MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2024.19.1.10134

Evaluating Porins OmpK35 and OmpK36 mRNA Expression in Ciprofloxacin-Resistant *Klebsiella pneumoniae*

Indah Sulistiyawati[1,2*], Wahyu Siswandari[3], Daniel Joko Wahyono[4]

[1]PhD Student in Biological Science Doctoral Program, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia
[2]Biological Science Bachelor Program, Faculty of Science and Technology, Universitas Nahdlatul Ulama Purwokerto, Indonesia
[3]Department of Clinical Pathology, Faculty of Medicine, Universitas Jenderal Soedirman, Purwokerto, Indonesia
[4]Department of Molecular Biology, Faculty of Biology, Universitas Jenderal Soedirman, Purwokerto, Indonesia

*Corresponding author email: indahsulistiyawati.s2@gmail.com

Received October 27, 2023; **Accepted** December 26, 2023; **Available online** March 20, 2024

ABSTRACT. Porin messenger RNA (mRNA) expression significantly influences porin permeability and reduces antibiotic penetration. *Klebsiella pneumoniae* has developed resistance to several antibiotics. Decreased expression levels of OmpK35 and OmpK36 porins result in changes in the porin profile and even functional loss of porins. This research aimed to analyze the relative expression of the outer membrane porins, OmpK35 and OmpK36. The research methodology involved MIC assays and quantification of OmpK35 and OmpK36 mRNA using RT-qPCR. The relative expression of OmpK35 and OmpK36 in clinical isolate *K. pneumoniae* is 0.8925 and 0.5877, respectively. The porin permeability of OmpK35/K36 mRNA positively correlated with the MIC values of OmpK35 (p-value = 0.029) and OmpK36 (p-value = 0.016), respectively.

Keywords: Ciprofloxacin, *Klebsiella pneumoniae*, OmpK35/K36, porin permeability

INTRODUCTION

Antibiotic resistance is still a global health problem. *Klebsiella pneumoniae* resistance has been reported in several hospitals in Indonesia. In 2018, Soeradji Tirtonegoro Hospital reported *K. pneumoniae* resistant to ampicillin, cefazolin, ciprofloxacin (Nirwati et al., 2019). Recent research reports that the prevalence of ciprofloxacin-resistant *K. pneumoniae* at Prof. Margono Soekardjo Hospital increased during August-September 2022 by 48.5% (Sulistiyawati et al., 2023). Bacteria have developed various mechanisms of resistance to antibiotics. The well-known mechanisms include changes in drug targets, enzyme inactivation, drug modification, and prevention of drug penetration or accumulation. An efficacious antibiotic must reach intracellular sites with critical concentrations that can inhibit a particular target. This is a challenge in controlling Gram-negative bacteria because of the complex membrane envelope (Silver, 2016). The membrane envelope is a common antibiotic barrier found in Gram-negative bacteria. The membrane envelope can block the penetration of antibiotics into bacterial cells (Blackwell, 2020).

The antibiotic must cross the outer membrane (OM) of Gram-negative bacteria to reach its target location. This membrane is an asymmetrical double layer containing lipopolysaccharide polymers (LPS) (Winterhalter, 2021). Antibiotic-containing hydrophilic solutes enter cells through the porin pathways. Porins are the main route or pathway of entry for hydrophilic antibiotics. Porins show a general preference for solute size. The characteristic properties of antibiotic activity against Gram-negative bacteria can be classified according to solubility and molecular weight. It reveals the highest activity for small polar molecules below 600 Dalton (Da) (Winterhalter, 2021). It also shows that porin is the main entry point that has a size exclusion limit of about 600 Da. For example, in *Escherichia coli*, there are three common trimeric porins, namely OmpF, OmpC, and PhoE. By their nature, OmpF and OmpC prefer cations to anions, whereas PhoE prefers anions. Each type of porin shows a general preference for the size and amount of solute to be accepted. OmpF porins allow slightly greater solute permeation than OmpC (Bajaj et al., 2017). Low permeability necessitates a large dosage of the medicine, which results in hazardous side effects (Winterhalter, 2021).

Porins in bacteria have long been studied. Porins may also participate in mechanisms of resistance to antibiotics. Some clinical isolates of Gram-negative modify porins to increase their resistance to the presence of antibiotics. It decreases antibiotic permeation through the outer membrane. Lactam

diffusion analysis revealed that *Klebsiella pneumoniae* expressing OmpK35 and OmpK36 created larger permeable channels. It is greater than their homologies in *E. coli*, i.e., OmpF and OmpC. OmpK35 is responsible for the formation of diffusion channels. It has a high permeability to lipophilic drugs (Sugawara et al., 2016). Antibiotic resistance in *K. pneumoniae* is frequently associated with mutational loss of OmpK35. Continuous translocation of β-lactam through porin channels can induce antibiotic resistance genes in plasmids. In addition, the hydrolysis of β-lactamases becomes inefficient, resulting in a high level of resistance.

Shakib et al., (2012) report that *K. pneumoniae* that does not express extended-spectrum beta-lactamases (ESBL) can produce OmpK35 and OmpK36 porins. Most isolates of *K. pneumoniae* expressing ESBL produced only OmpK36. However, current research finds some ESBL-producing clinical isolates that lack the OmpK35 and OmpK36 porins. The loss of OmpK36 also resulted in a moderate increase in fluoroquinolone resistance. This change correlates with the alternation of the porin profile, which makes it less permeable. This condition contributes to the emergence of antibiotic resistance in *K. pneumoniae*. Thus, the OmpF and OmpK35 analogues were replaced with the OmpC and OmpK36 analogues. *K. pneumoniae* strains with ESBL expression and OMP loss are more resistant to carbapenem (Hamzaoui et al., 2018).The substitution of porin-constituent amino acids leads to a narrowing of the porin channel. It has an impact on changes in channel size and their specificity to β-lactams (Blackwell, 2020). This research aimed to analyze the relative expression of the outer membrane porins, OmpK35 and OmpK36.

EXPERIMENTAL SECTION

Materials and Instruments

The following materials and instruments for research: Pure RNA isolation kit reagents (Zymo Research ZR Fungal/Bacterial RNA MiniPrep™, California), RT-qPCR cDNA Synthesis Kit (Toyobo ReverTra Ace® qPCR RT Master Mic), Bioline Sensi FAST Lo-ROX Kit reagents, and RT-qPCR Machine.

Sample Criteria

The samples used in this study were specimens from; blood, sputum, urine, pus, feces, pleural fluid from patients of RSUD Prof. Dr. Margono Soekardjo who conducted bacterial culture examination with complete data. *K. pneumoniae* isolates grown from specimen examination are tested by establishing a bacterial identification diagnosis. The number of samples used was 58 samples that met the inclusion criteria, and continued with antibiotic sensitivity testing.

Porin Membrane Permeability Activity

The minimum inhibitory concentration (MIC) was measured using a quantitative method. The required solvent is adjusted for ciprofloxacin as an indicator antibiotic. In the quinolone group, water is used as a solvent. The antibiotic is dissolved in the solvent to a final volume of 1 mL. The antibiotic was mixed with 19 mL of warm MHA medium (temperature 45–50 ℃), then poured into a petri dish (9 cm in diameter). Inoculum preparation: A 0.5 McFarland suspension is prepared (the McFarland bacterial suspension has a density of 5 x 105 CFU/mL). Preparation of broth medium: 0.5 McFarland suspension diluted 100x (9.9 mL broth media + 0.1 mL suspension) to a density of 106 CFU/mL. The suspension is poured into wells containing antibiotics (50 µL bacterial inoculum plus 50 µL liquid medium with antibiotics). The antibiotic suspension used is ciprofloxacin lactate 200 mg/100 mL (PT. Finusolprima Farma International Bekasi-Indonesia). The bacterial inoculum was adjusted to a 0.5 McFarland suspension. The absorbance wavelength of 630 nm is in the range of 0.08 to 0.13. Inoculum was added to liquid or solid media with antibiotics to maintain adequate cell density (CFU/mL). During the test, the medium was aerobically incubated at 35±1℃ for 18–24 hours. The resultant MIC value is the lowest antibiotic concentration at which bacterial growth is inhibited. The concentrations of ciprofloxacin tested were 1, 2, and 4 mg/L. The MIC value was measured following Kowalska-Krochmal & Dudek-Wicher (2021).

Relative Expression Activity of OmpK35/K36 mRNA

The relative expression levels of mRNA from the OmpK35 and OmpK36 genes encoding outer membrane proteins were analyzed using reverse transcription quantitative PCR (RT-qPCR) in ciprofloxacin-resistant *K. pneumoniae*. Isolates that are sensitive to ciprofloxacin were used as controls. The variable was the crossing point (CP) value for cDNA amplification between isolates from different groups. The CP value of the gene detection is normalized with the CP value for the ATP gene (the reference gene in qPCR). Isolates were cultivated on Trypticase Soy Broth (TSB) medium and incubated for 2–3 hours (Fajardo-Lubián et al., 2019).

RNA extraction

Total RNA extraction was performed following the protocol of Zymo Research ZR Fungal/Bacterial RNA MiniPrepTM, California. *K. pneumoniae* cell pellets were resuspended in 800 µL RNA Lysis Buffer and homogenized. The mixture is transferred into the ZR Bashing Bead Lysis Tube and centrifuged at 3000 rpm for 1 minute. The supernatant is transferred into a Zymo-SpinTM IIICG column attached to a collection tube and then centrifuged at 3000 rpm for 1 minute. The filtrate is accommodated, and 96% ethanol is added in a volume ratio of 1:1, then homogenized. The mixture is then transferred into the Zymo-Spin TM IICR Column attached to a collection tube and centrifuged at a speed of 3000 rpm for 1 minute. Discard the filtrate. A total of 400 µL of RNA prep buffer was added to the column and centrifuged at

3000 rpm for 1 minute. Discard the filtrate. A total of 700 µL of RNA wash buffer was added to the column and centrifuged at 3000 rpm for 1 minute. Discard the filtrate. A total of 400 µL of RNA wash buffer was added to the column and centrifuged at 3000 rpm for 1 minute to ensure complete removal of the wash buffer. Then carefully move the column into a nuclease-free tube. In the final stage, 50 µL of RNase-Free Water is added directly to the column matrix and centrifuged at 3000 rpm for 1 minute.

cDNA synthesis

The synthesis was performed using the cDNA RT-qPCR Synthesis Kit (Toyobo ReverTra Ace® qPCR RT Master Mic). The reverse transcription reaction is carried out during the RNA denaturation stage by incubating the RNA solution at 65 ℃ for 5 minutes and then storing it on ice. The PCR mix consisted of 2 µL of 5xRT Master Mix reagents, 1 µL of template RNA, and nuclease-free water. The mixture was incubated at 37 ℃ for 15 minutes, then at 50 ℃ for 5 minutes. The mixture is heated to 98 ℃ for 5 minutes, then stored at -20 ℃.

cDNA analysis with RT-qPCR

The analysis was performed using the Bioline Sensi FAST Lo-ROX Kit reagent. The composition of the reaction mixture is as follows: 2x SensiFAST SYBR®Lo-Rox 10 µL, 10 µM forward primer 0.8 µL, 10 µM reverse primer 0.8 µL, cDNA template up to 8.4 µL, and H2O up to a total volume of 20 µL. PCR conditions are as follows: denaturation at 95 ℃ for 5 seconds, annealing at 60–65 ℃ for 10 seconds, and extension at 72 ℃ for 20 seconds. The cycle is repeated 40 times. An analysis was performed to determine the differences in the relative expression of the two porin channel genes (OmpK35 and OmpK36). The primers used for expression analysis are listed in **Table 1**. The data was analyzed using the $2^{(-\Delta\Delta C\_T)}$ method (Livak & Schmittgen, 2001).

RESULTS AND DISCUSSION

K. pneumoniae, which has been successfully isolated and identified from August-October 2022 at RSUD Prof. Dr. Margono Soekardjo Purwokerto, has special characteristics with resistant distribution in male patients 55.5% and female patients 45.71% (Sulistiyawati et al., 2023). The study provides a picture of the resistance profile of *K. pneumoniae* to antibiotics there are 5 major groups of antibiotics, namely ampicillin, ceftriaxone, gentamycin, ciprofloxacin, and ceftazidime. The specific profile of antibiotic-resistant *K. pneumoniae* is illustrated by porin activation and expression.

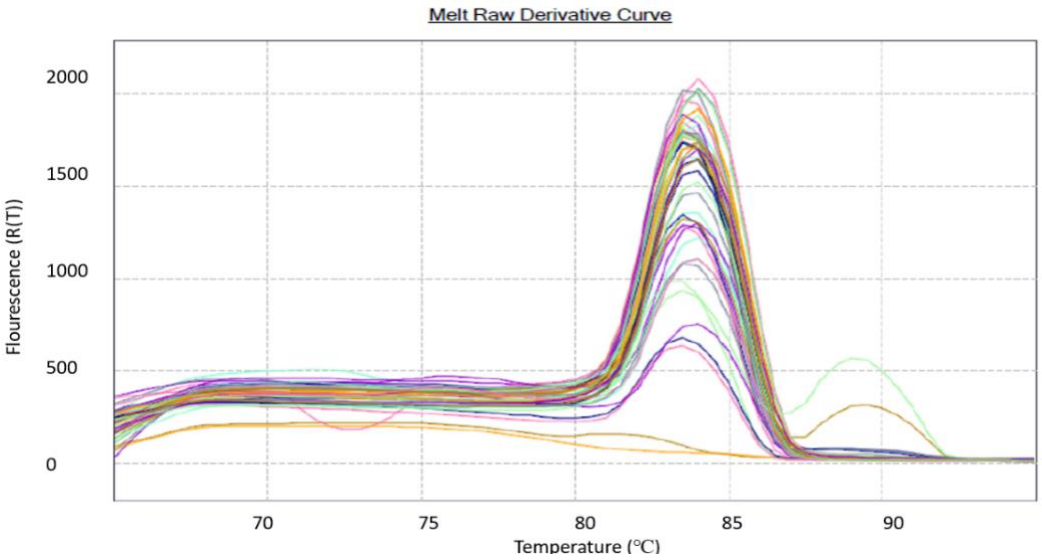
RNA samples were extracted using the Bioline Sensi FAST Lo-ROX Kit. Measurement of extracted RNA concentration using a nanodrop spectrophotometer obtained an average RNA concentration of 480.81 ng/µL. All samples were uniformized by adjusting the lowest concentration before entering the cDNA synthesis stage. Measurements of cDNA concentration and purity were performed at wavelengths of 260 and 280 nm (A260/280). The measured cDNA concentration averaged 1,393 ng/µL, with average cDNA purity ratios of 1.876 and 1.862.

Amplification of the OmpK35/K36 gene and reference gene (ATP) with qPCR showed that the primers used had fairly good specificity. Although some curves have more than one melting peak that indicates contamination and the formation of primary dimers (primary secondary structures), the majority show a picture of curves that coincide with one peak point (**Figure 1, *a***). The amplification curve is sigmoid, with a straight line indicating the amplification threshold. The threshold line is the minimum threshold at which the device can detect gene amplification in 40 cycles. Meanwhile, the cycle threshold (CT) value is the intersection between the threshold line and the amplification curve, showing the number of cycle sequences of each gene that began to be detected by the machine (**Figure 1, *b***). Figure 3 shows the sigmoid curve at OmpK35/36 amplification intersecting with the threshold line. The intersection of the sigmoid curve with the threshold line shows the CT value of the OmpK35/K36 gene with the ATP reference gene (**Table 2**).

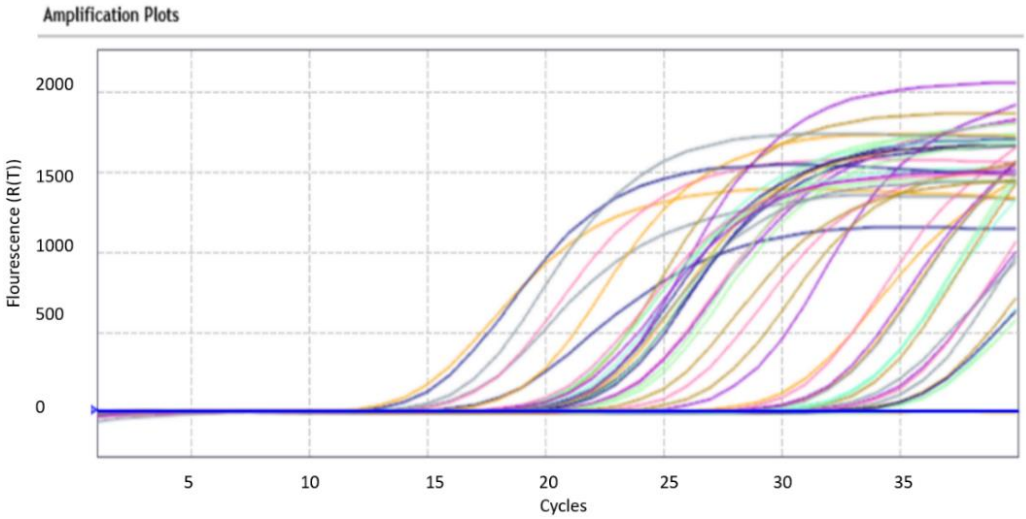
Fold change values in two groups of genes, OmpK35 and OmpK36, obtained by the formula $2^{-\Delta\Delta CT}$ result in average expression values of 0.8925 and 0.5877, respectively. Fold change values < 2 (less than 2) indicate low expression of the OmpK35 and K36 genes. An independent statistical t-test showed no significant difference in the expression of the OmpK35 gene with K36 ($p > 0.05$).

Table 1. Primers used in porin mRNA relative expression studies

Gene	Primer Set	Sequence	Reference
OmpK35	OmpK35-F	GAAGGTTCCCAGACCACAA	(Matovina et al., 2021)
	OmpK35-R	ACGGCCATAGTCGAATGAAC	
OmpK36	OmpK36-F	GACCAGACCTACATGCGTGTA	(Matovina et al., 2021)
	OmpK36-R	GTATTCCCACTGGCCGTAAC	
ATP	ATPs-F	TGGTTCTCGGTCTGCTGTTC	(Matovina et al., 2021)
	ATPs-R	TGGAATTTCCCTGGGTACGCC	



(a)



(b)

Figure 1. OmpK35/K36 primary melt curve graph **(a);** amplification curve graph OmpK35/K36 **(b).**

Table 2. C_T values of OmpK35, OmpK36, and ATP in the sample

No.	Gene	C_T value (Mean±SD)	ΔC_T* value (Mean±SD)
1.	OmpK35	23.19±6.38	-0.648±4.62
2.	OmpK36	27.70±6.83	3.502 ± 4.62
3.	ATP	25.62±6.18	

Note: The mean total C_T ATP is 25.62.
*ΔC_T Value = C_T OmpK35/K36 – Mean total C_T ATP

Table 3. Relative quantification analysis and independent t-test of OmpK35 and OmpK36 gene expression

No.	Gene	Mean ΔC_T	$\Delta\Delta C_T$ value	Fold change*			Independent t-test
				Mean	Lower	Upper	
1.	OmpK35	-0.6486	2.9644	0.8925	0.0003	3.4822	0.095
2.	OmpK36	3.5022	3.8902	0.5877	0.0002	2.3005	

Note: $\Delta\Delta C_T$ value = Mean ΔC_T OmpK35 resistant sample – Mean ΔC_T control sample
*Fold change is the result of the formula: $2^{-\Delta\Delta C_T}$

Table 4. Ciprofloxacin MIC value with resistance variable parameter

		MIC (mg/L)		
		1	2	≥4
Absorbance value (OD)	Minimum	0.55	0.06	0.03
	Maximum	1.25	0.93	1
	Mean (average)	0.58	0.49	0.28
Bacterial cell count	Minimum	3	1	1
	Maximum	75	72	79
	Mean (average)	32	19	34
Inhibition (%)	Minimum	3.51	7.23	2.95
	Maximum	91.31	90.57	93.92
	Mean (average)	31.51	34.72	47.22

The relationship between relative expression of RNA OmpK35/K36 and porin permeability (MIC value) in this study was analyzed using SPSS Pearson correlation statistics. This study showed that the relative expression of OmpK35 RNA correlated with MIC values (1, 2, and 4 mg/L) in *K. pneumoniae* against ciprofloxacin, namely $p = 0.029 < 0.05$ with regression values $R = 0.355$.

The relative expression of OmpK36 RNA correlates with the MIC value (1, 2, and 4 mg/L) in *K. pneumoniae* against ciprofloxacin, which is $p = 0.016 < 0.05$ with a regression value of $R = 0.481$. The analysis illustrates that the value of $p<0.05$ means a correlation between the expression of the OmpK35/K36 gene and the MIC value of ciprofloxacin against *K. pneumoniae*. The degree of relationship of the R-value in the expression OmpK35 $R = 0.355$ means that it is weakly correlated, while in the expression OmpK36 $R = 0.481$ it is moderately correlated (**Table 5**).

Porins have an important role in the interaction between bacteria and the environment. Porins are present in large quantities in the outer membrane of Gram-negative bacteria. Many porins were identified in several Enterobacteriaceae groups, e.g., OmpC and OmpF porins. This study analyzed the presence of OmpK35 and OmpK36 in *K. pneumoniae*, which are homologous to OmpC and OmpF. OmpK35 and OmpK36 facilitate porin channels as a means by which various antibiotics can p enetrate the cell wall of *K. pneumoniae.* As has been reported in previous studies, the absence of porin expression and the tendency to decrease its expression are important causes of *K. pneumoniae*'s resistance to some antimicrobials. Several mechanisms of *K. pneumoniae* resistance are associated with a loss or decrease in porin expression combined with ESBL production (Zhang et al., 2015).

The fold change value obtained from the relative quantification of outer membrane proteins OmpK35 and OmpK36 is 0.8925 and 0.5877, respectively. This indicates a low expression value or absence of expression. In antibiotic-resistant *K. pneumoniae*, the majority of isolates showed decreased expression of OmpK35/K36 when compared to controls. These results are in line with (Shakib et al., 2012), who reported that clinical isolates of antibiotic-resistant β-lactam *K. pneumoniae* (ESBL) simultaneously lacked porin expression of OmpK35 and OmpK36. High porous expression is exhibited by sensitive *K. pneumoniae*. Zhang et al. (2015) reported that 33 strains of hypervirulent *K. pneumoniae* were highly resistant to carbapenem with MIC values greater than 32 μg/mL, accompanied by decreased expression of OmpK35/36 and their ability to produce ESBL.

A study at Croatian University Hospital from 2012–2014 showed a decrease in the expression of nonselective main porin channels OmpK35 and OmpK36 in ertapenem-resistant ESBL-producing *K. pneumoniae* (Matovina et al., 2021). Studies comparing porin expression of OmpK35 and OmpK36 in isolates resistant or intermediately resistant to several lactamase antibiotics confirm that decreased expression of OmpK35/36 porin channels is involved in the development of resistance. The presence of mutations in these genes or decreased or lost porin expression has previously been reported as a mechanism of resistance to some (Bi et al., 2017; Palmeiro et al., 2019).

Table 5. Pearson correlation analysis of OmpK35/K36 RNA expression and MIC values

Gene	R-value	F Count	p-value
OmpK35	0.355	5.204	0.029*
OmpK36	0.418	6.556	0.016*

Note: *The p-value ($p < 0.05$) indicates a correlation between the two factors, dependent and independent variables.

The results of the statistical analysis showed that there was no significant difference in the relative expression of the OmpK35 and OmpK36 genes ($p > 0.05$). Matovina et al., (2021) found that the expression of OmpK36 and OmpK35 porins was not statistically significant. Doménech-Sánchez et al., (2000) reported that decreased expression of OmpK35/K36 in clinical isolates of *K. pneumoniae* showed higher antibiotic resistance than strains with high expression of OmpK35/K36. Similarly, Zhang et al., (2015) reported that carbapenem-resistant *K. pneumoniae* isolates that produce ESBL decreased OmpK35/K36 expression and were highly virulent in mouse lethality tests.

Porins are present in many outer membranes by forming nonspecific diffusion channels that allow small polar molecules (<600 Da) to diffuse across the membrane barrier. The change or loss of expression of the main nonspecific porin is related to its ability to produce broad-spectrum β-lactamase. Decreased outer membrane permeability can increaseβ-lactamase production 3-fold in *K. pneumoniae* KP3800 (Kim et al., 2020). Papagiannitsis et al., (2013) observed the porin variant profiles of OmpK35 and OmpK36. Carbapaneme-resistant *K. pnuemoniae* had no specific differences in the expression of the two porin variants, OmpK35 and OmpK36.

The results of the analysis showed a weak correlation between the relative expression of OmpK35/K36 RNA and porin permeability (MIC value) ($p<0.05$). Similarly, Sugawara et al., (2016) found that deletion of the OmpK35 gene resulted in a significant increase in MIC values for various β-lactam antibiotics, despite low OmpK35 expression levels. The main porins of *K. pneumoniae*, OmpK35 and OmpK36, produce channels larger than their homologues (OmpF and OmpC) in *E. coli*. OmpK35 porins in particular show very high permeability to hydrophobic compounds (e.g., benzylpenicillin) and large compounds (e.g., cefepime). Modification of outer membrane permeability (OM) is particularly important in *K. pneumoniae* resistance. Antibiotic molecules must use OmpK35 and OmpK36 porins, the number of which is reduced and modified to decrease membrane permeability (Moya & Maicas, 2020; Villa et al., 2014). In this study, a decrease in the relative expression of porin mRNA in OmpK35/K36 correlated with porin permeability (MIC value). It is indicated by an increase in the MIC value to a maximum value, i.e., 4 mg/L. Chevalier et al., (2000) reported that the increase in MIC values was eightfold higher in *K. pneumoniae* KP55 isolates compared to KP63. This is due to a decrease in Omp expression and target modification. Wassef et al., (2015) also concluded that *K. pneumoniae* resistance is characterized by a mechanism of loss of OmpK35/36 or a decrease in its expression, which can increase the value of the MIC against antibiotics.

Porin expression in *K. pneumoniae* had an impact on patient treatment. With a decrease in the relative expression of mRNA porin Omp K35/K36 in *K. pneumoniae* makes it increasingly resistant to ciprofloxacin. This case indicates the need for alternative use of combination drug therapy given to patients. The use of appropriate combinations of antibiotics is recommended to minimize the increase in resistance in *K. pneumoniae*.

CONCLUSIONS

Clinical isolates of ciprofloxacin-resistant *K. pneumoniae* showed decreased relative expression of OmpK35 and OmpK36 mRNAs. The relative expression of OmpK35/K36 RNA has a weak correlation to porin permeability. This indicates increased resistance of *K. pneumoniae* to ciprofloxacin.

ACKNOWLEDGMENTS

This research was supported by the "Beasiswa Unggulan" Grant from the Ministry of Education, Culture Research and Technology. The author thanks Hendro Pramono, Dini Riyandini, and Anwar Rovik for their discussion and editing manuscript.

REFERENCES

Bajaj, H., Acosta Gutierrez, S., Bodrenko, I., Malloci, G., Scorciapino, M. A., Winterhalter, M., & Ceccarelli, M. (2017). Bacterial outer membrane porins as electrostatic nanosieves: exploring transport rules of small polar molecules. *American Chemical Society (ACS) Nano*, *11*(6), 5465–5473. https://doi.org/10.1021/acsnano.6b08613

Bi, W., Liu, H., Dunstan, R. A., Li, B., Torres, V. V. L., Cao, J., Chen, L., Wilksch, J. J., Strugnell, R. A., Lithgow, T., & Zhou, T. (2017). Extensively drug-resistant *Klebsiella pneumoniae* causing nosocomial bloodstream infections in China: Molecular investigation of antibiotic resistance determinants, Informing therapy, and clinical outcomes. *Frontiers in Microbiology*, *8*(JUN). https://doi.org/10.3389/fmicb.2017.01230

Blackwell, W. (2020). *Bacterial resistance to antibiotic-from molecules to man* (B. B. Bonev & M. B. Nocholas (eds.); First edit). John Willey & Sons, Inc.

Chevalier, J., Pagès, J. M., Eyraud, A., & Malléa, M. (2000). Membrane permeability modifications are involved in antibiotic resistance in *Klebsiella pneumoniae*. *Biochemical and Biophysical Research Communications*, *274*(2), 496–499. https://doi.org/10.1006/bbrc.2000.3159

Doménech-Sánchez, A., Pascual, A., Suárez, A. I., Alvarez, D., Benedí, V. J., & Martínez-Martínez, L. (2000). Activity of nine antimicrobial agents against clinical isolates of *Klebsiella pneumoniae* producing extended-spectrum β-

lactamases and deficient or not in porins [9]. *Journal of Antimicrobial Chemotherapy*, *46*(5), 858–860. https://doi.org/10.1093/jac/46.5.858

Fajardo-Lubián, A., Ben Zakour, N. L., Agyekum, A., Qi, Q., & Iredell, J. R. (2019). Host adaptation and convergent evolution increases antibiotic resistance without loss of virulence in a major human pathogen. *PLoS Pathogens*, *15*(3), 1–30. https://doi.org/10.1371/journal.ppat.1007218

Hamzaoui, Z., Ocampo-Sosa, A., Maamar, E., Fernandez Martinez, M., Ferjani, S., Hammami, S., Harbaoui, S., Genel, N., Arlet, G., Saidani, M., Slim, A., Boutiba-Ben Boubaker, I., & Martinez-Martinez, L. (2018). An outbreak of NDM-1-producing *Klebsiella pneumoniae*, associated with OmpK35 and OmpK36 porin loss in Tunisia. *Microbial Drug Resistance*, *24*(8), 1137–1147. https://doi.org/10.1089/mdr.2017.0165

Kim, T. H., Tao., X., Moya., B., & Jiao., Y. (2020). Novel cassette assay to quantify the outer membrane permeability of five β-lactams simultaneously in carbapenem-resistant *Klebsiella pneumoniae* and enterobacter cloacae. *American Society for Microbiology MBio*, *11*(1), 1–15.

Kowalska-Krochmal, B., & Dudek-Wicher, R. (2021). The minimum inhibitory concentration of antibiotics: Methods, interpretation, clinical relevance. *Pathogens*, *10*(2), 1–21. https://doi.org/10.3390/pathogens10020165

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Matovina, M., Abram, M., Repac-Antić, D., Knežević, S., & Bubonja-Sonje, M. (2021). An outbreak of ertapenem-resistant, carbapenemase-negative and porin-deficient ESBL-producing *Klebsiella pneumoniae* complex. *Germs*, *11*(2), 199–210. https://doi.org/10.18683/germs.2021.1257

Moya, C., & Maicas, S. (2020). *Antimicrobial resistance in Klebsiella pneumoniae strains: Mechanisms and outbreaks*. 11. https://doi.org/10.3390/proceedings2020066011

Nirwati, H., Sinanjung, K., Fahrunissa, F., Wijaya, F., Napitupulu, S., Hati, V. P., Hakim, M. S., Meliala, A., Aman, A. T., & Nuryastuti, T. (2019). Biofilm formation and antibiotic resistance of *Klebsiella pneumoniae* isolated from clinical samples in a tertiary care hospital, Klaten, Indonesia. *BMC Proceedings*, *13*(Suppl 11), 1–8. https://doi.org/10.1186/s12919-019-0176-7

Palmeiro, J. K., de Souza, R. F., Schörner, M. A., Passarelli-Araujo, H., Grazziotin, A. L., Vidal, N. M., Venancio, T. M., & Dalla-Costa, L. M. (2019). Molecular epidemiology of multidrug-resistant *Klebsiella pneumoniae* isolates in a Brazilian tertiary hospital. *Frontiers in Microbiology*, *10*(July), 1–11. https://doi.org/10.3389/fmicb.2019.01669

Papagiannitsis, C. C., Giakkoupi, P., Kotsakis, S. D., Tzelepi, E., Tzouvelekis, L. S., Vatopoulos, A. C., & Miriagou, V. (2013). OmpK35 and OmpK36 porin variants associated with specific sequence types of *Klebsiella pneumoniae*. *Journal of Chemotherapy*, *25*(4), 250–254. https://doi.org/10.1179/1973947813Y.0000000075

Shakib, P., Ghafourian, S., Zolfaghary, M. R., Hushmandfar, R., Ranjbar, R., & Sadeghifard, N. (2012). Prevalence of OmpK35 and OmpK36 porin expression in beta-lactamase and non-beta-lactamase-producing *Klebsiella pneumoniae*. *Biologics: Targets and Therapy*, *6*, 1–4. https://doi.org/10.2147/btt.s27582

Silver, L. L. (2016). A Gestalt approach to Gram-negative entry. *Bioorganic and medicinal chemistry*, *24*(24), 6379–6389. https://doi.org/10.1016/j.bmc.2016.06.044

Sugawara, E., Kojima, S., & Nikaido, H. (2016). *Klebsiella pneumoniae* major porins OmpK35 and OmpK36 allow more efficient diffusion of Beta-Lactams than their *Escherichia coli* homologs OmpF dan OmpC. *Journal of Bacteriology*, *198*(23), 3200–3208. https://doi.org/10.1128/JB.00590-16.Editor

Sulistiyawati, I., Wahyono, D. J., & Siswandari, W. (2023). Prevalence and diversity of ciprofloxacin-resistant *Klebsiella pneumoniae* clinical isolates. *Proceeding ICMA-SURE*, *2*(1), 38. https://doi.org/10.20884/2.procicma.2023.2.1.7724

Villa, L., Feudi, C., Fortini, D., Garciá-Fernández, A., & Carattoli, A. (2014). Genomics of KPC-producing *Klebsiella pneumoniae* sequence type 512 clone highlights the role of RamR and ribosomal S10 protein mutations in conferring tigecycline resistance. *Antimicrobial Agents and Chemotherapy*, *58*(3), 1707–1712. https://doi.org/10.1128/AAC.01803-13

Wassef, M., Abdelhaleim, M., Abdulrahman, E., & Ghaith, D. (2015). The role of OmpK35, OmpK36 porins, and production of β-lactamases on imipenem susceptibility in *Klebsiella pneumoniae* clinical isolates, Cairo, Egypt. *Microbial Drug Resistance*, *21*(6), 577–580. https://doi.org/10.1089/mdr.2014.0226

Winterhalter, M. (2021). Antibiotic uptake through porins located in the outer membrane of Gram-negative bacteria. *Expert Opinion on Drug Delivery*, *18*(4), 449–457. https://doi.org/10.1080/17425247.2021.1847080

Zhang, D., Micek, S. T., & Kollef, M. H. (2015). Time to appropriate antibiotic therapy is an independent determinant of postinfection ICU and hospital lengths of stay in patients with sepsis. *Critical Care Medicine*, *43*(10), 2133–2140. https://doi.org/10.1097/CCM.0000000000001140