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Research Article

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Characteristics of Klebsiella Pneumonia St4 Coharboring Qnrb1, Aac-Ib-Cr, CTX-M-15 and SHV 11 in A Tunisian Hospital

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Abstract

Background: *Multiresistant Klebsiella pneumonia are predominant cause of hospital acquired infection. This work describes the molecular epidemiology of these isolates in Tunisian Hospital.*

Methods: *Between October 2010 and June 2013, 50 non-duplicated clinical K.pneumoniae were selected based on nalidixic acid (NA) resistance and were characterized. Isolates were identified using APi 20E system. Susceptibility testing was determined using the disc diffusion method and the micro dilution technique to determine the MIC of ciprofloxacin.*

PMQR and ESBL genes were detected by PCR and positive results were confirmed by direct sequencing of PCR products. Multilocus sequence typing (MLST) was performed to determine the genetic relationship among isolates. Conjugation and transformation were done to know if PMQR and ESBL were carried with one or two plasmids.

Results: *20 PMQR harboring K.pneumoniae representing 40% of all NA resistant isolates were characterized. Among PMQR positive K.pneumoniae 13 were resistant to amoxicillin, amoxicillin/clavulanic acid, ticarcilline, piperacillin, cefaloridine, cefotaxime (CTX) and ceftazidime. The rate of resistance to gentamicin, tobramycin and amikacin were 85%, 95% and 25% respectively.*

Out of 20 K.pneumoniae (60%) were qnr positive (1 qnrA6 and 11 qnrB1) and (60%) were aac-Ib-cr positive. 33.3% harbored the aac-Ib-cr and qnrB1 determinants. Out of all PMQR positive strains, 65% harbored ctx-M-15 gene. It was associated to shv11 in three cases and tem1 in two cases. The predominant types were ST4 (35%) and ST15 (20%). ST 101(15%) and ST 147 (10%) come in second order. one case of each ST14,ST86,ST336 and ST307 were also observed. qnrB1, aac-Ib-cr, Ctx-m-15 can be carried with more than one plasmid in the same bacteria.

Conclusion: *The co-existing of different genes conferring resistance among the same and different family of antibiotics is a big threat to patient because it limits the therapeutic process. This phenomenon is a problem of concern that needs to improve the resistance surveillance of multi gene carrying K pneumonia.*

Keywords: PMQR, ESBL, MLST, Pneumonia, Conjugation, Transformation

Background

Pathogenic *Klebsiella pneumonia resistant* to quinolones and β-lactam drugs is widely recognized as important bacteria causing array of diseases [1]. Antibiotic sensitivity assays have revealed high frequencies of quinolone drug resistance in extended spectrum beta lactamases (ESBL) producing clinical isolates [1]. The distribution of *qnr* and ESBL alleles varies among countries. In 1998, the first plasmid-mediated mechanism of resistance to quinolones was described in multiresistant *K.pneumoniae* harbouring the *qnr*A gene on pMG252 plasmid and associated to the cephalosporin's Fox-5 [2, 3]. Several studies have found the *qnr* determinants mainly *qnrA* and *qnrB* that were more frequent in ESBL producing *Enterobacteriacae*; the genes were carried by large plasmids (40-320Kb) in Class1 integrin type sul1,*qnr S* was more associated to *tem-1* and *lap-1*[4-8].

The *aac-Ib-cr* gene was found in various integrin's, especially on IncF11 plasmid expressing *ctx-m-15* that have spread rapidly so that ctx-m-15 has become predominant ESBL in many countries around the world [9 -18].

Aac-Ib-cr has been associated with other PMQR genes including *qnrA1*[19,20,21], *qnrB1* [6], *qnrB2* [17,22], *qnrB4* [19,23], *qnrB6* [19,23], *qnrB10* [24], *qnrS1* [19,23], *qnrS2* [25] and *qepA* [23] and with other β-lactamases including, ctx-m-1 [26], ctx-m15 [6], ctx-M14 [19], CTX-M-24[19], DHA-1 [23], SHV-12[23].

Very few studies have reported on the problem of the coexisting of PMQR and ESBL in *K. pneumonia* in Africa. [4, 6]

In Tunisia, *qnrA6* and CTX-m-15 were the predominant allele among *K.pneumonia*e isolates [4, 27, and 28]. *QnrB1* coexisted with ctx-m-15, SHv-28 and Tem-1 in 125 Kb plasmid through the study of Dahmer et al in a Tunisian hospital [4].

Despite the high prevalence of these isolates in nosocomial infections, large studies to investigate the molecular epidemiology of these isolates in Africa are still lacking and the sequence type of coharboring PMQR and ESBL *K.pneumoniae* are not yet known.

We therefore conducted the first epidemiological study in Africa to determine the molecular epidemiology of PMQR and ESBL co-producing *k.pneumoniae*. This is also the first report of aac-Ib-cr gene among *K.pneumoniae* despite its huge distribution in several countries.

Methods

Sample Selection

In total, 50 non-duplicate clinical *K.pneumoniae* isolates recovered between October 2010 and June 2013 at Fattouba Bourguiba hospital (Tunisia) were selected on the basis of nalidixic acid resistance.

The K.pneumoniae isolates were identified by bio typing by using API20E system ((bioMérieux SA, Marcy-etoile, France).

Antimicrobial Susceptibility Testing

The antibiotics susceptibilities of the *K.pneumoniae* isolates were determined on Mueller-Hinton agar by the standard disk diffusion procedure as described by the Antiprogram Committee of the French Society for Microbiology (http://www.sfm.asso.fr).

The micro broth dilution technique was used to determine the MIC for ciprofloxacin.

PMQR and ESBL Screening and DNA Sequencing

The *qnrA, qnrB, and qnrS, qnrC, qnrD, aac-Ib, qepA, gyrA, Ctx-M1G, SHV and Tem* genes were detected by PCR with the primers described previously [29-37].

Positive results were confirmed by direct sequencing of PCR products.

Analysis and comparison of nucleotide and amino acid sequence data were carried out using Lasergene software ((version 7.1; DNASTAR, Wisconsin, USA) and programs available from the national Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Multi Locus Sequence Typing (MLST)

The genetic relationship among *K. pneumoniae* isolates was assessed based on the Multilocus Sequence Typing Method as described previously on the website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html). PCR for seven housekeeping genes; *rpoB, gapA, mdh, pgi, phoE, infB and tonB* was conducted and products were directly sequenced. Analysis was carried out as described on the website.

Location of PMQR and ESBL genes

Conjugation

The transferability of *qnr, aac-Ib-cr and ctx-m-15* genes between the *K.pneumoniae* isolates and the *E. coli* J53Az^r recipient were carried out in LB broth. Cultures of donor and recipient cells in logarithmic phase (0.5 ml of each) were added to 4 ml of fresh LB broth and incubated overnight without shaking. Tran's conjugants were selected on Trypticase soy agar (TSA) plates containing sodium azide (100 µg/ml; Sigma Chemical Co., St. Louis, Mo.) for counter selection and cefotaxima (10 µg/ml) to select for plasmid-encoded resistance.

To determine if quinolone and ESBL resistance were transferred, colonies were replicated onto TSA with and without ciprofloxacin (0.06 _g/ml). MICs of ciprofloxacin for the donor, recipient, and Trans conjugant strains were measured by E-test.

Transformation

For transformation reactions, Top 10 competent cells and plasmid extracted from Trans conjugants were taken to ice for 2 min. 5 to 10µl of plasmid were taken and added to 100µl of competent cells. The mixture was taken to ice for 30 min, then to 42°C for 1 min. After this it was taken to ice again for 3 min without mixing. After this step 900µl of LB broth were added to each mixture then taken to 37°C for one hour with cheeking.

100µl of transform ants were plated on 3 LB agar containing separately Amp (1µg/ml), Cip (0.06µg/ml) and Ctx (1µg/ml).

Results

Distribution of PMQR Positive Isolates

Out of 50 *K pneumonia* resistant to quinolones, 20 (40%) harbored PMQR over a period of three years. Table I characterize these isolates.

Table I: Features of PMQR Positive Klebsiella Pneumonia Isolates

Strain code	Date of isolation	Sample	Unit	PMQR	ST	MIC of CIP µg/ml	ESBL genes
154	3/12/10	Blood	Pediatric	qnr B1 aac-Ib-cr	4	128	blaCtx-m-15 blashv11
2116	18/03/11	Blood	Pediatric	qnr B1 aac-Ib-cr	4	512	blaCtx-m-15
3142	31/05/11	Urine	Nephrology	aac-Ib-cr	101	256	blaCtx-m-15
4144	27/05/11	Pus	ICU	qnr A6	147	512	-
5189	28/12/11	Blood	Pediatric	qnr B1 aac-Ib-cr	4	32	blaCtx-m-15
6208	7/04/12	Blood	Surgery	qnr B1 aac-Ib-cr	14	16	blaCtx-m-15 blashv11
7228	29/01/13	Blood	ICU	aac-Ib-cr	147	>1024	blaCtx-m-15 blaShv11
880	25/01/11	Urine	nephrology	aac-Ib-cr	15	512	blaCtx-m-15 blatem1
99	30/10/10	Pus	Pediatric	qnr B1	86	32	-
10127	09/05/11	Urine	nephrology	aac-Ib-cr	15	128	blaCtx-m-15
11113	15/03/11	Urine	nephrology	aac-Ib-cr	15	256	blaCtx-m-15
12231	30/01/13	Urine	nephrology	aac-Ib-cr	101	256	blaCtx-m-15
1387	9/02/11	T.S	ICU	aac-Ib-cr	101	>1024	blaCtx-m-15
1442	22/11/10	Urine	Nephrology	aac-Ib-cr	15	256	blaCtx-m-15
15247	19/04/13	T.S	ICU	qnr B1	336	128	-
16223	26/01/13	Urine	Cardiology	qnr B1	307	256	-
1791	24/02/11	Pus	Pediatric	qnr B1	4	128	blaCtx-m-15 blaTem1
18190	29/12/11	Urine	Pediatric	qnr B1	4	64	-
1924	16/11/10	Pus	Pediatric	qnr B1	4	64	-
20198	17/01/12	Urine	Pediatric	qnr B1	4	64	-

All of PMQR harbouring *K. pneumonia* were from inpatients. Samples were gathered from different organs and systems: urine (45%), blood (25%), pus (20%) and respiratory samples (10 %). They were collected from patients admitted in Pediatrics (40%), nephrology (30 %), intensive care units (20 %), cardiology (5 %), and surgery (5 %).

During the survey period of quinolone resistance in *K. pneumonia*, all of the strains had high MIC of ciprofloxacine (16-1024µg/mL). A higher rate of resistance to commonly used non-quinolones was observed. Among PMQR harbouring *K.pneumoniae* isolates 13 were resistant to Amoxicillin, Amoxicillin/clavulanic Acid, ticarcillin, piperacillin, cefaloridine, cefotaxim and ceftazidim. As well as, they possessed an ESBL by phenotypic testing. The rate of resistance to gentamicin, tobramycin and amikacin were 85%, 95% and 25% respectively. Resistance to trimethoprim/sulfoxides was 60%. All isolates were sensitive to imipenem using the disc diffusion method.

PMQR and ESBL Alleles

Out of 20 PMQR harboring K.pneumoniae, 60 % were found to contain *qnr* type determinants. The breakdown of the determinants

detected was as follows: 1 *qnrA6* and 11 *qnrB1*. The rate of aac *(6')-IB-cr* gene was 60 % also.

Among the aac (6')-IB-cr positive strains, there were 4 (33.3%) which were also qnr positive.

Among the twenty PMQR positive *K.pneumoniae,* 13 (65%) harbored the $bla_{CTX\text{-}M\text{-}15}$ determinant. The previous gene was associated to the bla_{SHV11} gene in three cases and coexisted with the bla_{TEM1} gene in two cases.

PMQR and Ctx-M-15 genes transferability

Conjugation

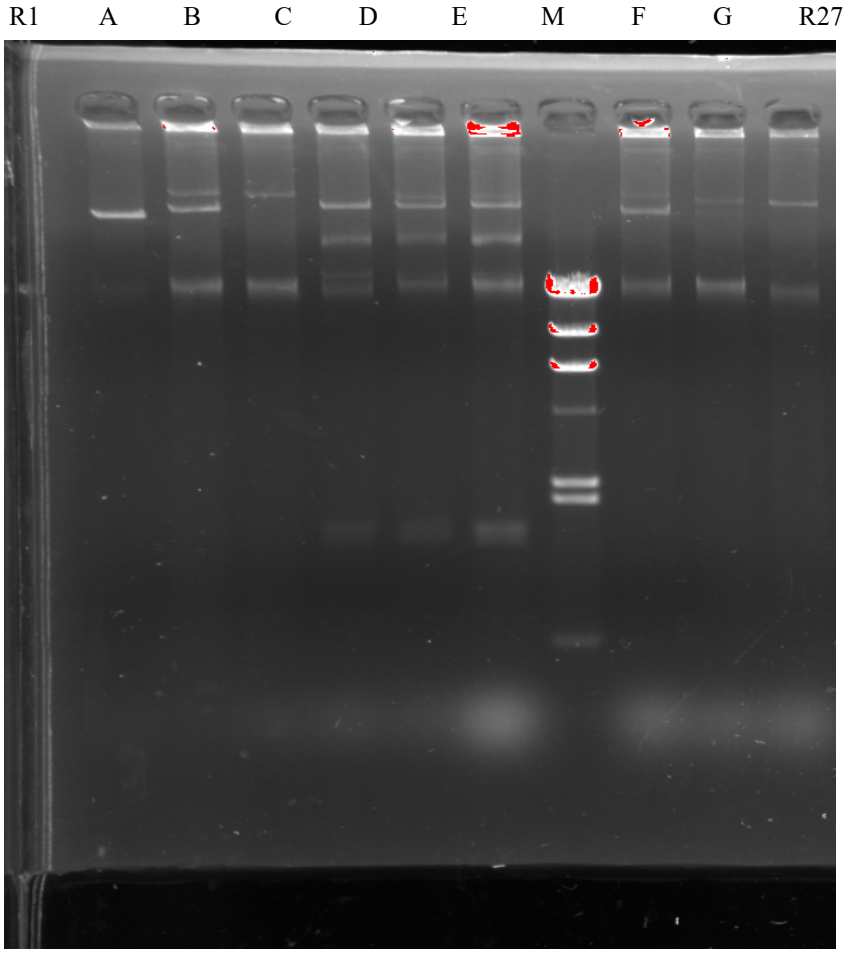

Figure 1: Plasmid Profile Extracted From Trans Conjugants

A: transconjugants 154_{c+I} of donor 154 inoculated in CTX-+azide plate. **B:** transconjugants154_{c+I} of donor 154 inoculated in CIP+azide plate. **C:** transconjugants 5189c of donor 5189 inoculated in CTX+azide. **D:** transconjugant 5189c+I of donor 5189 inoculated in ctx+azide plate. **E:** transconjugants 5189 c+I inoculated in Cip+azide plate. **F:** transconjugants 2116_{c+I} inoculated in ctx+azide plate, G: transconjugants 2116_{c+I} inoculated in ctx+azide plate.

Notes:
c+I: the same transconjugants give colonies on CTX+azide plate and on CIP +azide plate.
c: the transconjugant give colonies on CTX+azide plate only.

All the conjugation reactions between *Ecoli* J53 and the donor 154 or the donor 2116 has given transconjugants able to give colonies on CTX+azide plates and Cip+azide plates.

Genetic Relatedness

Seven ST4 *K.pneumoniae* variant were isolated in the Pediatric Unit. Four isolates belonging to ST15 were found in the nephrology unit. Three strains assigned to ST101 were recovered from the nephrology unit (n=2) and the ICU (n=1). Tow ST147 *K.pneumoniae* variant were also isolated from the ICU. One case of each ST14, ST86, ST336 and ST307 were isolated from Surgery, Pediatric, ICU and Cardiology respectively

However, conjugation reactions between *E.coli* J53 and the donor 5189 have given two types of transconjugants. First type: when inoculated from CTX+azide plate to Cip+azide plate they give colonies. Second type gives colonies only on CTX+ azide plates.

Table 2: Genes Detected In Trans Conjugants and the Corresponding Ciprofloxacin MIC

Donor	**154**		**5189**			**2116**	
Trans conjugants	A	B	C	D	E	F	G
Qnr B1	+	+	-	+	+	+	+
Aac-Ib-cr	+	+	+	+	+	+	+
Ctx-m-15	+	+	+	+	+	+	+
MIC Cip µg/ml	0,5	0,5	0,064	0,5	0,5	0,5	0,5

Transformation

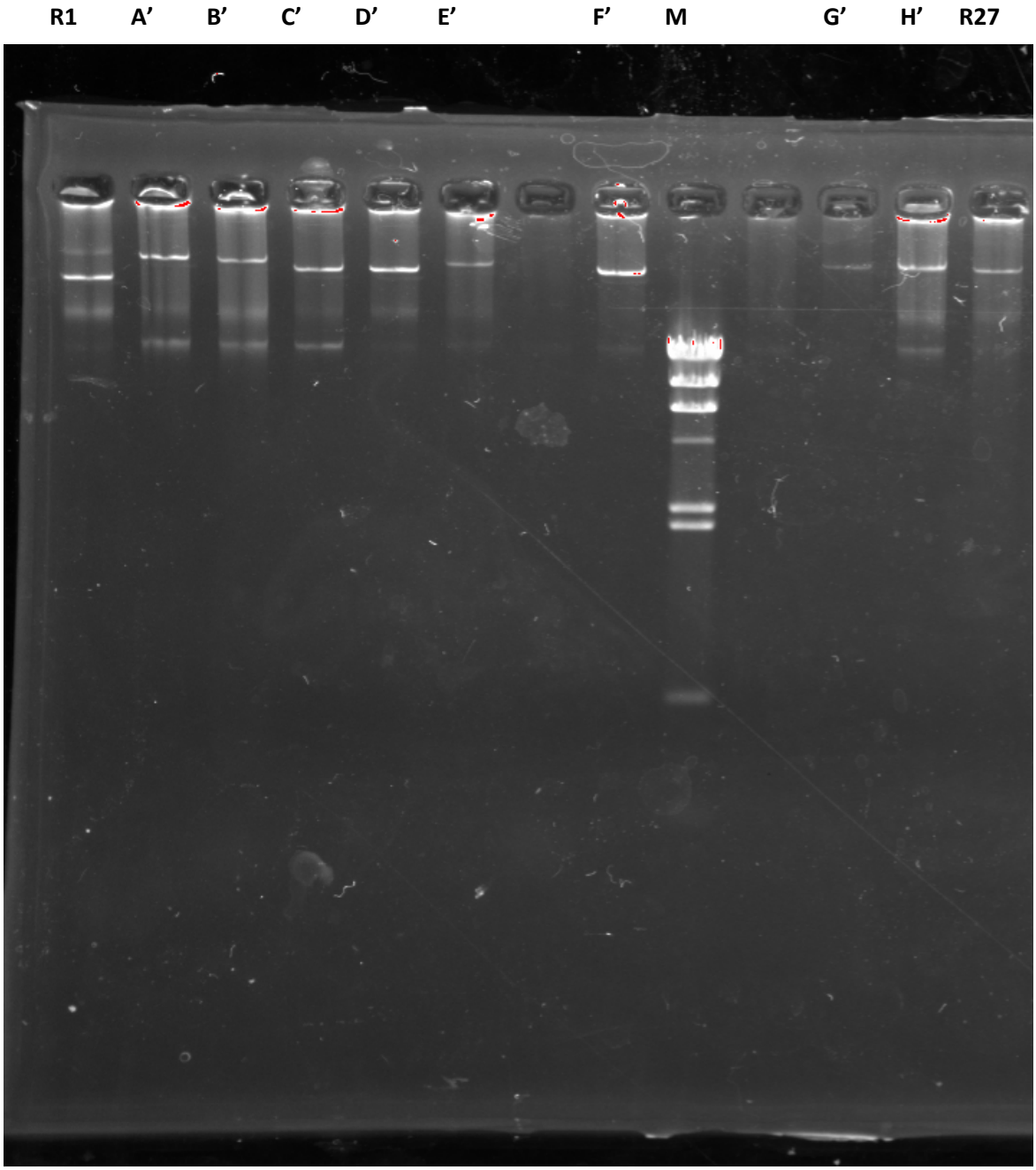

Figure 2: Plasmid Profile Extracted from Transform ants
A': transformant with plasmid A, B': transformant with plasmid B. C': transformant with plasmid C, D': transformant with plasmid D, E': transformant with plasmid D, F': transformant with plasmid E, G': transformant with plasmid F, H': transformant with plasmid G.

Table III: Genes Detected On Transform Ants and the Corresponding Ciprofloxacin MIC

Plasmid donor	154		5189				2116	
Transformants	A	B	C	D	E	F	G	H
Qnr B1	+	+	-	-	+	-	+	+
Aac-Ib-cr	+	+	+	+	+	+	+	+
Ctx-m-15	+	+	+	+	+	+	+	+
MIC cip µg/ml	0,19	0,19	0,008	0,006	0,19	0,008	0,19	0,19

Plasmid extraction from transform ants has shown the harbor of one plasmid in each one. Consequently, all genes detected in one transform ant were carried in the same plasmid. Therefore, the donors 154 and 2116 are carrying qnrB1, aac-Ib-cr and CTX-m-15 in the same plasmid. However, the donor number 5189 contains two types of plasmids, the first one is carrying the three cited genes (transform ant E) and the second one is carrying aac-Ib-cr and CTX-m-15.

Discussion

The results of this study provide insights into the molecular epidemiology of PMQR and ESBL producing *K. pneumonia* isolates in the university hospital Fattouma Bourguiba in Monastir, Tunisia, thereby representing the first study to characterize PMQR and ESBL producing *K. pneumonia* in Tunisia.

During our three years study, most of the *K.pneumoniae* was isolated from patients with urinary tract infection at inpatient department. Blood was the second site colonized by these bacteria. It was shown that multidrug resistant *K.pneumoniae* is one of the important nosocomial and community acquired opportunistic bacteria causing urinary tract infection, pneumonia, septicemia etc [1].

The majority of the isolates characterized in this study were from pediatric units. Infections caused by PMQR and ESBL harboring organisms in neonates and pediatric wards are usually reported to be hospital acquired and associated with invasive procedures [38, 39 and 40]. The predominance of these isolates in these units could be explained by the high use of the third generation of cephalosporin, as cefotaxim was the most prescribed drug in pediatric unit during the study period. Comparing to other units, the MIC of ciprofloxacin (16-64µg/ml) was lower in pediatric wards as ciprofloxacin is usually contraindicated in children and neonates. High rates of ciprofloxacin resistance were detected in the ICU, the second ward contaminated with multidrug resistant *Pneumonia*. These units have high rates of empirical treatment using fluoroquinolones, 3rd generation cephalosporin and carbapenems.

Among quinolone resistant *K.pneumonia*e, 40% only harbored the PMQR genes. It has been demonstrated that quinolone resistance in Enterobacteriaceae family usually results from mutations in genes carried by chromosomally encoded type II topoisomerases, efflux pump or poring related protein [41].

PMQR such as *qnr* and aac *(6')-IB-cr* may facilitate the spread and increase the prevalence of quinolone resistant strains. To date, *qnr* genes have been widely detected in Africa, Asia, America and Europe. The co-harbor of *aac (6')-IB-cr* and *qnr* genes have been reported in several studies. In *K. pneumonia* the aac (6')-IB-cr gene was related to *qnr* B1 [6], to *qnr* B32 [5], to *qnrS1*, *qnrB6* and *qnrB4* [42].

It was also shown that PMQR and ESBL could be transferred in several studies and this is amplify the infection risk due to these strains of *K.pneumoniae* [1, 4, 5 and 42].

This is the first report in Tunisia of coexisting aac (6')-IB-cr and *qnrB1* in *K.pneumoniae.* Moreover, these two PMQR coexisted with bla CTX-m15 and shv11 in three cases and with ctx-m15 and tem1 in tow cases. To the best of our knowledge, this is the first report of coexisting qnrB1, *aac-Ib-cr, ctx-m-15* and shv11/tem1 in *K.pneumoniae*. Referring to conjugative reactions, *qnrB1, aac-Ib-cr and ctx-m15* were co transferred from tow donors.

Plasmids extracted from the corresponding transform ants were the same in size and there were only one plasmid for each transform ant. This is confirming that these three genes were carried by the same plasmid. The third donor gave Trans conjugants with qnrB1, aan-IB-cr and ctx-m-15 or Trans conjugants with aac-Ib-cr and ctx-m-15 only. This is had already influence on MIC of ciprofloxacin on Trans conjugants. Effectively, the MIC of ciprofloxacin on Tran's conjugants with qnrB1 and aac-Ib-cr genes was higher (0.5µg/ml) then those with *aac-Ib-cr* only (0.064µg/ml). This is mean the expression of qnrB1 in resistance to ciprofloxacin is match more important than the expression of *aac-Ib-cr.* The transformation of top 10 with plasmid extracted from Tran's conjugants coming from the third donor has shown two types of transform ants; the first type is carrying one plasmid harboring the three cited genes (transform ant E), the second type is carrying one smaller plasmid harboring aac-Ib-cr and ctx-m-15. Referring to the

previous data the donor 1 and 3 are harboring one plasmid that carry 3 genes and the third donor harbor tow type of plasmids the first carry the 3 genes and the second carry aac-Ib-cr and ctx-m-15. Bought type of donor with one or two plasmid are prohibiting the patient health and this is need important survey of the epidemiology of these strains.

This is the first epidemiological report in Tunisia using MLST to type strains of *K.pneumoniae* harboring PMQR. The results revealed mainly sequence type 4, 15,101 and 147 compared to previous reported ST 101, ST 107 and ST147 [27]. There is a significant distribution of the ST and the corresponding unit. The ST4 *K.pneumoniae* were found in pediatric wards, the ST 15 in nephrology unit, ST147 in ICU and the ST101 between the nephrology and the ICU. This phenomenon can be explained by the nosocomial spread of strains with the same sequence type among patients of the same unit. The study of Filippa et al, have demonstrated the spread of an unusual *K.pneumoniae* isolated from Nigerian patient among 10 French patients in the ICU of French hospital [6]. The ST4 and ST 15 *K.pneumoniae* were first described in France, but until 2013, the ctx-m-15 does not exist in France and it was introduced among this country because of a strain coming from Nigerian patient [6]. The nosocomial contamination between patients could explain the harbor of new genes in ST4 and ST15 *K.pneumoniae*. The ST101 and ST147 *K.pneumoniae* were first reported respectively in Poland and Hungary. Since that these sequence type have been reported to occur worldwide and are associated with multidrug resistant K.pneumoniae [43, 44]. The ST14 found in the surgery unit was described previously in Africa [28]. The ST86, ST336 and ST307 are strange to Africa and this is the first report of these types in Tunisia since they were described respectively in France, America and Netherlands. Tunisia has important relationship with the cited countries and this can explain the first appearance of these ST types *K.pneumoniae.*

Conclusion

This study highlights the need to improve the antimicrobial resistance surveillance in Tunisia for *K.pneumoniae* to monitor the new types of resistance mechanisms emerging in different ward in the same or different hospitals. As well as, the factors responsible for the selection and dissemination of the high conjugative plasmids encoding the *qnr* genes, *aac-Ib-cr* determinant and different ESBL must be considered for the antibiotic policies and controlled to prevent other outbreaks in future

Declarations

Abbreviations

Ethics Approval and Consent to Participate

Not applicable because strains were already collected thinks to the clinical microbiological laboratory of Fattouma Bourguiba Hospital.

Consent for Publication

Not applicable

Availability of Data and Material

Not applicable

Competing Interests

The authors declare that they have no competing interests

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Authors' Contributions

MNY carried the bacteria collection and typing antibiotic susceptibility, PCR screening, conjugation and transformation reactions, sequence blasting and writing the manuscript. IDR is project designer and co-supervisor of research work. QG Laboratory supervisor, she controlled the analysis, interpretation of data and controlled the manuscript. MM provided the necessary materials for collection, typing and doing the susceptibility tests. MA project designer and general supervisor of research group. MW General Supervisor of research experiments and controlled the manuscript. All authors read and approved the final manuscript.

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Not applicable

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