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Review Article

Plasmid-Mediated Resistance Genes among Ciprofloxacin-Resistant *Enterobacteriaceae* Isolates in Neonatal and Pediatric Intensive Care Units

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Abstract
Ciprofloxacin resistant *Enterobacteriaceae* due to plasmid-mediated quinolone resistance genes (PMQR) is a rising problem worldwide. Objectives: The aim of this study was to determine the prevalence of ciprofloxacin resistant *Enterobacteriaceae* isolates in neonatal and pediatric intensive care units (NICUs and PICUs) through the detection of *qnrA, qnrB,* and *qnrS* in clinical isolates. Methods: A total of 329 *Enterobacteriaceae* isolates were collected from patients attending Misr children hospital. Identification was performed by biochemical reactions, whereas antimicrobial susceptibility testing was done by disk diffusion test. Detection of minimum inhibitory concentration (MIC) of ciprofloxacin was done using E-test method. Multiplex polymerase chain reaction (PCR) was performed for the detection for *qnrA, qnrB* and *qnrS* genes simultaneously. Results: The prevalence of ciprofloxacin resistance among *Enterobacteriaceae* isolates was 15.2%. *Klebsiella pneumoniae* was the main pathogen isolated from both ICUs, but significantly higher in NICU than PICU (88% and 60%) respectively. This was followed by *Escherichia coli* and *Enterobacter cloacae* with higher isolation rates in PICU (24% and 16%) than in NICU (4% and 8%). The *qnr* genes among all ciprofloxacin resistant *Enterobacteriaceae* isolates were present at 68%. Among *qnr* -positive isolates; *qnrS* and *qnrB* were detected at 82.4% and 11.8% respectively. In conclusion: The high prevalence of *qnr* genes detected in *Enterobacteriaceae* from neonatal and pediatric patients is of serious concern, as ciprofloxacin, although with limited indications in these age groups, it is usually used in life-threatening infections in which they may represent the only effective antibiotic. In addition, this finding highlights the possibility of horizontal transmission of these genes to other pathogenic bacteria.

Key Words: Plasmid-mediated quinolone resistance, *Enterobacteriaceae*, multiplex PCR, intensive care units, neonatal unit.

INTRODUCTION

Healthcare-associated infections (HAI), especially within neonatal (NICUs) and pediatric intensive care unit (PICU), represent a major health problem that may reach 2-5 times higher than in the general inpatient hospital population. It is usually associated with high morbidity, mortality and increase of health cost [1, 2, 3].

As newborns are devoid of efficient mature immune system including structural barriers and a protective endogenous microbial flora, neonatal infections are major causes of morbidity and mortality in this age group worldwide [4, 5]. As these infections expose them to prolonged parenteral nutrition, intravascular catheterization, serous medical conditions; as depending on respiratory ventilators, gastrointestinal surgery, and the use of broad spectrum antibiotics [6, 7]. This complies with the most common HAI in NICUs; that are bloodstream infections, then ventilator associated pneumonias, surgical site infections and less common catheter associated urinary tract infections, ventricular shunt and skin and soft tissue related infections [7].

PICU is also a unique fragile environment within the hospital. Its patients often require some degree of invasive monitoring and cardiopulmonary assistance. Again, these patients usually show varying degrees of immune defeciencies arising from young age, underlying disease, also with higher rates of central line-associated bloodstream infections [8].

Empirical antibiotic therapy is often used in NICUs and PICUs that can lead to selective pressure for antibiotic resistance [9]. In neonatology, the use of ciprofloxacin is saved to life-threatening infections in which the clinical benefits overweight the potential risks [10]. Ciprofloxacin has been successfully prescribed in several pediatric infections as well. It is the only fluoroquinolone that is included on the list of "Essential Medicines for Children" prepared by the World Health Organization (WHO)[11].

Recently resistance to ciprofloxacin among Enterobacteriaceae family has been reported ominously, ususally explained with the presence of plasmid-mediated quinolone resistance genes (PMQR). PMQRs are implicated in quinolones resistance as qnr proteins protect the quinolone targets; DNA gyrase and topoisomerase IV from the inhibitory activity of quinolones. Although PMQRs confer low-level resistance, they select for mutations in gyrase and topoisomerase genes that usually results in high-level resistance [12, 13, 14, 16].

The aim of this study was to determine Enterobacteriaceae that are responsible for nosocomial infections and its antibiotic sensitivity patterns in PICUs and NICUs and to determine prevalence of ciprofloxacin resistant *Enterobacteriaceae* clinical isolates through the molecular detection of PMQRs; *qnrA*, *qnrB*, and *qnrS*.

MATERIALS AND METHODS

Bacterial isolates

A total of 333 clinical isolates belonging to the family *Enterobacteriaceae* were collected from patients admitted to pediatric (121/329) and neonatal (208/329) intensive care units in Misr children hospital during the period from June 2017 to February 2018. Among the *Enterobacteriaceae* isolates from pediatric (PICU) and neonatal intensive care units (NICU); ciprofloxacin resistant was detected in 15.2% (50/329). They were isolated from different clinical infections as blood stream, respiratory, urinary tract, surgical wound, central line and umbilical catheter infections. Samples were cultured on MacConkey agar and identified by routine biochemical tests [17]. Bacterial isolates was stored at - 20°C for further study.

Antimicrobial Susceptibility Testing (AST)

Antimicrobial susceptibility was done using Kirby-Bauer disk diffusion (KBDD) method according to Clinical and Laboratory Standards Institute (CLSI) guidelines [18] using antibiotics discs; ciprofloxacin (CIP; 5 µg), levofloxacin (LEV; 5 µg), nalidixic acid (NA; 30 µg), amikacin (AK; 30 µg), gentamicin (CN; 10 µg), ceftriaxone (CRO; 30 µg), ceftazidime (CAZ; 30 µg), cefotaxime (CTX; 30 µg), cefoperazone (CEP; 75 µg), amoxicillin/clavulanic (AMC; 30 µg), ampicillin/sulbactam (SAM; 20 µg) and imipenem (IPM; 10 µg) (Mast Diagnostics, U.K.). *E. coli* 25922 served as the quality control in the antimicrobial susceptibility test. The AST of the tested isolates was interpreted as per CLSI [18].

Minimum inhibitory concentration (MIC) of ciprofloxacin was determined by E-test (AB Bio Disk Solna, Sweden). It was performed according to the manufacturer's instructions. CLSI interpretive criteria for ciprofloxacin were used in interpretation [18].

Phenotypic Detection of Extended Spectrum β-Lactamases (ESBLs)

Enterobacteriaceae isolates were screened for ESBL by the KBDD method where isolates with reduced susceptibilities to CTX (zone diameter of <27 mm) and/or CAZ (zone diameter of <22 mm) were considered possible ESBL-producers according to CLSI guidelines [18].

Phenotypic confirmation of potential ESBL-producing isolates was performed using disc approximation method and results were interpreted according to CLSI recommendations [18]. The standard strain *E. coli* ATCC 25922 was used as negative control for the assay.

DNA extraction and *qnr* genes amplification

Primers with 100% homology to all retrieved *qnrA*, *qnrB* and *qnrS* with no match to human genes have been selected to be synthesized (Table1). DNA Extraction of *Enterobacteriaceae* isolates was performed by boiling method [19]. PCR reactions for *qnrA*, *qnrB*, and *qnrS* pairs have been carried out in multiplex using negative controls with no DNA sample. DNA extract of each isolate was added to each PCR tube to reach 50µl. PCR master mix that contained; dream Taq DNA polymerase, 2 x Dream Taq green buffer, Nucleotides mix (0.4 mM each), and 4 mM MgCl2. Amplification was carried out with the following thermal cycling conditions; initial denaturation at 95°C for 5 min, followed by 25 cycles at 95°C for 105 sec, annealing at 56°C for 15 sec, and extension at 72°C for 15 sec (Biometra, UK) [20]. DNA product was analysed by electrophoresis in a 2% agarose gel containing 0.05 mg ethidium bromide. The gel was photographed using the gel documentation system (Cleaver, UK).

Statistical Methods

Data were statistically described in terms of frequencies (number of cases) and relative frequencies (percentages). A probability value (Pvalue) less than 0.05 was considered statistically significant. All statistical calculations were done using computer programs Microsoft Excel 2013 (Microsoft Corporation, NY., USA) and SPSS (Statistical Package for the Social Science; IBM SPSS statistic) version 20 for Microsoft Windows.

RESULTS

The prevalence of ciprofloxacin resistance among *Enterobacteriaceae* isolates was 15.2% (50/329). They were equally isolated from the PICU and NICU (25 isolates each). Ciprofloxacin resistant *Enterobacteriaceae* isolates were determined in NICU and PICU in 12.1% (25/208) and 20% (25/121) respectively. The clinical isolates from the NICU were recovered from 17(68%) male and 8 (32%) females; their age ranged from 3 to 21 days with mean age of 8.48 ± 5.6. The clinical isolates from the PICU were recovered from 16 (64%) males and 9 (36%) females; their age ranged from 3 months to 5.5 years with mean age of 2.2 ± 1.88.

The majority of bacterial isolates from NICU were recovered from blood cultures (60%; 15/25). The remaining isolates were taken from endotracheal tube (20%; 5/25), central lines (3/25; 12%), wound swab (4%; 1/25), and an umbilical catheter (1/25; 4%). The isolates from PICU were recovered equally from blood specimens and endotracheal tube (each 44%, 11/25), then wound swab (8%; 2/25) and urine (4%; 1/25).

Identification of Clinical Isolates:

The distribution of ciprofloxacin resistant *Enterobacteriaceae* isolates according to the species and specimen type in NICU and PICU is shown in table 2. *Klebsiella pneumoniae* (*K. pneumoniae)* was the most frequently isolated species in NICU and PICU in 88% (22/25) and 60% (15/25) respectively. However, *K. pneumoniae* was more significantly isolated from NICU than PICU (Pvalue 0.02). On the other hand, *E. coli* and *Enterobacter cloacae (E. cloacae)* isolation rates were higher in PICU (24% and 16%) than in NICU (4% and 8%), with P value 0.049 and 0.3 respectively.

Antimicrobial Susceptibility Testing (AST)

Antibiotic susceptibility pattern of 50 ciprofloxacin resistant *Enterobacteriaceae* isolates to different antibiotics recovered from NICU and PICU is shown in table 3.

Ninety-eight percent (98%; 49/50) of ciproflocacin resistant *Enterobacteriaceae* isolates by KBDDwhich demonstrated very high MIC (>32 μg/ml) to ciprofloxacin. One *E. coli* isolate (2%) retrieved from PICU showed intermediate susceptibility (MIC= 3μg/ml). Carbapenem-resistant *Enterobacteriaceae* (CRE) isolates were determined in 68% (34/50). CRE was more frequently isolated from NICUs than from PICUs in rates of 88% and 48% respectively (P value 0.002). ESBL-production among ciprofloxacin resistant *Enterobacteriaceae* isolates was detected in 6% (3/50). They were all recovered from PICU (12%; 3/25). They were 2 *E. coli* (8%; 2/25) isolates and one isolate of *K. pneumoniae* (4%; 1/25).

Detection of *qnr* genes

The overall prevalence of *qnr* genes was 68% (34/50) in ciprofloxacin resistant *Enterobacteriaceae* isolates. They were recovered from NICUs and PICUs. Among *qnr* -positive isolates, the *qnrS* and *qnrB* were detected in 82.4% (28/34) and 11.8% (4/34) respectively. Both *qnrB* and *qnrS* were determined simultaneously in two *K. pneumoniae* isolates (5.8%; 2/34). However, *qnrA* was not detected in any of the studied isolates. The *qnr* genes were detected mainly in *K. pneumoniae* in 91.2% (31/34) followed by *E. coli* in 5.9% (2/34) and *E. cloacae* in 2.9% (1/34) isolates. The *qnrS* gene was determined significantly in *K. pneumoniae* isolates in a rate of 79.4% (27/34) (P value 0.03).

In NICU, *qnr* genes were detected in 76% (19/25) of ciprofloxacin resistant *Enterobacteriaceae* isolates. Among 19 *qnr*-positive isolates, *qnrS* was detected significantly in 94.7% (P value 0.00003) while both *qnrB* and *qnrS* genes were determined simultaneously in one *K. pneumoniae* isolate (5.3%; 1/19).

In PICU, *qnr* genes were detected in 60% (15/25) of ciprofloxacin resistant *Enterobacteriaceae* isolates. *QnrS* and *qnrB* were detected in 66.7% (10/15) and 26.6% (4/15) respectively, and both *qnrB* and *qnrS* genes were determined simultaneously in one *K. pneumoniae* isolates (6.7%; 1/15). *QnrB* gene was detected in one ESBL-producer *K. pneumoniae* isolates (1/3; 33.3%), while *qnrS* gene was not detected in any ESBL-producer isolate.

The alliance between the detection of *qnr* and the resistance to other antibiotics was evaluated

among the 50 ciprofloxacin resistant *Enterobacteriaceae* isolates. Amikacin was the only antibiotic showed statistically significant association between its resistance and the presences of *qnr* genes (P value 0.00003).

PMQR and their distribution among ciprofloxacin resistant Enterobacteriaceae isolates according to specimens and species of the isolate in NICU and PICU are illustrated in table 4 and figure1.

DISCUSSION

Multidrug-resistant organisms causing HAI are of serious concern among healthcare professionals, especially within ICUs [21]. As patients in ICU, being exposed to various invasive procedures, make them more vulnerable to infections [22]. Although ICUs account for less than 10% of the total number of beds in most hospitals, however more than 20–30% of all nosocomial infections are acquired in the ICU with high rates of antimicrobial resistance and mortality when compared with other hospital wards [15]. A rise in nosocomial infections has been reported markedly recently especially from NICUs and PICUs.

Long term ICU stay where; broad spectrum antibiotics intake, chronic underlying conditions, and the exposure to invasive procedures and devices are common risk factors that make these patients more vulnerable to infections caused by multidrug-resistant strains of bacteria [23]. Although fluoroquinolones use in children is limited to severe or life threatening infections, they are considered as an important class of antibiotics due to their broad spectrum of activity and good tissue penetration [11].

Although PMQRs confer low-level fluoroquinolones resistance, they select for mutations in gyrase and topoisomerase genes those results in the spread of high-level resistant strains. Also PMQR genes are usually located on the same plasmid with the ESBL genes [24]. So there is concern that the horizontal transmission of PMQR will eventually lead to more spread in ESBL resistance [25].

The aim of this study was to determine the *Enterobacteriaceae* responsible for nosocomial infections and its sensitivity profiles in PICUs and NICUs and to determine prevalence of ciprofloxacin resistant *Enterobacteriaceae* clinical isolates. The molecular detection of PMQR determinants *qnrA*, *qnrB*, and *qnrS* to estimate the magnitude of these resistance genes was assessed in this study.

In this study, the overall prevalence of ciprofloxacin resistant among *Enterobacteriaceae* isolates was 15%. Ciprofloxacin resistant *Enterobacteriaceae* isolates were determined in NICU and PICU in 12.1% (25/208) and 20% (25/121) respectively. Higher rates were detected in another two Egyptians studies by **Shehab El-Din *et al*, and Fahmey** [26,27] who reported a prevalence of 38.9% (21/54) and 30.3% (33/109) in NICUs respectively. Other studies from Saudi Arabia, Bangladesh and Nepal showed higher prevalence rates; 29.2%, 42%, 52% and 62.5% respectively [28, 29, 2,14]. However, lower rate of 6% was determined in a NICU in a Tunisian study [30]. Comparative figure from China was reported, in which among the 243 isolates from pediatric patients, 51 (20.99%, 51/243) were resistant to fluoroquinolones [25].

In NICU, the use of ciprofloxacin in life-threatening infections, although rare, but it is vindicated by the clinical benefits that overweight the probable risks [10]. It is especially primarily indicated in cases of cystic fibrosis and severe infections. In addition ciprofloxacin is considered superior to standard treatments for children with typhoid fever, severe shigella dysenteries, and enterobacteria meningitis [31].

The high prevalence of fluoroquinolone resistance in this study is could be explained by the high usage of fluoroquinolones to treat other infections such as urinary tract infections, as this drug used to be sold over the counter in Egypt. It is also known that the mother's vaginal flora may be harboring such resistant organisms and may transmit to their newborns [13].

In the current study, the majority of bacterial isolates from NICU were retrieved from blood cultures (60%), while isolates from PICU were recovered equally from blood cultures and endotracheal tubes (each 44%). This was in accordance with an Egyptian study by **Mohamed and El Seifi** [32] who reported that the main neonatal HAI was bloodstream infection (58.0%), followed by pneumonia (46.0%). Another study by **Tauhid et al** [2] concluded that the first four weeks of life were the most vulnerable period of getting infection so neonates are more susceptible to develop bloodstream infection than children aged above 28 days.. Other studies also reported that the most common HAI in NICUs and PICUs are bloodstream infections, and then ventilator associated pneumonias [8, 33, 32, 7].

In this study, *K. pneumoniae* was the most frequently isolated species in NICU and PICU at

rates of 88% and 60% respectively. This was in agreement with several studies from Egypt, china and Nepal, in which *K. pneumoniae* represented 72.7% (8/11), 61% (92/151) and 60% (14/24) respectively [26, 34, 35]. *K. pneumoniae* is one of the most common causes of nosocomial infections in adults and children and is associated with a high mortality rate[36]. In 2017, the WHO included ESBL-producing *K. pneumoniae* in the list of the most dangerous superbugs along with *Acinetobacter baumannii* and *Pseudomonas aeruginosa* [37]. On the other hand, *E. coli* and *E. cloacae* isolation rates were higher in PICU (24% and 16%) than in NICU (4% and 8%) respectively. *E. coli* was isolated in different rates ranging from 9.1% - 39% in many studies [26, 14, 2, 32, 34]. Several studies from India, Nepal and Egypt reported a wide isolation rate of *E. cloacae* ranging from 2.3%, 17.9% and 22.5% respectively [38,14, 27]. This variation could be due to the diversity of organisms causing sepsis from region to another that could change over time. This could be attributed to the pattern of antibiotic use and alterations in lifestyles from region to region [26].

In the present study, carbapenem-resistant *Enterobacteriaceae* (CRE) was isolated more frequently from NICUs than PICUs in rates of 88% and 48% respectively. **Nour et al.** [39] stated that CRE was determined in 58 out of 158 (36.7%) infants with sepsis in NICU. However, lower rates of 8.7% were reported in other studies [2,40]. *K. pneumoniae* and *E. cloaca* were resistant to imipenem in 81.5% and 50% respectively while *E. coli* isolates were 100% sensitive to imipenem. **Tauhid et al.** [2] found that all *E.coli* isolates were sensitive to imipenem. Many studies found that, the most common *Enterobacteriaceae* exhibiting carbapenem resistance was *K. pneumoniae* followed by *Enterobacter* species, while other members of *Enterobacteriaceae* were detected less frequently [41, 42]. Increasing reports of CRE infections worldwide, in concomitant with the complex pediatric population suggest the future emergence of CRE as significant nosocomial pathogens in pediatric centers [42].

The prevalence of ESBL-producing ciprofloxacin resistant *Enterobacteriaceae* isolates were 6%. ESBL-producer isolates were all recovered from PICU. In other studies, higher rates were detected in neonates and pediatric patients in 52% and 57.9% respectively [43, 44]. However, this number may be underestimated, as most of our isolates were imipenem resistant (68%) and phenotypic detection of ESBL is not reliable in presence of other β-lactamases as production of carbapenemases, although not investigated in the current study, is the main mechanism of carbapenem resistance [45].

In this study, the overall prevalence of *qnr* genes were 68% in ciprofloxacin resistant *Enterobacteriaceae* isolates recovered from NICU and PICU. PMQR in *Enterobacteriaceae* has been widely reported in North and South America and European countries, but only a few reports on pediatric isolates. In recent study in India, 81% of the isolates obtained from blood cultures of septicaemic neonates carried at least one of the PMQRs [13]. **Chmielarczyk et al**. [46] reported that among 80 isolates from NICU, 27.5% carried at least one PMQR determinant. In China, PMQR genes were detected in 21.82% of quinolone resistant isolates from pediatric patients [25], while in a Tunisian study, the detection rate was 14.4% [47] and in a Korean study, the PMQR was found in 9.7% [48]. These findings suggested that PMQR genes might not be directly associated with the selective pressure caused by the direct use of quinolones in pediatric patients. Whereas it could be linked to the marked use of cephalosporin much more than quinolones, suggesting that other resistance genes may have a greater effect on PMQR prevalence than the low-level quinolone resistance conferred by PMQR itself [25].

In the present study, the *qnrS* and *qnrB* genes were detected in 82.4% and 11.8% respectively among PMQR positive isolates. Both *qnrB* and *qnrS* genes were determined simultaneously in 5.8%. In both NICU and PICUs, *qnrS* gene was the most frequent gene detected (94.7%, 66.7% respectively). This result was consistent with previous study in China, where the most frequently detected *qnr* gene was *qnrS* 58.2% (32/55), followed by *qnrB* in 16.4% (9/55) in PMQR-positive isolates from pediatric patients [25]. In another study, *qnrS* was the most prevalent in 50% (6/12) followed by *qnrB* in 25% (3/12) [49]. **Wang et al.** [50] reported that *qnrS* and *qnrB* were detected in 66.3% (61/92) and 27.2% (25/92) respectively. This result is also similar to **Jiang et al** [51] report, in which the prevalence of *qnrS* and *qnrB* among PMQR positive isolates was 31% (9/29) and 27.6 (8/29) respectively. In a European survey *qnrS* was the most prevalent among clinical *Enterobacteriaceae* isolates [52]. However, some studies reported higher prevalence of *qnrB* than *qnrS* in PMQRs positive isolates [13, 53].

In the current study, *qnrA* was not detected among the studied isolates. Our data are in accordance with previous reports, which did not detect *qnrA* [25, 30, 53, 46]. However, **García - Fulgueiras et al.** [24] found *qnrA* in 50% (2/4) of isolates from PICU.

In this study *qnrB* was detected in one ESBL-producer *K. pneumoniae* isolates while *qnrS* gene was not detected in any ESBL-producer isolate. This finding was similar to Uruguay study where *qnrB* gene was detected in one ESBL-producer *K pneumonuae* isolate [24]. **Poirel et al.** [52] reported that *qnrS* was mostly identified in non-ESBL-positive strains. In another study, all *qnr* positive isolates were ESBL producer except 2 *qnrS* positive *K. pneumoniae* isolates were non-ESBL producers [25].

CONCLUSION

The present study reveals a high prevalence of PMQR genes among the clinical isolates of *Enterobacteriaceae* from neonatal and pediatric patients. This confirms the pervasiveness of antibiotic resistance among these vulnerable age groups. These resistance determinants reinforces the necessity for permanent surveillance programs aimed at the phenotypic detection and molecular characterization of ESBLs, CRE and PMQR. Understanding the local epidemiology for prevalent bacterial species and their antimicrobial susceptibility profiles in NICUs and PICUs is essential for establishing a proper empiric therapy guidelines and a subsequent effective antimicrobial stewardship program. Strict infection control measures and following standard antibiotic prescription guidelines is mandatory to improve patients' outcomes and to slow down the development of antimicrobial resistance that eventually may reduce hospitalization costs.

Conflict of interest

We have no conflict of interest to declare.

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