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N. O. Vrynchanu[1], L. B. Zelena[2], N. I. Humeniuk[1], E. M. Vazhnichaya[3]

Antivirulent activity of macrolides against *P. aeruginosa*

[1]State Institution "Institute of Pharmacology and Toxicology of the National Academy of Medical Sciences of Ukraine", Kyiv

[2]D. K. Zabolotny Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, Kyiv

[3]Poltava State Medical University, Poltava

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Respiratory diseases are a serious health problem worldwide. In the European Union respiratory diseases (influenza, chronic obstructive pulmonary disease, pneumonia, and asthma) are the main cause of mortality in patients. In particular, in 2022, about 363,500 people died from respiratory diseases in the EU countries, which is 7% of all mortality in the population [1]. A significant place among the inflammatory processes of both the upper and lower respiratory tracts belongs to the bacterial microbiota, including *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiella pneumoniae,* etc. [2]. One of the groups of antimicrobial preparations (AMPs) used in respiratory diseases are azithromycin and clarithromycin (second-generation macrolides), which are characterized by the presence of a macrocyclic lactone ring containing ≥ 12 elements in the molecule [3]. The mechanism of the antibacterial effect of macrolides is realized by inhibiting protein synthesis in the microbial cell due to binding to the 23S part of the 50S subunit of the bacterial ribosome, which leads to disruption of the translocation of aminoacyl-tRNA from the A-site to the P-site of the ribosome [4, 5]. Macrolide antibiotics differ in pharmacokinetic parameters [6], in particular in their concentration in tissues, blood plasma, and interstitial fluid, which affects the duration of therapy.

Given their sufficient oral bioavailability, tissue penetration, and broad spectrum of activity against most pathogens of respiratory tract diseases, macrolides have long been used as first-line drugs for respiratory infections [7]. However, despite the effectiveness of these antimicrobial agents for a long time, there is currently a limitation on their use as first-line drugs for empirical antibiotic therapy of infectious diseases of the upper and lower respiratory tract due to the problem of antibiotic resistance. In particular, according to data [8], the number of clarithromycin-resistant strains of *H. influenzae* increased from 0.7% (2015–2019) to 12.6% (2022–2023). Currently, the use of macrolides in medical practice is regulated by the Standard of Medical Care "Rational Use of Antibacterial and Antifungal Drugs for Therapeutic

and Prophylactic Purposes" (Ministry of Health of Ukraine, 2023) [9].

Macrolide antibiotics, in addition to their ability to affect planktonic cells of Gram-positive microorganisms, have an antibiofilm effect, in particular against *P. aeruginosa* biofilms. By affecting Quorum sensing (QS), azithromycin and clarithromycin inhibit the adhesion of *P. aeruginosa* cells to the surface, disrupt the synthesis of exopolysaccharides and virulence factors [10–12]. Macrolides also inhibit the synthesis of key virulence factors of the *P. aeruginosa*: elastase, protease, pyocyanin, flagella and pili, which lead to disruption of biofilm formation and reduced tissue damage to the macroorganism [13]. The antivirulent effect of these drugs is also realized by modulating the host immune response, reducing inflammation, which is important in chronic infections, in particular in cystic fibrosis complicated by *P. aeruginosa* at late stages of the disease [14]. According to clinical trials, the use of azithromycin leads to a slight but sustained improvement in respiratory function, a decrease in the risk of exacerbations, and an increase in the remission period to 6 months. However, information regarding the mechanisms of action of macrolides on the genetic regulation of their antivirulence effect is limited and requires comprehensive research.

The aim of the study – to evaluate the effect of macrolide antibiotics azithromycin and clarithromycin on virulence factors and activity of genes associated with them in *P. aeruginosa*.

Materials and methods. Azithromycin (Ningxia Qiyvan pharmaceutical CO., LTD, China) and clarithromycin (Zhejiang guobang pharmaceutical CO., LTD, China) were used in the study. The solvent was 95% ethanol, the concentration of the stock solution was 1 mg/ml. All other chemicals were obtained from commercial sources.

The antivirulence properties of macrolides were investigated using the test strain *P. aeruginosa* 449 and the nutrient media Luria-Bertani broth (Conda, Spain), Luria-Bertani agar and Tryptone Soya Broth (TSB, HiMedia, India). The clinical isolate *P. aeruginosa* 449 is resistant to cefepime and sensitive to aztreonam, cefoperazone, ciprofloxacin, gentamicin and amikacin, as determined by [15].

The minimum inhibitory concentration (MIC) of macrolides was determined by the serial microdilution method according to [16]. The study used a 1-day culture grown at 37 °C for 18–24 h.

The antivirulence properties of macrolides against *P. aeruginosa* were assessed by their effect on hemolytic and protease activity, bacterial motility and expression of genes responsible for QS-dependent processes.

The effect of macrolide AMPs on the hemolytic activity of the test strain *P. aeruginosa* was carried out according to [17] at concentrations of: 0.15 MIC, 0.25 MIC and 0.5 MIC. The amount of released hemoglobin in the supernatant was estimated on the "Adsorbance Microplate Reader ELx × 800" (BioTek, USA) at a wavelength of 540 nm. Positive control was erythrocytes lysed with 0.1% sodium dodecyl sulfate solution.

The effect of azithromycin and clarithromycin on the protease activity of *P. aeruginosa* was studied using agar with 1.5% casein [18]. Bacterial culture was grown in liquid TSB medium with the addition of the test substances at concentrations of 0.15 MIC, 0.25 MIC and 0.5 MIC or without them (control). Incubation duration – 18 h at 37 °C. After the end of the incubation period, the overnight culture (50 µl) was added to the agar wells and incubated

for 16–18 h at 37 °C. The effect of macrolide antibiotics on the protease activity of *P. aeruginosa* was assessed by the diameter of the proteolysis zones, which were detected using 5% solution of trichloroacetic acid.

To determine the ability of macrolides to affect the motility of the test strain *P. aeruginosa*, a 16-h culture was incubated with azithromycin or clarithromycin (0.5 MIC and 2.0 MIC) for 30–45 min at 37 °C. The study was carried out according to [19, 20]. The motility of *P. aeruginosa* cells was assessed by the diameter of the zone formed by bacteria migrating from the inoculation point.

Swimming-migration of *P. aeruginosa* cells was studied using 0.3% agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.3% agar). A cell suspension was added to Petri dishes with agar medium and incubated for 16–20 h at room temperature.

Swarming migration was determined on the surface of 0.5 % agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.5% agar, 1 M $MgSO_4$, 0.5% glucose). Bacterial cells (2 µl) were added to the upper part of the semi-solid agar. Incubation time was 16–24 h at 37 °C.

Twitching migration of bacteria was studied using 1% agar medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 1% agar). *P. aeruginosa* cells were added to the bottom of the dish through the agar layer. After incubation for 24–48 h at 37 °C, the growth zone at the interface between the agar and the glass surface was recorded after staining the attached *P. aeruginosa* cells with a crystal violet solution.

Analysis of gene expression in *P. aeruginosa* under macrolide treatment was carried out by quantitative real-time polymerase chain reaction (qRT-PCR). Total RNA was isolated from a 24-h suspension of bacterial cells cultured with or without azithromycin or clarithromycin (0.5 MIC) using TRIzol Reagent (Invitrogen) followed by DNase I treatment. Amplification was performed using LunaR Universal One-Step RT-qPCR Kit (New England BioLabs Inc.) on QuantStudio™ 3 Real-Time PCR System (Applied Biosystems). The reaction mixture contained 10 µl of 2x Luna Universal One-Step Reaction Mix, 1 µl of 20x Luna WarmStart® RT Enzyme Mix, 10 pmol of each primer (forward and reverse; see Table 1) and 2 µl of bacterial RNA. The total volume of 20 µl was adjusted with nuclease free water.

The qRT-PCR cycling conditions were: 1 min at 55 °C, 1 min at 95 °C, followed by 45 cycles of 10 s at 95 °C, 15 s at 55–58 °C, 1 min at 72 °C. Expression of the target genes was normalized to the expression of 16S rRNA gene used as the reference control. Relative gene expression levels were calculated using $2^{-\Delta\Delta Ct}$ method [27].

The ANOVA method was used to evaluate the research results. Statistical processing was performed using the computer program "Statistica 6.0" (StatSoft. Inc., USA). The research data are presented in the form of M ± m, where M is the mean value, m is the standard error of the mean.

Results and discussion. The study of the antimicrobial activity of macrolides showed that the MIC of azithromycin against the clinical strain *P. aeruginosa* 449 was 25 µg/ml, and that of clarithromycin was 100 µg/ml.

Effect of macrolides on protease and hemolytic activity. One of the virulence factors of the *P. aeruginosa* are extracellular proteases, including alkaline protease (AprA), protease IV (PIV), elastase A (staphylolysin, LasA) and elastase B (pseudolysin, LasB), large exoprotease A (LepA), *P. aeruginosa*'s aminopeptidase (PAAP),

Table 1

Oligonucleotides used in molecular genetic analysis

Primer	Sequence (5'–3')	Reference
lasI (Fw) *lasI* (Rv)	5'-CGCACATCTGGGAACTCA-3' 5'-CGGCACGGATCATCATCT-3'	[21]
lasR (Fw) *lasR* (Rv)	5'-CTGTGGATGCTCAAGGACTAC-3' 5'-AACTGGTCTTGCCGATGG-3'	[21]
rhlR (Fw) *rhlR* (Rv)	5'-GCCAGCGTCTTGTTCGG-3' 5'-CGGTCTGCCTGAGCCATC-3'	[21]
pqsR (Fw) *pqsR* (Rv)	5'-CTGATCTGCCGGTAATTGG-3' 5'-ATCGACGAGGAACTGAAGA-3'	[21]
exoS (Fw) *exoS* (Rv)	5'-GGCGGATGCGGAAAAGTAC-3' 5'-CTGACGCAGAGCGCGATT-3'	[22]
toxA (Fw) *toxA* (Rv)	5'-GGTAACCAGCTCAGCCACAT-3' 5'-TGATGTCCAGGTCATGCTTC-3'	[23]
exoA (Fw) *exoA* (Rv)	5'-GACAACGCCCTCAGCATCACCAGC-3' 5'-CGCTGGCCCATTCGCTCCAGCGCT-3'	[24]
aprA (Fw) *aprA* (Rv)	5'-GCTTCAGCCAGAACCAGAAGAT-3' 5'-TCGACACATTGCCCTTCAAC-3'	[25]
16S-1 (Fw) *16S-2* (Rv)	5'-CTGTCGTCAGCTCGTGTTGT-3' 5'-TTCATGGAGTCGAGTTGCAG-3'	[26]

Note. Fw – forward primer; Rv – reverse primer.

MucD and small Pseudomonas protease (PASP), which play a leading role in the pathogenesis of the disease, cause proteolytic damage to host tissues and negatively affect the innate immunity of the host [28, 29]. Proteases play a key role in the formation of biofilms; impairment of their synthesis is important in lower respiratory tract infections, particularly in cystic fibrosis [30]. Protease synthesis is inhibited by tetracycline, chloramphenicol, and polymyxin B, with tetracycline acting at subinhibitory concentrations (sub-MIC), while the others are active at concentrations ≥ MIC [31].

The effect of macrolide antibiotics azithromycin and clarithromycin on the protease activity of *P. aeruginosa* 449 is shown in Figure 1.

The results of the experiments showed (Figure 1) that the protease activity of the *P. aeruginosa* under the action of azithromycin and clarithromycin decreases compared to the control by 10.1–19.7% and 19.7–24.8%, respectively. Our data on the effect of macrolides on the *P. aeruginosa* protease activity are consistent with the literature. In particular, according to [13] azithromycin is an inhibitor of protease synthesis, and in sub-MIC it disrupts the functioning of QS systems. Another macrolide antibiotic, clarithromycin, also inhibits protease production [31].

The pathogenesis of opportunistic infections caused by *P. aeruginosa* is multifactorial, as evidenced by a large number of cell-associated and

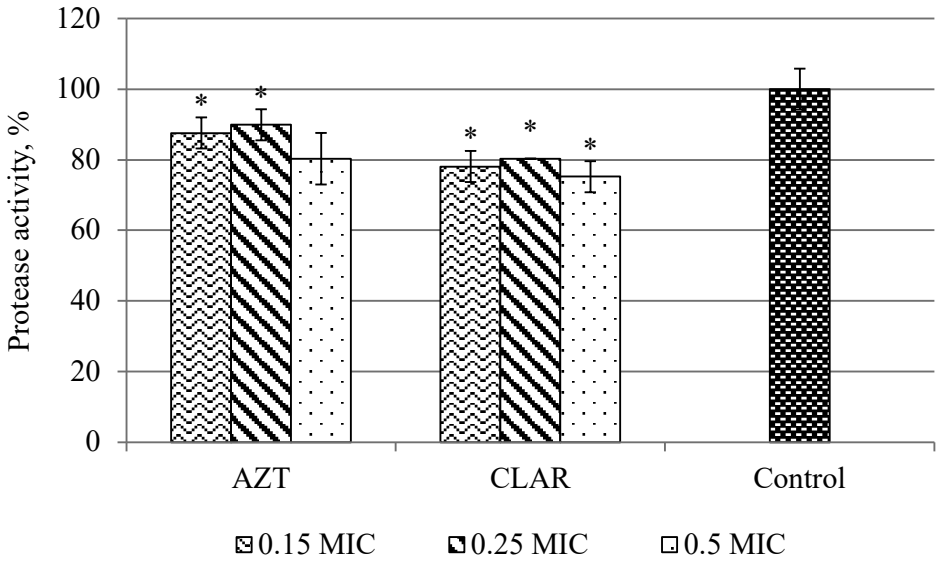

Fig. 1. Protease activity of P. aeruginosa 449 under the action of macrolides (% relative to the control, taken as 100%)
*Note. AZT – azithromycin; CLAR – clarithromycin; *p < 0.05 compared to control; m value for 0.25 MIC of clarithromycin is 0.*

extracellular virulence factors, some of which contribute to colonization, while others contribute to bacterial invasion. The most potent toxigenic factor of *P. aeruginosa* is hemolysins, which exert a toxic effect on host cells and disrupt the function of immune cells. The ability of *P. aeruginosa* to initiate respiratory infection is associated with the degree of hemolysin production [32].

When studying the effect of macrolides on the hemolytic activity of *P. aeruginosa*, it was found that the most pronounced inhibitory effect of azithromycin was registered at concentrations of 0.15 MIC and 0.25 MIC: the activity of extracellular hemolysins decreased by 10.1 times and 10.4 times, respectively ($p < 0.05$) (Figure 2). When the concentration of azithromycin increased to 0.5 MIC, the activity of hemolysins decreased by 1.3 times ($p > 0.05$).

According to the results of the study, azithromycin inhibits the activity of cell-associated hemolysins of *P. aeruginosa*: at 0.15 MIC, their production decreases by 2.9 times, at 0.25 MIC – by 3.7 times, at 0.5 MIC – by 3.4 times compared to the control. According to the data obtained, clarithromycin also inhibits the activity of hemolysins, but is inferior to azithromycin. Under its influence at a concentration of 0.15 MIC and 0.5 MIC, the hemolytic activity of *P. aeruginosa*, caused by extracellular hemolysins, decreases by 2.7 and 1.8 times, respectively, the activity of cell-associated hemolysins (within the range from 0.15 MIC to 0.5 MIC) decreases by 1.4–1.8 times, compared to the control.

Effect of macrolides on motility. In the formation of biofilms on a biotic or abiotic surface, an important role is played by mechanisms that provide bacteria with movement along the substrate, which is one of the factors of adhesion

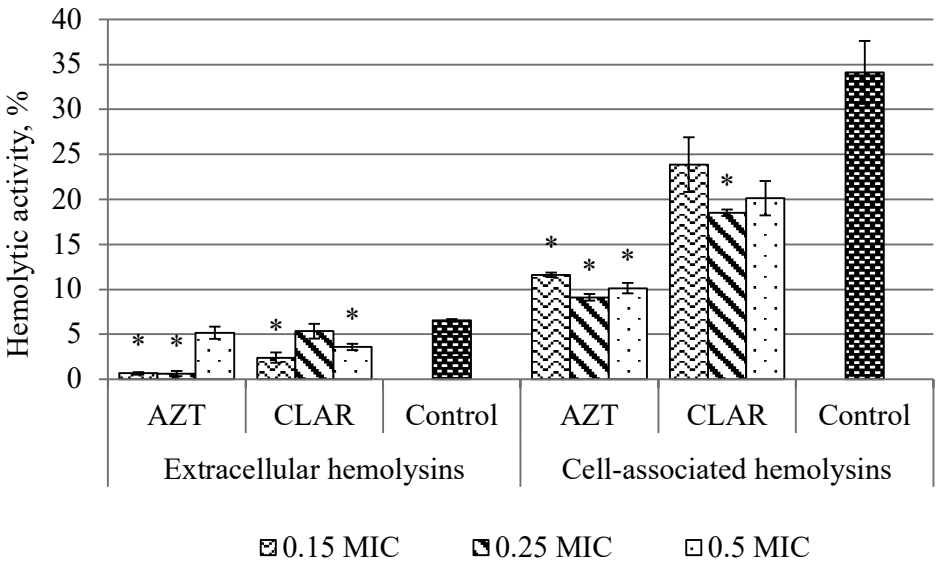

Fig. 2. Hemolytic activity of P. aeruginosa 449 under the action of macrolides (% relative to positive control)
*Note. AZT – azithromycin; CLAR – clarithromycin; *p < 0.05 compared to control.*

and biofilm formation. In a liquid medium, microorganisms move using flagella, which allows them to respond to attractants and repellents. For solid surfaces or tissues, colonization pili are necessary, which are also responsible for intercellular aggregation. Flagella and type IV pili are important for swarming; these structures are involved in mucus colonization. *P. aeruginosa* strains with altered swarming motility have defects in biofilm formation, which indicates its important role in the early stages of biofilm formation. Conversely, strains with a swarming phenotype were more resistant to antibiotics (ciprofloxacin, gentamicin, polymyxin) [33, 34].

The results of the study of the macrolides effect on the motility of *P. aeruginosa* are given in Table 2.

According to the data in Table 2, the studied macrolides affect the motility of the *P. aeruginosa* cells: the inhibitory effect depends on the type of migration, the drug and its concentration. In particular, the migration zone of *P. aeruginosa* in the thickness of the agar medium ("swimming") at 0.5 MIC decreases under the influence of azithromycin by 59.2% and clarithromycin – by 20.4% compared to the control ($p < 0.05$). At 2.0 MIC, a more pronounced inhibition of the motility is observed: under the influence of azithromycin: the diameter of the zones decreases by 70.1% ($p < 0.05$), while clarithromycin – by 10.4% ($p > 0.05$). According to the results of the study, azithromycin and clarithromycin inhibit the swarming-migration of *P. aeruginosa* cells: at a concentration of 0.5 MIC the inhibition is 35.8% and 41.6%, respectively, at 2.0 MIC – 64.0% and 30.2% versus control ($p < 0.05$). It was found that macrolides do not lead to statistically significant changes in the twitching-migration of cells, however, under the influence of azithromycin at 0.5 MIC, the motility decreases by 22.9%, and the AMPs

Table 2

Effect of azithromycin and clarithromycin on the P. aeruginosa motility

Conditions of the experiment	Zones of the microorganisms motility, mm		
	swimming	twitching	swarming
Azithromycin 0.5 MIC	13.7 ± 1.67*	3.7 ± 0.21	25.5 ± 1.44*
Azithromycin 2.0 MIC	10.0 ± 1.0*	3.5 ± 0.34	14.3 ± 0.67*
Clarithromycin 0.5 MIC	26.7 ± 2.33*	5.3 ± 1.02	23.2 ±1.09*
Clarithromycin 2.0 MIC	30.0 ± 0.57	3.8 ± 0.48	27.7 ± 2.19*
Control	33.5 ± 0.05	4.8 ± 0.57	39.7 ± 0.25

*Note. MIC – minimum inhibitory concentration; *p < 0.05 compared to control.*

of azithromycin and clarithromycin at 2.0 MIC – by 27.1% and 20.8%, respectively.

Expression of genes regulating the virulence factors synthesis. P. aeruginosa is able to adapt to the adverse environment in the host organism by producing various factors that contribute to infection and disease development, including the AprA protein, exoenzyme S (ExoS), and exotoxin A (ExoA).

The synthesis of the membrane-associated protein AprA, which is secreted into the environment by the Apr system through the type 1 secretion system (T1SS) [35] is regulated by the *aprA* gene; the synthesis of the bifunctional protein ExoS, secreted through the type 3 secretion system (T3SS), is regulated by the *exoS* gene. ExoS blocks phagocytosis and elimination of bacteria, which is associated with a high frequency of dissemination in the bloodstream [36, 37]. The most dangerous is exotoxin A (ExoA), secreted through the type 2 secretion system T2SS, causing disruption of cellular protein homeostasis and apoptosis. The synthesis of exotoxin A is regulated by the *exoA* gene [38, 39].

The study assessed the expression of the *aprA*, *exoS*, *exoA*, and *toxA* genes (*toxA* is the precursor of exotoxin A) under the influence of subinhibitory concentrations of azithromycin and clarithromycin.

According to the data obtained (Figure 3), under the action of azithromycin, the expression of the *aprA* gene decreases by 5.4 times, *exoA* – by 4.7 times, the *toxA* gene expression is practically not detected, the *exoS* expression increases by 13.5 times compared to the control. Changes in gene expression were also noted under the action of clarithromycin: *aprA* and *toxA* are almost not detected, the *exoA* gene expression decreases by 10.4 times, the *exoS* gene expression does not change.

Thus, macrolides are able to inhibit the expression of genes that regulate the synthesis of exotoxins AprA and ExoA. The inhibitory effect of azithromycin on the synthesis of exotoxins was noted

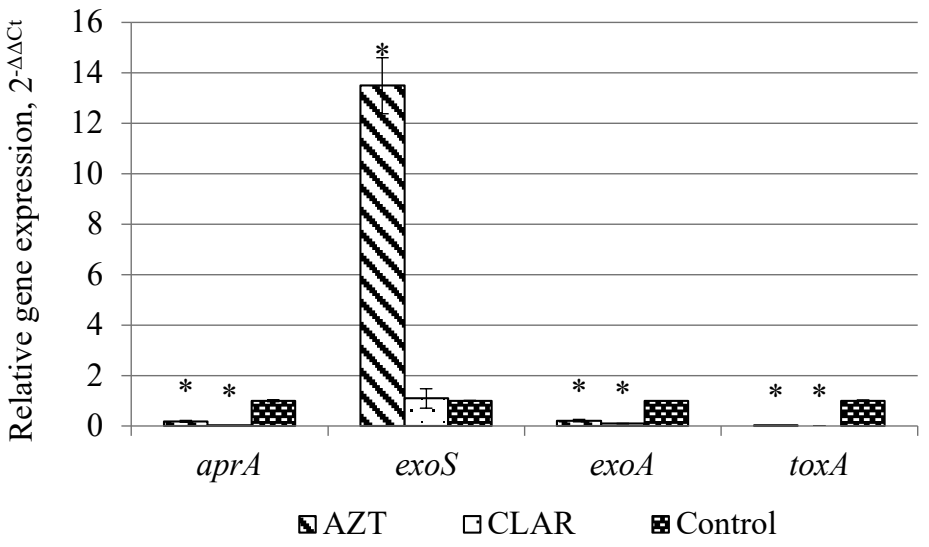

Fig. 3. Relative expression level of genes ($2^{-\Delta\Delta Ct}$) regulating the synthesis of virulence factors in P. aeruginosa 449 under the action of azithromycin and clarithromycin
*Note. AZT – azithromycin, CLAR – clarithromycin, *p < 0.05 compared to control.*

in [40]. In our studies, a significant increase in the expression of the *exoS* gene under the action of azithromycin was found ($p < 0.05$), which indicates the possible activation of the production of exotoxin S, a "contact" exotoxin that enters directly into the cytoplasm of the macroorganism cell ("macromolecular syringe"). The mechanism of contact intoxication allows the *P. aeruginosa* to counteract the effects of the immune system, since ExoA does not enter the extracellular environment and cannot be neutralized by antibodies. An increase in the synthesis of the exoenzyme ExoS under the action of azithromycin was noted in [41].

The effect of macrolides on Quorum sensing systems. In the pathogenesis of the disease caused by *P. aeruginosa*, an important role is played by the QS system, a communication network that uses chemical signals (acyl-homoserine lactones (AHLs) and quinolones) to regulate gene expression, in particular virulence factors, biofilm formation and adaptation to stressful environmental conditions. Since macrolides are able to inhibit the synthesis of virulence factors associated with the QS system, the study assessed the functioning of the QS systems (LasI/LasR, RhlI/RhlR and PQS) at a concentration of 0.5 MIC.

The results of the experiments showed (Figure 4) that azithromycin and clarithromycin affect the expression of the Las-, Rhl- and PQS-system genes of the QS test strain *P. aeruginosa* 449. Under the influence of azithromycin, the expression of the *lasI* gene decreases by 2.5 times, the *rhlI* gene – by 2.6 times, and the *rhlR* gene – by 7.1 times. The study showed an increase in the expression of the *pqsR* and *lasR* genes (by 2.1 times and 3.7 times, respectively, compared to the control).

According to the data obtained, under the influence of clarithromycin, the expression of the *lasI* gene decreases by

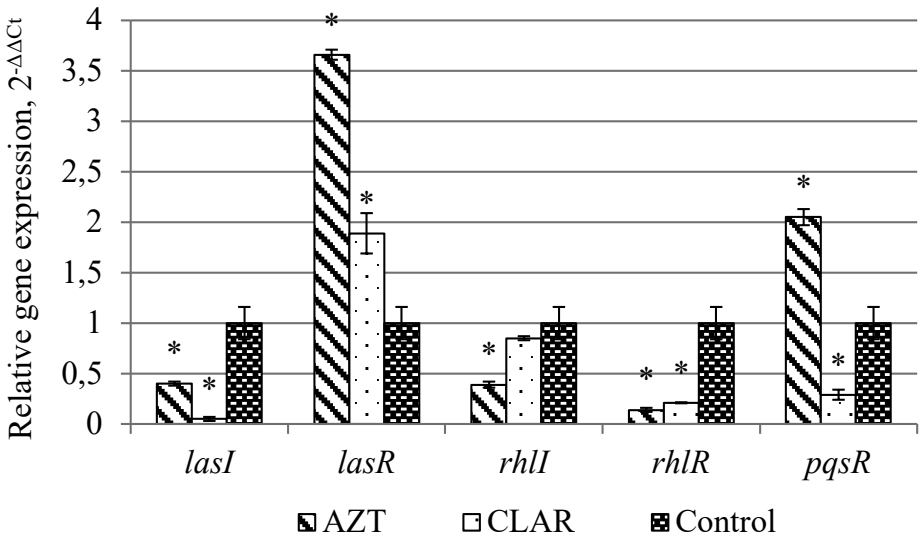

Fig. 4. Relative expression level of genes ($2^{-\Delta\Delta Ct}$) regulating Quorum sensing systems in P. aeruginosa 449 under the action of azithromycin and clarithromycin
*Note. AZT – azithromycin, CLAR – clarithromycin, *p < 0.05 compared to control.*

20 times, the *pqsR* gene – by 3.4 times, the *rhlI* gene – by 1.2 times, and the *rhlR* gene by – 4.8 times compared to the control. The expression of the *lasR* gene under the influence of 0.5 MIC of clarithromycin increases by 1.9 times.

A decrease in the expression of the *lasI* gene and an increase in the expression of *lasR* can lead to a decrease in the synthesis of virulence factors, such as elastase, by bacterial cells, since the *lasI* gene is responsible for the synthesis of the AHL QS signaling molecule. High levels of the LasR protein without sufficient AHL can lead to impaired function of the QS system: a decrease in the ability to form biofilms and impaired synthesis of virulence factors by cells. Bacteria with impaired Las QS system function may be more competitive in certain environments, particularly in wounds, and impaired autoinducer synthesis may prevent biofilm formation on the wound surface, but the inflammatory response in the wound may be enhanced. According to literature data [42], antibiotics at concentrations below the MIC (ciprofloxacin, amikacin, ceftazidime, meropenem) act as modulators of biochemical signals, reprogramming regulatory networks, preserving stress-adapted subpopulations that activate QS systems and stress resistance in later growth phases. It should be noted that in static environments, biofilms can increase the expression of virulence factors, while in natural conditions, on the contrary, their formation may be suppressed, since biofilms are pressured by various environmental factors that, by disrupting the spatial structure, affect the *las*, *rhl* and *pqs* regulons [43].

Of note is the increased expression of the *pqsR* gene under the influence of azithromycin. PqsR acts as a receptor for the *Pseudomonas* quinolone signal and controls the production of 2-alkyl-4-quinolone molecules, which are important for the manifestation of bac-

terial pathogenicity. The expression of the *pqsR* gene is positively controlled by the QS regulator LasR. Activation mediated by LasR occurs at the distal promoter site and can be antagonized by the CysB regulator. The proximal promoter site also promotes *pqsR* transcription, but initiation at this site is inhibited by a negative regulatory sequence element and, potentially, by members of the H-NS family MvaT and MvaU [44]. Since PqsR is a key transcriptional regulator in the QS system of *P. aeruginosa*, its increased expression leads to the activation of the synthesis of virulence factors. This response of *P. aeruginosa* to the action of sublethal concentrations of antibiotics is clearly recorded both in the stationary phase of growth and in the phase of cell death, when the growth of a subpopulation of adapted bacteria is observed. These results of the study complement the data on the mechanism of influence of macrolides on QS systems. It is known that azithromycin disrupts the synthesis of autoinducers, which are the final signals in the QS system of *P. aeruginosa* [45-47]. The effect of macrolides azithromycin and clarithromycin on the expression of genes regulating QS systems and the synthesis of virulence factors of *P. aeruginosa* is shown in the scheme (Figure 5).

In P. aeruginosa, 4 QS systems are known: Las, Rhl, PQS and IQS, which are interconnected and participate in the synthesis of virulence factors and biofilm formation. The Las system affects the functioning of other systems and the production of enzymes with proteolytic activity, exotoxin A, maturation and development of biofilm. The Rhl system regulates the synthesis of pyocyanin, rhamnolipids, HCN, bacterial motility, PQS – the synthesis of exoenzymes, lectin, early stages of biofilm formation, and eDNA release [48, 49]. The antivirulent effect of macrolides azithromycin and clarithromycin is realized by influencing Las, Rhl and PQS of the QS system. Under their action, there is a decrease in the expression of the lasI gene, which correlates with the suppression of protease activity and transcriptional activity of the aprA, exoA and toxA genes, which regulate the synthesis of alkaline protease and exotoxin A. Macrolides disrupt the functioning of the Rhl system, reducing the expression of the rhlR, rhlI genes (azithromycin only) and, accordingly, suppress hemolytic activity in the P. aeruginosa. Under the action of macrolide AMPs, a different effect on the PQS system was noted: azithromycin increases the expression of the pqsR gene and, accordingly, exoS, which regulates the synthesis of the "contact" toxin ExoS, under the influence of clarithromycin, the activity of the pqsR gene, on the contrary, is suppressed, and the exoS gene is unchanged. Under the action of both AMPs, azithromycin and clarithromycin, bacterial motility was inhibited, despite the different effects on the PQS system. The data obtained may indicate another mechanism of violation of the microorganisms' migration, in particular through the Rhl system or a direct effect on flagella and pili, which ensure the movement of bacteria and adhesion to the substrate.

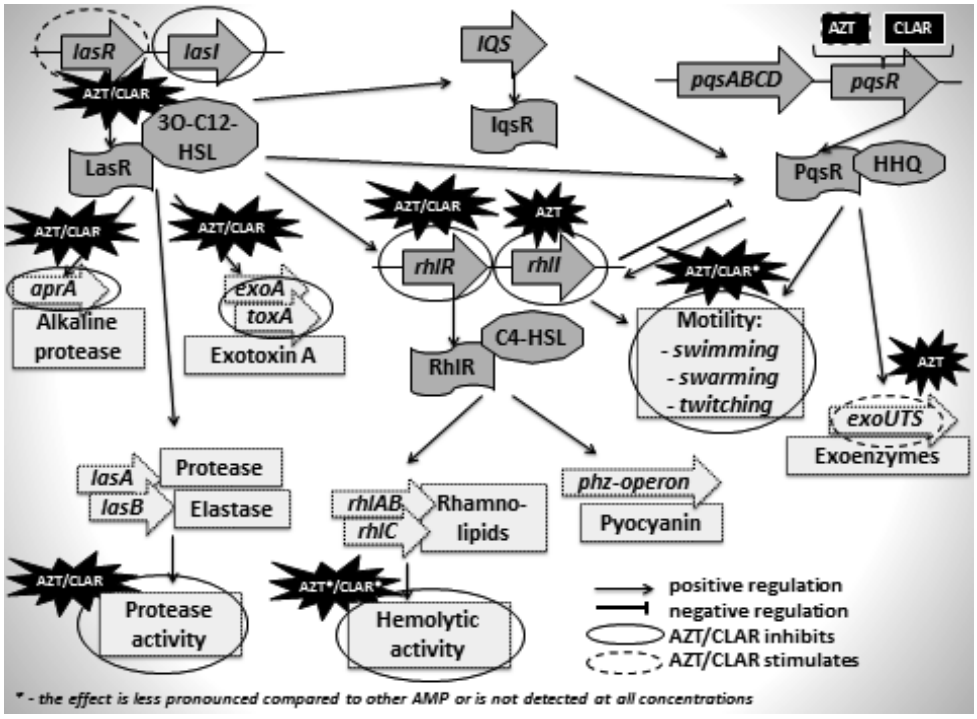

Fig. 5. Probable mechanism of macrolides' influence on regulatory pathways of QS systems and synthesis of virulence factors of P. aeruginosa

Conclusions

1. Azithromycin and clarithromycin disrupt QS-dependent processes in the *P. aeruginosa* cells, which is confirmed by changes in hemolytic, protease activity and motility of bacteria.
2. Macrolides, azithromycin and clarithromycin, change the expression of genes that regulate the synthesis of exotoxins AprA and ExoA: the expression of the *aprA* and *exoA* genes decreases by 5.4 and 4.7 times, respectively. Under the action of azithromycin, the expression of the *exoS* gene increases (by 13.5 times, compared to the control), which indicates a possible activation of the production of the exotoxin ExoS.
3. The antivirulent properties of macrolide antibiotics azithromycin and clarithromycin are realized by influencing LasI/LasR, RhlI/RhlR and PQS of the Quorum sensing system, which play a key role at all stages of the infectious process development.
4. Macrolides are able to modulate bacterial regulatory networks, affecting virulence and changing the level of expression of QS system genes in different phases of microbial growth.

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N. O. Vrynchanu, L. B. Zelena, N. I. Humeniuk, E. M. Vazhnichaya

Antivirulent activity of macrolides against *P. aeruginosa*

Second-generation macrolides (azithromycin and clarithromycin) are widely used in respiratory diseases. In addition to antibacterial activity against planktonic cells of gram-positive microorganisms, they have antibiofilm and antivirulent effects, in particular against *Pseudomonas aeruginosa*. However, information on the genetic regulation of this antivirulent effect is limited and requires comprehensive research.

The aim of the study – to evaluate the effect of macrolide antibiotics azithromycin and clarithromycin on virulence factors and activity of genes associated with them in *P. aeruginosa*.

The minimum inhibitory concentration (MIC) of macrolides against the clinical test strain *P. aeruginosa* 449 was determined by the serial microdilution method. The antivirulent properties of azithromycin and clarithromycin were evaluated in the range of concentrations from 0.15 MIC to 2.0 MIC, determining hemolytic and protease activity, bacterial motility and expression of genes responsible for Quorum sensing-dependent processes. The effect of macrolides (0.5 MIC) on gene expression in *P. aeruginosa* was studied using quantitative real-time polymerase chain reaction by the $2^{-\Delta\Delta Ct}$ method. The ANOVA method was used for statistical processing of the results.

It was established that the macrolides azithromycin and clarithromycin exhibit antivirulent properties against *P. aeruginosa*. Under the action of azithromycin and clarithromycin, the protease activity of bacteria decreases by 10.1–24.8% compared to the control. The inhibitory effect of azithromycin on the hemolytic activity of *P. aeruginosa* caused by extracellular hemolysins was recorded at 0.15 MIC and 0.25 MIC (10.1- and 10.4-fold reduction), and on cell-associated hemolysins at 0.15–0.5 MIC (2.9–3.7-fold reduction). The effect of clarithromycin on the hemolytic activity of bacteria is inferior to azithromycin. The effect of macrolides on bacterial motility depends on the type of migration, the drug, and its concentration, but the most pronounced effect was observed on swimming- and swarming-migration. The data obtained indicate that azithromycin and clarithromycin cause changes in the expression of genes that regulate the synthesis of virulence factors. Under their influence, the expression of the *aprA*, *exoA*, and *toxA* genes is significantly reduced. The transcriptional activity of the *exoS* gene under the action of azithromycin increases (by 13.5 times), clarithromycin does not change. Macrolides reduce the expression of the *lasI*, *rhlI* (azithromycin only), *rhlR*, *pqsR* (clarithromycin) genes by 2.5–20 times and increase the activity of the *lasR*. *pqsR* (azithromycin) genes by 1.9–3.7 times, which are involved in the functioning of Quorum sensing systems in *P. aeruginosa*.

Thus, the antivirulent activity of azithromycin and clarithromycin against *P. aeruginosa* is realized by influencing the hemolytic, protease activity and motility of bacteria. Macrolide antibiotics, by changing the level of gene expression, are able to disrupt the synthesis of virulence factors and the functioning of Quorum sensing systems, which play a key role at all stages of the development of the infectious process.

Ключові слова: azithromycin, clarithromycin, antibacterial agents, biofilms, Quorum sensing, virulence factors, Pseudomonas aeruginosa, genes expression

Н. О. Вринчану, Л. Б. Зелена, Н. І. Гуменюк, О. М. Важнича

Антивірулентна активність макролідів щодо *P. aeruginosa*

Макроліди II покоління (азитроміцин і кларитроміцин) широко застосовуються при захворюваннях дихальних шляхів. Окрім антибактеріальної активності щодо планктонних клітин грампозитивних мікроорганізмів, їм притаманна антибіоплівкова та антивірулентна дія, зокрема щодо *Pseudomonas aeruginosa*. Однак інформація стосовно генетичної регуляції цієї антивірулентної дії обмежена та потребує комплексного дослідження.

Мета дослідження – вивчити вплив макролідних антибіотиків азитроміцину та кларитроміцину на фактори вірулентності й активність генів, асоційованих із ними, у *P. aeruginosa*.

Мінімальну інгібуючу концентрацію (MIK) макролідів щодо клінічного тест-штаму *P. aeruginosa* 449 визначали методом серійних мікророзведень. Антивірулентні властивості азитроміцину та кларитроміцину оцінювали в межах концентрацій від 0,15 MIK до 2,0 MIK, визначаючи гемолітичну, протеазну активності, рухливість бактерій та експресію генів, відповідальних за Quorum sensing-залежні процеси. Вплив макролідів (0,5 MIK) на експресію генів у *P. aeruginosa* досліджували за допомогою кількісної полімеразної ланцюгової реакції у реальному часі методом $2^{-\Delta\Delta Ct}$. Для статистичної обробки результатів використовували метод ANOVA.

Встановлено, що макроліди азитроміцин і кларитроміцин виявляють антивірулентні властивості щодо *P. aeruginosa*. За дії азитроміцину та кларитроміцину протеазна активність бактерій знижується на 10,1–24,8 % порівняно з контролем. Інгібувальний ефект азитроміцину щодо гемолітичної активності *P. aeruginosa*, обумовленої позаклітинними гемолізинами, зареєстрований при 0,15 MIK та 0,25 MIK (зменшення у 10,1 і 10,4 разу), клітинно-асоційованими гемолізинами – за впливу 0,15–0,5 MIK (зменшення у 2,9–3,7 разу). Дія кларитроміцину на гемолітичну активність бактерій поступається азитроміцину. Вплив макролідів на рухливість бактерій залежить від типу міграції, препарату та його концентрації, проте найвираженіший ефект відмічено на swimming- та swarming-міграцію. Отримані дані свідчать, що азитроміцин і кларитроміцин обумовлюють зміни в експресії генів, які регулюють синтез факторів вірулентності. За їхнього впливу значно знижується експресія генів *aprA*, *exoA* та *toxA*. Транскрипційна активність гена *exoS* за дії азитроміцину збільшується (у 13,5 разу), кларитроміцину – не змінюється. Макроліди знижують у 2,5–20 разів експресію генів *lasI*, *rhlI* (лише азитроміцин), *rhlR*, *pqsR* (кларитроміцин) та збільшують у 1,9–3,7 разу активність генів *lasR*. *pqsR* (азитроміцин), які беруть участь у функціонуванні систем Quorum sensing у *P. aeruginosa*.

Таким чином, антивірулентна активність азитроміцину та кларитроміцину щодо *P. aeruginosa* реалізується шляхом впливу на гемолітичну, протеазну активність і рухливість бактерій. Макролідні антибіотики, змінюючи рівень експресії генів, здатні порушувати синтез факторів вірулентності та функціонування систем Quorum sensing, які відіграють ключову роль на всіх етапах розвитку інфекційного процесу.

Key words: азитроміцин, кларитроміцин, антибактеріальні засоби, біоплівки, Quorum sensing, фактори вірулентності, Pseudomonas aeruginosa, експресія генів

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Контактна особа: Врінчану Ніна Олексіївна, доктор медичних наук, лабораторія фармакології протимікробних засобів відділу фармакології, ДУ «Інститут фармакології та токсикології НАМН України», буд. 14, вул. Антона Цедіка, м. Київ, 03057. Тел.: + 38 0 44 456 42 56. Електронна пошта: nvrynchanu@gmail.com