Association of Efflux Pump and OMPs with Antibiotic Susceptibility Among ESBL-Producing *Klebsiella Pneumoniae* Clinical Strains in Malaysia

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Research Article

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Abstract

Multidrug-resistant (MDR) *Klebsiella pneumoniae (K. pneumoniae)* poses a serious public health threat. *K. pneumoniae* strains that produce extended-spectrum beta-lactamases (ESBL) are becoming increasingly reported in nosocomial and community-acquired infections. Besides resistance genes, integrons, and plasmids, altered membrane permeability caused by porin loss and energy-dependent efflux have also contributed to antibiotic resistance in *K. pneumoniae*. The objective of this study was to determine the correlation between the reduction of antibiotic susceptibility and overexpression of efflux pump as well as the lack of outer membrane proteins (OMPs) among clinical ESBLs resistant *K. pneumoniae*. The expression levels of *ramA*, *acrA*, *ompK*35 and *ompK*36 in 12 MDR *K. pneumoniae* strains with varying MICs levels were analyzed using quantitative real time-Polymerase Chain Reaction (qRT-PCR). The role of efflux pump on antibiotic resistance was also studied by using minimum inhibitory concentration (MICs) method with//without efflux pump inhibitor. The result indicated that the strains with highest resistance to cefotaxime showed the lowest level of *ompK*35 and *ompK*36 genes expression while the strains with lowest MIC level of resistance to cefotaxime showed the highest level of expression of *acrA* and *ramA*. Our finding also revealed the effect of efflux pump inhibitor phenyl-arginine-b-naphthylamide (PAβN) on the MIC levels of ceftazidime, amoxicillin-clavulanate and cefotaxime which were significantly reduced around 1–7 folds MIC levels. These results suggest that Efflux pump system and deficiently of OMPs contributing role in antibiotic susceptibility which should be taken seriously to prevent the treatment failure due to antimicrobial resistance.

Introduction

*Klebsiella pneumonia*e is a one of the most important hospital-acquired pathogens causing a wide range of infections such as pneumonia, urinary tract infections and septicaemia (De Oliveira et al., 2008). The overuse of expanded-spectrum cephalosporins for the treatment of these organisms has led to the emergence of ESBLs production in *K. pneumoniae* strains reported worldwide (Ali et al., 2018; Keynan & Rubinstein, 2007; Ben Hamouda et al., 2003; Romero et al., 2007). In Malaysia, Palasubramaniam et al. (2005) reported the first nosocomial outbreak of ESBL- producing *K. pneumoniae* associated with SHV-5 ESBL enzyme. Other ESBL types have also been reported between 2009–2019 (CTX-M, SHV, TEM) (Lim et al., 2009; Al Marzooq et al., 2015; Low et al., 2017, Mobasseri et al., 2020).

The cell envelope of Gram-negative bacterium consists of three layers: the outer membrane, the peptidoglycan cell wall, and the inner membrane. The outer membrane porin (Omp) is a trimeric membrane protein that functions as water-filled protein channels for transportation of small hydrophilic molecules such as iron, nutrients, and antibiotics (including β-lactams) across the outer membrane. Porins also function as the receptors for phages, bacteriocins and in conjunction with peptidoglycan and lipopolysaccharide (LPS) in maintaining the structure the cells (Tsai et al., 2011).

The role of porins in Gram-negative bacteria has been studied intensively. Two major porins, OmpC and OmpF which are found in *E. coli* and *Salmonella* serovars, have been known as homologue to OmpK35

and OmpK36 in *K. pneumoniae*, (Doménech-Sánchez et al., 2009; Tsai et al., 2011). In general, OmpF has slightly larger pore than OmpC allowing more molecules to pass through (Jiang et al., 2009; Tsai et al., 2011). In ESBL-producing *K. pneumoniae*, the expression of *OmpK36* was significantly higher than *OmpK35*. In some strains, the *OmpK35* porin has not been expressed at all. The low or non-expression of *ompK35* has increased the antibiotic resistance of ESBL-producing *K. pneumoniae* (Palasubramaniam et al., 2009). In addition, the role of OmpK36 in carbapenem-resistance has been elucidated in reports worldwide (Uz-Zaman et al., 2014; Malek et al., 2019; Wise et al., 2018).

Energy-dependent efflux is also a contributing factor to antibiotic resistance in *K. pneumoniae* (Padilla et al., 2010). One of the efflux systems involved in this resistance phenotype is the AcrAB multidrug efflux system that encoded by the AcrRAB operon. In AcrRAB operon, AcrAB repressor is encoded by *acrR*, while *acrA* and *acrB* encode a periplasmic lipoprotein, AcrB connects with TolC, an outer membrane protein which belongs to a family of envelope proteins and are found in all Gram-negative bacteria (Padilla et al., 2010). The increasing *AcrAB* efflux pump expression in MDR-resistant *K. pneumoniae* strains was reported to be caused by mutation in *AcrR, AcrAB* repressor, or overexpression of *RamA* (transcriptional regulator) (Schneiders et al., 2003). Correlation between reduced susceptibility to antibiotics and *AcrA* overexpression have been reported in several studies (Mazzariol et al., 2002; Schneiders et al., 2003; Hasdemir et al., 2004; Padilla et al., 2010).

To better manage the increase of the antimicrobial resistance in worldwide, there is a need to investigate other mechanisms that might contribute to resistance. Therefore, the objectives of this study were to determine the correlation between reduced susceptibility and overexpression of efflux genes among 12 ESBL-producing *K. pneumoniae* isolated from a hospital in Johor Bahru, Malaysia. In addition, the effects of the presence and expressions of OMPs on the resistance levels of the strains were also investigated.

Materials And Methods

Bacterial strains and PCR detection of OmpK35, OmpK36, acrA and ramA genes

Twelve Malaysian *K. pneumoniae* strains were previously isolated and confirmed as ESBL-producer phenotypically and genotypically (Mobasseri et al., 2020). Genomic DNAs of these 12 *K. pneumoniae* strains was extracted by using genomic DNA extraction kit (YEASTERN Biotech Co., Ltd.). PCR detection for *ompK*35, *ompK*36, *acrA* and *ramA* genes was performed using established primers (Ruzin et al., 2008; Landman et al., 2009). All PCR amplified products were purified and sent for DNA sequencing to confirm their identity.

Determination of susceptibility to antibiotics with and without activities of efflux pumps

Minimum inhibitory concentration of ceftazidime, amoxicillin-clavulanate and cefotaxime were determined by E-test (bioMerieux) according to the CLSI guidelines (CLSI 2018). The tests were performed with and without the efflux pump inhibitor, PAβN (26.3 mg/L). *E. coli* ATCC 25922 strain was used as quality control (Hasdemir et al., 2004).

Determination of gene expression levels of efflux pump and porin associated genes contribution to ESBL resistance by Quantitative Real Time– Polymerase Chain Reaction (qRT-PCR)

Total RNA for 12 selected ESBL-producing strains were obtained from late-exponential-phase cultures using RNeasy kit (Qiagen, Germany), the extracted RNA was quantified using NanoDrop (IMPLEN, Germany) and adjusted to 25 ng. The RNA was then subjected to transcription using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, Foster City, California, USA) and gene expression for *ompK*35, *ompK*36, *ramA* and *acrA* as previously described by Ruzin et al. (2008) and Landman et al. (2009). A non-ESBL producing *K. pneumoniae* strain KP2014C40 was used as the calibrator in relative quantification of *ramA*, *acrA*, *ompK*35 and *ompK*36 genes and *rrs*E was used as the reference gene (Ruzin et al., 2008). The PCR reaction was run on Applied Biosystem Vii A 7 Real-Time PCR system (Applied Biosystem, Foster City, California, USA).

Results And Discussion

*K. pneumoniae*is a common pathogen causing nosocomial and community-acquired infections such as septicaemia, pneumonia, and wound infection (De Oliveira et al., 2008). Previous studies reported the association of OMPs and efflux pumps with multidrug resistant phenotypes in clinical strains of *K. pneumoniae* (Doménech-Sánchez et al., 2009; Tsai et al., 2011; Padilla et al., 2010; Landman et al., 2009). In this study, we showed correlation between overexpression of efflux pump and lacking of OMPs with ESBLs resistance in twelve ESBL-producing *K. pneumoniae* strains.

The minimal inhibitory concentrations (MIC) for ceftazidime, cefotaxime and amoxicillin-clavulanate for the 12 ESBL-producing strains with and without PAβN (efflux pumps inhibitor) are summarized in Table 1. MIC results for ESBL-producing strains showed the range of resistance to cefotaxime (MIC from > 256 µg/mL to 16 µg/mL), cefotaxime + PAβN (MIC from > 128 µg/mL to 1 µg/mL), ceftazidime (MIC from > 256 µg/mL to > 8 µg/mL), ceftazidime + PAβN (MIC from > 96 µg/mL to > 0.75 µg/mL), amoxicillin-clavulanate (MIC from > 256 µg/mL to > 4 µg/mL), and amoxicillin-clavulanate + PAβN (MIC from > 32 µg/mL to > 2 µg/mL). Overall, the MIC levels in these ESBL-producing strains were reduced 2–5 folds for cefotaxime and ceftazidime and 1–7 folds for amoxicillin-clavulanate (Table 1) (Fig. 1). A previous study by Pages et al., (2009) indicated that PAβN is able to block the efflux pumps involved in antibiotic expel in *K. pneumoniae.* MICs decreased substantially with the presence of PAβN which observed with the different ß-lactam molecules tested especially with cefoxitin, amoxicillin, piperacillin and cefepime. Other studies by Maurya et al. (2019) and Filgona et al., (2015) also reported the decreasing antibiotic resistance of *K. pneumoniae* by using PAβN as efflux pump inhibitor.

All targeted efflux pump and porin-associated genes were observed in the 12 ESBL-producing strains used in the determination of gene expression levels in this study. The results of the qRT–PCR analysis for *acrA* and *ramA* gene expression levels clearly indicated the correlation between reduced susceptibility to cefotaxime and *AcrA, ramA* gene overexpression. Strain KP2014C19 (cefotaxime MIC ranged 16 (µg/mL)) showed lowest level of gene expression for *acrA* and *ramA* (7.51 Δ*CT*, 6.07 Δ*CT*, respectively), while in

KP2014C68 (cefotaxime MIC ranged > 256 (µg/mL), gene expression levels of *acrA* and *ramA* dramatically increased to highest level of gene expression (38.24 Δ*CT*, 23.7 Δ*CT*, respectively) (Table 2) (Fig. 2). These data concurred with studies by Mazzariol et al., (2002) and Hasdemir et al., (2004) where decreasing susceptibility to antibiotics was correlated to the overexpression of *acrA* gene in several ESBL-producing clinical *K. pneumoniae* strains. In another study, Schneiders et al. (2003) found that increasing *acrAB* gene expression in ESBL-producing *K. pneumoniae* strains was caused by mutations in the AcrAB repressor, or overexpression of the transcriptional regulator ramA. Some studies reported the role of the efflux pumps mechanisms especially the efflux pump AcrAB-TolC in the resistance to other classes of antibiotics such as aminoglycoside, carbapenem and chloramphenicol in *K. pneumoniae* strains (Jabar & Hasson, 2019; Marsik & Nambiar, 2011; Hasdemir et al., 2004).

In this study, the *OmpK*35 and *OmpK*36 gene expression profiles showed that the strain KP2014C37 (cefotaxime MIC ranged 16 (µg/mL)) showed highest level of gene expression for *OmpK*35 and *OmpK*36 (1.02 Δ*CT*, 1.31 Δ*CT*, respectively), while gene expression levels of *OmpK*35 and *OmpK*36 have dramatically decreased to lowest level of gene expression (0.29 Δ*CT*, 0.35 Δ*CT*, respectively) in KP2014C13 (cefotaxime MIC ranged > 256 (µg/mL)) (Table 2) (Fig. 2). The expression of *ompK*35, *ompK*36 genes is a major factor in conferring resistance against ESBLs in *K. pneumoniae* (Uz-Zaman et al., 2014). The lack of OmpK35 has been reported as the major porin through which ceftazidime penetrates the Gram-negative outer membrane in many ESBL-producing *K. pneumoniae* strains while the absence or reduced expression of the two major porins (OmpK35 and OmpK36) in *K. pneumoniae* in combination with various β-lactamases has been implicated in carbapenem resistance by previous studies (Doumith et al., 2009; Uz-Zaman et al., 2014).

In conclusion, this study showed the correlation between the reduction of antibiotic susceptibility and expression of efflux pump and OMPs as well as role of efflux pump inhibitor among ESBL-producing *K. pneumoniae*. The presence of efflux pump inhibitor PAβN, has direct association with MIC levels of CAZ, CTX and AMC which is more significant in amoxicillin-clavulanate MIC levels (reduced 1–7 folds) followed by 2–5 folds reduction in ceftazidime, these results suggested that the major ESBL-resistance mechanism found in these strains is a PAβN-sensitive mechanism, namely, a drug efflux mechanism (Hasdemir et al., 2004). Studies on mechanisms and structure-function association of bacterial OMPs and efflux systems as well as the interactions between the pumps and other resistance mechanisms are needed to monitor the usage of antibiotics in hospital settings to control and prevent antimicrobial resistance issue.

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The strains have revived from hospital cultures and do not contain any information about patients.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIAL

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

COMPETING INTERESTS

Golnaz Mobasseri, Thong Kwai Lin and Cindy Shuan Ju Teh declare that they have no conflict of interest

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AUTHORS' CONTRIBUTIONS

All three authors have contributed to the laboratory works and writing of this article.

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AUTHORS' INFORMATION

Not applicable.

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Tables

Table. 1: The minimal inhibitory concentrations (MIC) of 27 ESBL-producing clinical strains with/without PaβN (efflux pumps inhibitor).

Strains	* Minimal inhibitory concentration (MIC) (μg/mL); with/without PAβN(efflux pumps inhibitor)					
	CTX (30 μg)	CTX (30 μg) +PAβN (20 mg/L)	CAZ (30 μg)	CAZ (30 μg) +PAβN (20 mg/L)	AMC (20/10μg)	AMC (20/10μg) +PAβN (20 mg/L)
KP2014C04	32/R	12/R	8/I	3/S	8/I	6/R
KP2014C06	256/R	96/R	16/R	6/S	64/R	32/R
KP2014C09	64/R	48/R	8/I	2/S	32/R	12/I
KP2014C10	256/R	128/R	16/R	8/I	32/R	24/R
KP2014C13	256/R	128/R	8/I	1.5/S	32/R	16/R
KP2014C15	32/R	12/R	8/I	2/S	32/R	12/I
KP2014C18	256/R	96/R	16/R	6/S	64/R	48/R
KP2014C19	16/R	8/R	8/I	0.75/S	8/I	6/S
KP2014C23	256/R	64/R	16/R	6/S	64/R	32/R
KP2014C26	128/R	64/R	32/R	12/I	32/R	12/I
KP2014C30	256/R	128/R	16/R	4/S	32/R	12/I
KP2014C37	16/R	1/S	8/I	1/S	4/S	2/S
KP2014C46	64/R	12/R	16/R	6/S	36/R	24/R
KP2014C47	256/R	32/R	256/R	96/R	256/R	32/R
KP2014C52	256/R	128/R	32/R	16/R	8/I	8/I
KP2014C54	64/R	12/R	32/R	8/I	32/R	12/I
KP2014C56	256/R	128/R	32/R	16/R	64/R	32/R
KP2014C62	256/R	128/R	32/R	6/S	8/I	12/I
KP2014C68	256/R	96/R	128/R	48/R	128/R	16/R
KP2014C69	256/R	64/R	32/R	16/R	32/R	12/I
KP2014C74	128/R	12/R	64/R	24/R	32/R	12/I
KP2014C83	128/R	24/R	16/R	4/S	8/I	12/I
KP2014C84	256/R	128/R	32/R	12/I	64/R	24/R
KP2014C90	64/R	8/R	16/R	3/S	32/R	12/I

KP2014C94	256/R	128/R	32/R	8/I	8/I	6/S
KP2014C96	128/R	64/R	16/R	6/S	32/R	12/I
KP2014C99	128/R	32/R	16/R	8/I	8/I	8/I

Table.2 : Gene expression level profiles of Omp 35, Omp 36, *ramA* and *AcrA* in 12 ESBL-producing clinical *K. pneumoniae* strains.

KP strains	M.I.C (µg/mL)	Omp35,Omp36 PCR	Omp35 qPCR	Omp36 qPCR	*acrA*,*ramA* PCR	*acrA* qPCR	*ramA* qPCR
KP2014C04	CTX:32	Omp35,Omp36	0.83	0.91	*acrA*, *ramA*	9.8	7.43
KP2014C06	CTX>256	Omp35,Omp36	0.37	0.44	*acrA*, *ramA*	31.57	29.33
KP2014C13	CTX>256	Omp35,Omp36	0.29	0.35	*acrA*, *ramA*	21.42	31.64
KP2014C19	CTX:16	Omp35,Omp36	0.92	1.27	*acrA*, *ramA*	7.11	5.12
KP2013C23	CTX>256	Omp35,Omp36	0.33	0.48	*acrA*, *ramA*	24.69	26.31
KP2014C37	CTX:16	Omp35,Omp36	1.02	1.31	*acrA*, *ramA*	7.51	6.07
KP2014C46	CTX:64	Omp35,Omp36	0.81	0.89	*acrA*, *ramA*	10.42	8.78
KP2014C56	CTX>256	Omp35,Omp36	0.45	0.32	*acrA*, *ramA*	27.18	30.09
KP2014C68	CTX>256	Omp35,Omp36	0.41	0.51	*acrA*, *ramA*	38.24	23.7
KP2014C74	CTX:128	Omp35,Omp36	0.78	0.75	*acrA*, *ramA*	16.08	19.7
KP2014C83	CTX:128	Omp35,Omp36	0.65	0.84	*acrA*, *ramA*	13.63	12.1
KP2014C99	CTX:128	Omp35,Omp36	0.79	0.69	*acrA*, *ramA*	19.01	10.29

Figures

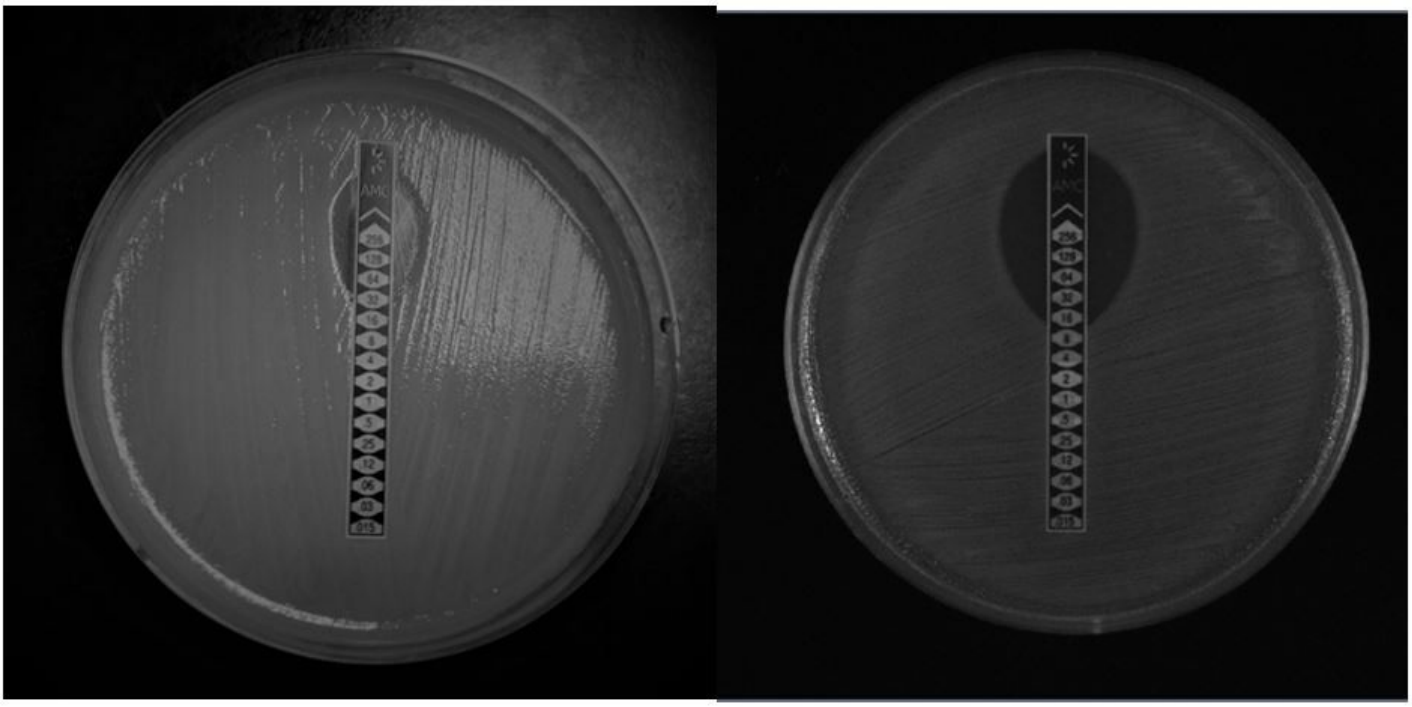

Figure 1

Representative pictures of MIC by E-test strips for amoxicillin-clavulanate (20/10 µg) in KP2014C13 with and without PAβN (efflux pumps inhibitor) (20 mg/L). Amoxicillin-clavulanate MIC with using PAβN: 32 (µg/mL). Amoxicillin-clavulanate MIC without using PAβN: 16 (µg/mL).

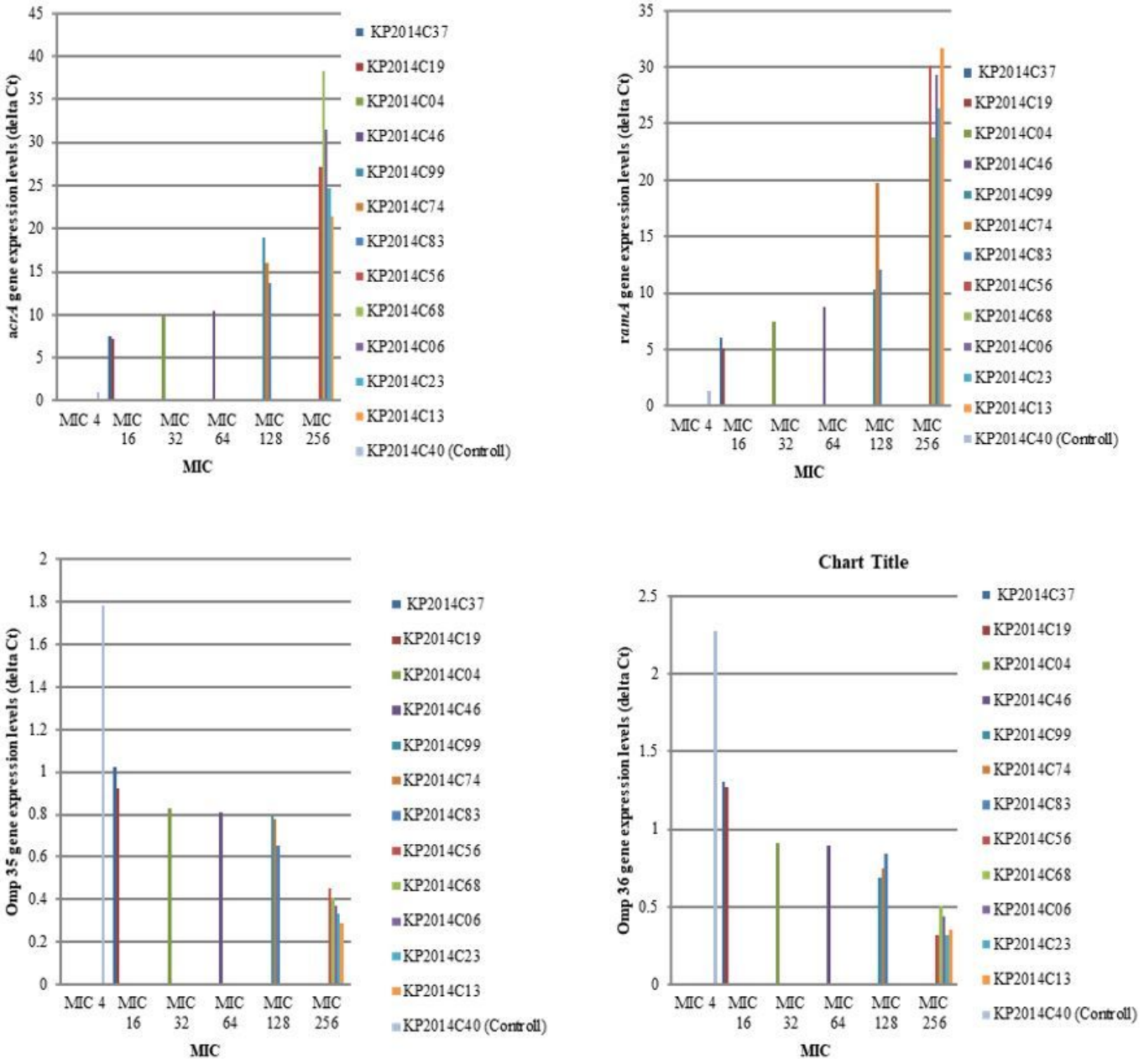

Figure 2

Gene expression level of Omp 35, Omp 36, ramA and acrA in 12 ESBL-producing K. pneumoniae strains based on different MICs.