have been no studies published on the prevalence of quinolones resistance *Proteeae* isolates and its association with PMQR genes. The aim of this study was to investigate the occurrence and diversity of PMQR among *Proteeae* isolates recovered from patients with significant bacteriuria.

METHODS

Collection of Specimens: A cross-sectional study was conducted in patients clinically suspected to have UTI during the period of six months from October 2016 to March 2017. The inclusion criteria for the study participants were all patients attended or admitted in the three major hospitals in Najaf.

Identification of *Proteeae* Isolates: The pure cultures of respective uropathogens were further subjected to species identification and confirmation. Identification of the causative organisms was based on Gram reaction, morphology, and conventional biochemical characteristics.

In addition, isolates were also confirmatory identified by using commercially available API 20 E kit and VITEK-2 compact system (BioMerieux/France).

Antimicrobial Susceptibility Testing: The antibiotic susceptibility tests were performed by Kirby-Bauer disc diffusion method on Mueller-Hinton agar. The following antibiotic discs tested were ampicillin (10 µg), amoxicillin(25 µg),piperacillin (25 µg), amoxicillin/ clavulanic acid (20/10 µg),piperacillin-tazobactam(100/10 µg), ticarcillin-clavulanic acid(75/10 µg),cefixime (5 µg), cefotaxime(30 µg), ceftazidime(30 µg), ceftriaxone(30 µg), cefoxitin(30 µg), aztreonam(30 µg), imipenem (10 µg), meropenem(10 µg), amikacin (30 µg),gentamicin (10 µg), netilmicin(30 µg), tobramycin (10 µg), nalidixic acid (30 µg), ciprofloxacin(5 µg), levofloxacin (5 µg), norfloxacin(10 µg) , ofloxacin (5 µg), moxifloxacin (5 µg), lomefloxacin(10 µg), gatifloxacin (5 µg), trimethoprim (5 µg), trimethoprim-sulfamethoxazole(25 µg) and nitrofurantoin (300 µg) (Mast Diagnostic, UK). The results wereinterpreted as sensitive, intermediate or resistant according to the guidelines of Clinical and Laboratory Standards Institute (CLSI, 2016).*E. coli* ATCC 25922 was used as thequality control for antimicrobial susceptibility tests.

Screening Test for Quinolones Resistance: According to CLSI (2016) recommendations, disc diffusion test was performed to detected quinolone resistance in tribe *Proteeae*isolates by using nalidixic acid (30 µg/disc) and ciprofloxacin (5 µg/disc).

Screening for the PMQR Genes: Genomic DNA extraction was performed for all isolated members of the tribe *Proteeae* using a Wizard Genomic DNA Preparation kit (Promega, USA) according to manufactures' instructions. Monoplex polymerase chain reaction (PCR) assay was performed to determine the presence of the *qnrA*, *qnrB*, *qnrS*, *qnrD*, and*aac(6`)-Ib-cr*, differentiated from the original *aac(6`)-Ib*gene by PCR digestion with *BstCI,* Genes in the PMQR producing isolates using thermocycler (T3000) and Taq Green Polymerase 2X Master Mix (Promega, USA). The sequences of primers targeting PMQR genes were published elsewhere are shown in Table 1.PCR conditions were as follows: an initial denaturationstep of2 min at 95 ˚C; 30 cycles of95 ˚C for 30 s, annealing at58.3˚C (*qnrB*), 56.2˚C (*qnrS* and *qnrD*) and extension at 72 ˚C for 50 sce (*qnrS* and *qnrD*),72 ˚C for 70 sce (*qnrB*) and a final extension step at72 ˚C for 5 min.*QnrA* 94˚C/5 min;32 cycles of 94/45 sec 53/45 sec 72/60 sec,and a final extension at72/10 min.*Aac(6′)-Ib-cr* 94˚C /4 min; 34 cycles of94/45 sec 55/45 sec 72/45 sec,and a final extension at 72/5 min. The amplifiedPCR products were resolved by electrophoresisin1.5 % agarose gel and visualized after staining withethidium bromide.

RESULTS

According to cultural characteristics and biochemical reactions, out of 1132 collected isolates of bacteria, 75 non-duplicate isolates were identified as tribe*Proteeae*, and this gave an occurrence rate of 6.6%. The present study revealed that *Proteus* spp. had the fourth (74, 6.5%) frequency ofoccurrence among the bacteria isolated from patients with significant bacteriuria.

Subsequent identification exhibited that 72 (96.0%) isolates were recognized as *P. mirabilis* and one (1.3%) isolate was identified as *P. vulgaris*. Additionally, one (1.3%) *P. penneri* isolate. Likewise, the present study investigated the presence of *M. morganii* in one (0.1%) urine specimens. The rate of this isolate in tribe*Proteeae* was (1.3%), (Figure 1).

Screening Test for Quinolones Resistance: Overall, 56.0% (42/75) of the isolates were nonsusceptible (resistant plus intermediate) to at least one of the two quinolones tested. Moreover, the present study found that 44.0% and 22.7% of urinary isolates were resistant to nalidixic acid and ciprofloxacin, respectively.

Antibiotic Susceptibility of Tribe *Proteeae* Isolates: The result of the antibiotic susceptibility testing of isolates to 29antibiotics, (belonging to twelve categories) revealeda high frequency ofthe resistance to the majority ofantibiotics tested (figure 2). By the present definition ofMDR, 30 (71.4%) ofthe isolates were classified as MDR, 4 (9.5%), 1 (2.4%), 1 (2.4%), 3 (7.1%), 8 (19.1), 4 (9.5) and 9 (21.4%) ofthem were resistant to 3, 4, 5, 6, 7, 8 and 9 antibiotic categories, respectively. Surprisingly, 12 (28.6%) isolates were resistant to more than or equal to ten categories of antibiotics meeting criteria for XDR isolates, of which 3 (7.1%) were resistant to ten antibiotic categories, and 9 (21.4%) resistant to eleven antibiotic categories (Table 2). PMQR determinants were found in 11 isolates (26.2%).The distribution of each PMQR gene is shown in Table (3).PCR revealed that the *qnr*-encoding genes were present in 7 (16.7%) ofthe quinolone non-susceptible isolates; among them, *qnrD* was the most commonly detected gene (4, 9.5%) (Figure 3), followed by *qnrA* (3, 7.1%) (Figure 4) either alone or in combination with*aac(6′)-Ib-cr* gene(Figure 5, A,B). Neither *qnrB* nor*qnrS* genes were found in any of the tested isolates.

DISCUSSION

Frequency of Tribe *Proteeae:* Tribe *Proteeae* was isolated from 75(6.6%) of the patients with significant bacteriuria. However, this study clearly showed that *Proteus* spp was the most commonly isolated organisms amongst the tribe *Proteeae* (6.5%). This result agrees with a similar study conducted in Belgium 6.1% (Smelov *et al*., 2016), Nigeria 6.8% (Otajevwo and Eriagbor, 2014) and India 8.9% (Pal *et al*., 2016). Three *Proteus* spp (*P. mirabilis*, *P. vulgaris*and *P. penneri*) were identified to be responsible for causing significant bacteriuria. *P. mirabilis* was the most common species isolated, accounting for 96.0 % and hence responsible for the majority of *Proteus* UTIs, and this supported the finding that *P. vulgaris*and *P. penneri*infections of the urinary tract are rare (Patrick *et al.,* 2010). *P. penneri*(formerly *P. vulgaris*bio-group 1) was recognized as a new species in 1982 but its role in clinical infection remains cryptic (Cantón *et al.,* 2006).In research from India, among all *Proteus* sppositive clinical specimens cultures from hospitalized patients an occurrence of 7.9% for *P. penneri*was reported (Pal *et al.,* 2014). The urinary tract is the major portal for *M. morganii* entry, followed by the hepato-biliary tract, skin and soft tissue, and blood (Sakaguchi*et al*., 2014).Based on the findings of the research study, significant bacteriuria frequency of *M. morganii*was found to be very few (1.3%), (Shatalovand Aleksey, 2015; Ekrem *et al.,* 2015).

Table (1): Primers used in this study (Alpha DNA) Korea

Gene name	Primer name	Primer sequence (5′-3′)	Product (bp)	Reference
qnrA	qnrA -F	ATTTCTCACGCCAGGATTTG	516	
	qnrA -R	GATCGGCAAAGGTTAGGTCA		Cattoir *et al.*, 2007
qnrB	qnrB -F	GATCGTGAAAGCCAGAAAGG	469	
	qnrB- R	ACGATGCCTGGTAGTTGTCC		
qnrS	qnrS -F	ACGACATTCGTCAACTGCAA	417	
	qnrS- R	TAAATTGGCACCCTGTAGGC		
qnrD	qnrD-F	CGAGATCAATTTACGGGGAATA	644	Cavaco *et al*., 2009
	qnrD-R	AACAAGCTGAAGCGCCTG		
aac(6′)-Ib-cr	aac(6′)-Ib-cr-F	TTGCGATGCTCTATGAGTGGCTA	482	Park *et al.*, 2006
	aac(6′)-Ib-cr-R	CTCGAATGCCTGGCGTGTTT		

Table (2): Distribution of MDR and XDR among (42) tribe *Proteeae* isolates had display reduced susceptibility to nalidixic acid and/or ciprofloxacin

Type of resistance	No. of isolates	No. of resistance to antibiotic categories (n=12)
MDR*(n=30, 71.4%)	4	3
	1	4
	1	5
	3	6
	8	7
	4	8
	9	9
XDR*(n=12, 28.6%)	3	10
	9	11
PDR*(n=0)	0	-

(*) MDR: multidrug resistance; XDR: extensive drug resistance; PDR: pan drug-resistant.

Table (3): Distribution of PMQR genes and their combinations among the 42 isolates of tribe *Proteeae* presented a reduced susceptibility to quinolones

Type of PMQR gene	No. (%) of isolates	No. of *Proteeae* carried PMQR gene (n= 42)	
		P. mirabilis	*P. penneri*
qnrA	2 (4.8)	2	-
qnrD	3 (7.1)	2	1
aac(6′)-Ib-cr	4 (9.5)	4	-
aac(6′)-Ib-cr and qnrD	1 (2.4)	1	-
aac(6′)-Ib-cr and qnrA	1 (2.4)	1	-
qnrB	0 (0)	-	-
qnrS	0 (0)	-	-
Total (%)	11 (26.2)	10 (23.8)	1 (2.4)

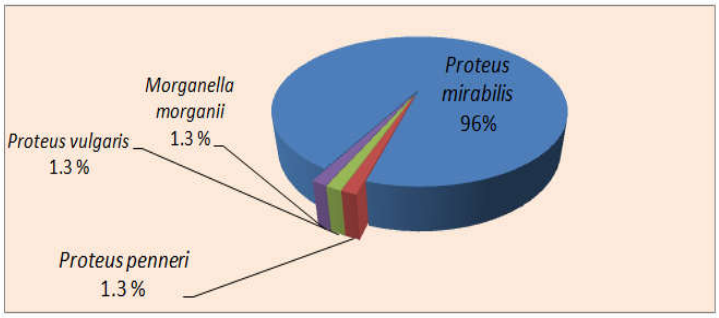

Figure (1): Frequency of tribe *Proteeae* isolated from patients with significant bacteriuria.

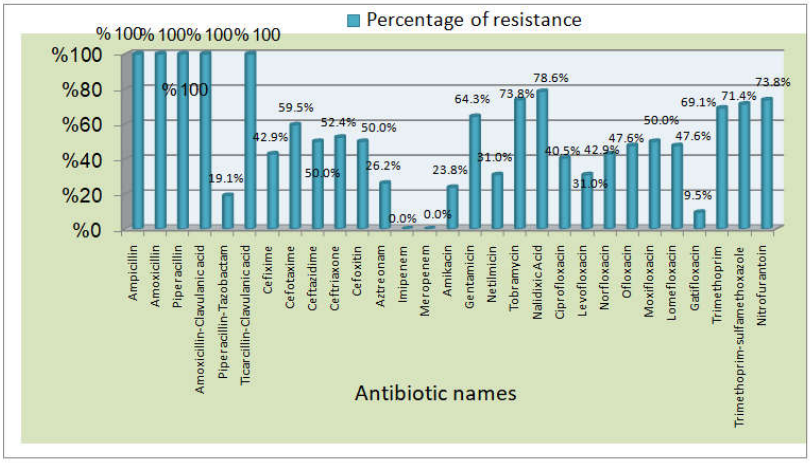

Figure (2). Antibiotics susceptibility profile of 42 isolates of quinolone resistanttribe *Proteeae*

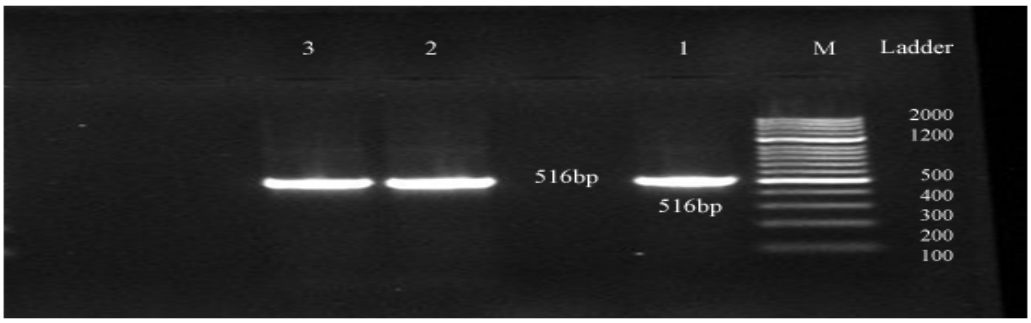

Figure 4. Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *Proteus* isolates and amplified with *qnrA*genes primers. The electrophoresis was performed at 70 volts for 1.5 hr. Lane (M), DNA molecular size marker (100 bp ladder), Lanes (1, 2 and 3) show positive results with *qnrA*(516 bp)

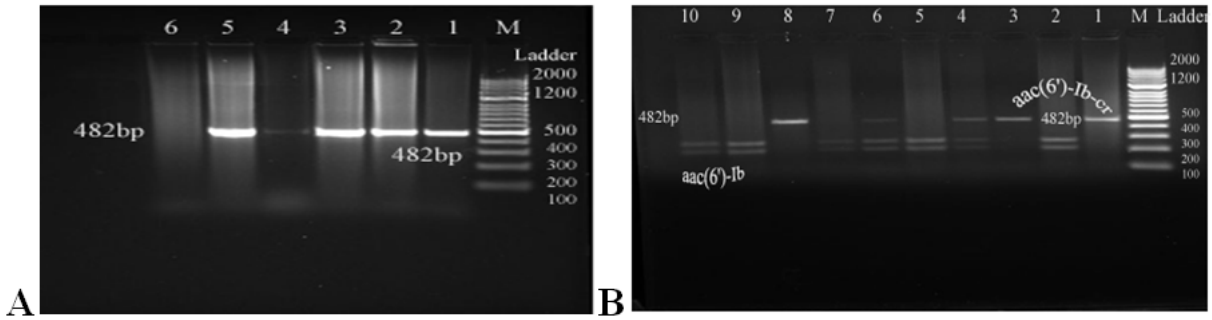

Figure (5a): Ethidium bromide-stained agarose gel of monoplex PCR amplified products from extracted DNA of *Proteus*spp isolates and amplified with *aac(6')-Ib* gene primers. The electrophoresis was performed at 70 volts for 1.15 hr. Lane (M), DNA molecular size marker (100 bp ladder), Lanes(1, 2, 3, and 5) show positive results with *aac(6')-Ib*(482 bp). Figure (5b): Ethidium bromide-stained agarose gel of PCR amplified products from extracted DNA of *Proteus*spp isolates and amplified with *aac(6')-Ib*positive genes after digested with *BstCI*. The electrophoresis was performed at 70 volts for 1.30 hr. Lane (M), DNA molecular size marker (100 bp ladder), Lanes (1, 3, 8) show positive results with *aac(6')-Ib-cr*, Lane (2, 5,7,9,10) *aac(6')-Ib* wild-type genes, Lane (4,6) partial digest with *BstCI*

In this research, the resistance rates to nalidixic acid and ciprofloxacin were 44.0% and 22.7% respectively, indicating a moderate rate of resistance to quinolone and fluoroquinolones by *Proteeae* isolates associated with significant bacteriuria in Najaf. Since the development of nalidixic acid in 1962, this antibiotic has been used for the treatment of UTIs for more than five decades (Aldred *et al*., 2014; Bisacchi, 2015). The resistance rate for nalidixic acid, therefore, was expected to be higher than for fluoroquinolones. Ciprofloxacin is often used for the treatment ofUTIs in Iraq. The decreased susceptibility to ciprofloxacin during the study period might be due to the inappropriate use and increased consumption of fluoroquinolones in Iraq in recent years. Other studies in most parts of the world have indicated that fluoroquinolones resistance in *Proteus* spp isolates is increasing (Cunha *et al*., 2016; Pal *et al*., 2016).

Antibiotic Resistance Profiles: In this study, tribe *Proteeae*was further classified into MDR, XDR and PDR based on Magiorakos *et al*., (2012) criteria, MDR was found in 71.4% of isolates. Infections by MDR *Proteus* spp has increased worldwide in the past few years, due to its rapid acquisition and dissemination of a wide variety of antibiotic resistance genes, as well as other members of the *Enterobacteriaceae* family (Wang *et al.,* 2014; Yang *et al.,* 2015). The appearance ofthis high percentage of MDR in this study is a major problem and must have to follow wise antibiotic policy to overcome those resistant organisms. However, several studies reported that significantly higher mortality rates in patients with MDR *P. mirabilis* bacteremia (Korytny *et al.,* 2016; Perween *et al*., 2016). To the best of study knowledge, this is the first study investigating the frequency of XDR *Proteeae* in Najaf. Out of 42 tribe *Proteeae* isolates, 12 (28.6%) exhibited XDR. These discoveries are alarming because infections with these XDR isolates maybe leave clinicians with no treatment options, leading to increased morbidity and mortality. The growing resistance of tribe *Proteeae* isolates and other Gram-negative organisms and the emergence of XDR isolates need to be restricted in Najafhospitals.

Frequency of PMQR Genes: Numerous studies have emphasized that, in recent years, resistance to fluoroquinolones has increased globally, particularly in members of the *Enterobacteriaceae* (Redgrave *et al*., 2014; Hariharan *et al*., 2015). Some of the national studies found that the fluoroquinolones resistance rates in *E. coli* and *K. pneumoniae* were high (Fayroz-Ali, 2012; Hadi, 2015). Nevertheless, very few reports regarding the prevalence ofPMQR are available in Iraq (Fayroz-Ali, 2012; Hadi, 2015). The present investigation did not find any reports on the presence of PMQR genes in tribe*Proteeae* isolates from Najaf. The current study identified, for the first time, the frequency of the*qnr A, qnrB, qnrS, qnrD, aac(6')-Ib-cr* variant among 42 uropathogens *Proteeae* isolates had display reduced susceptibility to quinolones in Najaf. In this cross-sectional study, it should be noted that the frequency of PMQR genes among urinary *Proteeae* isolates was 26.2% and maybe represents a potential reservoir for the spread of these genes in Najaf hospitals and community. Previously,

PMQR genes have been reported in some *Enterobacteriaceae* in Najaf. In these reports, PMQR genes were detected in 63.5% of *K. pneumoniae* isolates exhibited reduce susceptibility to quinolones (Hadi, 2015) and in 76.9% of the MDR uropathogenic *E. coli* (Al-Hilali, 2015). In contrast to national studies, most surveys report a higher occurrence of PMQR genes in *K. pneumoniae* compared to *E. coli* (Briales*et al*., 2012; Yang *et al*., 2015). In this study, the detection of *qnrA* associated with the reduced susceptibility to quinolones showed that this gene is disseminated among 7.1% of *Proteeae* isolates in Najaf. This result agrees with the most previous studies indicating that *qnrA*, although representing the *qnr* determinant identified initially, is not the most prevalent *qnr* determinant worldwide (Robicsek *et al.,* 2006; Minarini *et al.,* 2008). In a similar study in Najaf, *qnrA* was recognized in 2.7% of *K. pneumoniae* clinical isolates (Hadi, 2015). The present study investigated the emergence of the new PMQR gene, *qnrD* in four (9.5%) of urinary isolates of *Proteeae* (3 *P. mirabilis* and 1 *P. penneri*). The *qnrD* gene has never been found in other prevalence studies on PMQR genes from Najaf that addressed family *Enterobacteriaceae* not belonging to the tribe *Proteeae*, such as *K. pneumoniae* (Hadi, 2015; Al-Hilali, 2015). This fact strengthens the notion that the species belonging to the tribe *Proteeae* might constitute a natural reservoir of this gene (Zhang *et al.,* 2013). To the best of study knowledge, this is the first report of present *qnrD* in Iraq. In this study, of the 42 *Proteeae* isolates selected for detection of *aac(6')-Ib-cr* variant, 20 (47.6%) were positive for *aac(6')-Ib*. However, the amplicons of 6 (14.3%) were not susceptible to restriction digestion by *Bts*CI, implying that these isolates possessed the variant *aac(6')-Ib-cr*. This gene has been reported mostly from *K. pneumoniae* clinical isolates in Najaf (Hadi, 2015). Current investigation and the above studies found that the *aac(6')-Ib-cr* gene appears to be more predominant than any other *qnr* genes. These findings also are inconsistent with the results of previous studies, which indicated that *aac(6')-Ib-cr* gene was the most widespread PMQR (Yang *et al*.,2015).It is worth mentioning that *aac(6')-Ib-cr* variant was detected in one *P. mirabilis* isolate carrying the*qnrA* gene and in one *P. mirabilis* isolate carrying the *qnrD* gene (Literak *et al.,* 2010).

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