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Virulence genes and antibiotic resistance among clinical *Klebsiella pneumoniae* strains

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ABSTRACT

Introduction: *Klebsiella pneumoniae* is one of the etiological factors of nosocomial infections. Recently, infections caused by these bacteria have become more dangerous due to the acquired resistance to many antibiotics, severely limiting therapeutic options. The most common mechanism of resistance in *K. pneumoniae* rods is the production of extended-spectrum β-lactamases (ESBL). However, a more perilous mechanism is the production of carbapenemases. The course of the infection process is also influenced by various virulence characteristics, primarily enabling adhesion and aiding in avoiding host immune responses. Most genes encoding mechanisms of resistance and virulence are located on plasmids, facilitating their spread. The aim of the study was to detect selected virulence genes among clinical multidrug-resistant strains of *K. pneumoniae*.

Materials and methods: We determined the drug susceptibility of strains and confirmed the presence of antibiotic resistance mechanisms using phenotypic methods. Additionally, we assessed the presence of genes encoding selected resistance mechanisms and genes determining selected virulence factors. A total of 134 strains from various hospital units were used for the study.

Results: The highest percentage of strains was isolated from urine (46%). Among the isolates, 72% were from male patients. Fifty-seven percent of *K. pneumoniae* produced ESBL (KpESBL), while the remaining 43% carried the New Delhi metallo-β-lactamase (NDM) mechanism. The drug susceptibility of the KpESBL varied, with full sensitivity observed only in the case of antibiotics from the carbapenem group. New Delhi metallo-β-lactamase-producing *K. pneumoniae* showed sensitivity only to amikacin and gentamicin. In KpESBL strains, genes from the TEM family were most observed (74/76). Most of the strains had all 4 β-lactamase-encoding genes (61/76). In the group of strains producing carbapenemases, only the bla NDM gene was detected. Regardless of the resistance mechanisms, the tested strains most often had virulence genes related to the adhesion ability (fimH) and the structure of LPS (wabG).

Keywords: *Klebsiella pneumoniae*; virulence factors; multidrug resistance.

INTRODUCTION

Klebsiella pneumoniae is a Gram-negative enteric bacillus, part of the *Enterobacteriaceae* family. Most of these bacteria do not pose a threat to humans, but certain gastrointestinal strains can cause infections. Infections linked to *K. pneumoniae* are mainly associated with hospitalization, with colonization rates in hospital settings dependent on the length of stay. Among hospitalised patients, the prevalence in the nasopharyngeal cavity is approx. 19% and in stool samples, it is around 77% [1], although recent studies suggest that it may be closer to 20% [2]. The rise in colonization is primarily linked to the use of antibiotics, especially broad-spectrum ones [1].

Hospitals provide several opportunities for the transmission of pathogenic microorganisms. One source is the hands of healthcare staff and their interactions with patients. Medical equipment can also serve as a transmission source. Important risk factors for *K. pneumoniae* infections include cancer and other debilitating diseases, age (including newborns and the elderly), weakened immunity, stimulants (such as alcohol and smoking) and catheters [1, 3, 4, 5]. Infections caused by *K. pneumoniae* can be divided into nosocomial and non-hospital infections, though the majority are associated with hospitalization. Hospital-acquired infections include urinary tract infections (UTI), pneumonia, wound and gastrointestinal infections, and bacteremia, either primary or secondary, resulting from bacterial spread in the body. Other forms include liver abscesses, pneumonia, ocular infections and meningitis [6].

Klebsiella pneumoniae has evolved several mechanisms to facilitate colonization, evade the host immune response and persist in hospital environments. Virulence factors play crucial roles in various infections. Among the well-understood factors are the polysaccharide envelope, lipopolysaccharide (LPS), adhesins, siderophores, iron transport systems, and hemolysin [7]. These virulence factors are encoded both by

the core genome (the genome present in all representatives of the species) and genes found on mobile elements like plasmids.

The polysaccharide envelope, a critical virulence element, primarily defends against host responses, particularly phagocytosis [1]. This envelope includes strain-specific K antigens [7] with K1- and K2-serotyped strains exhibiting greater virulence compared to other serotypes, often causing severe infections in humans. Pathogens isolated in patients with UTI, pneumonia or bacteremia most often have serotype K2 [1]. Studies on hypervirulent strains showed that the vast majority of them had serotype K1. The plasmid or chromosomal regulator of the mucosal phenotype A – *rmp*A – is responsible for the increased production of envelopes. In addition, hypervirulent pathogens have been shown to possess another virulence gene, *mag*A, detected in most liver abscess isolates, which is associated with resistance to phagocytosis and the bactericidal effect of serum [8].

Another important virulence factor of *K. pneumoniae* is LPS, which is part of the bacterial outer membrane. Lipopolysaccharide consists of 3 subunits: lipid A, core and antigen O. Lipid A is a potent activator of inflammation. The O antigen, on the other hand, is involved in complement protection [7, 9]. Lipopolysaccharide, in addition to protecting the bacterium from complement action, induces a strong immune response in the host. A phenomenon that protects against recognition by the host immune system is the modification of lipid A *in vivo* to a form that is not recognized. The modified lipid A increases the virulence of *K. pneumoniae* without inducing an immune response [10].

Genes responsible for normal LPS synthesis include the *uge* gene, encoding UDP galacturonate 4-epimerase, and the *wab*G gene, encoding GalA transferase. The *uge* gene is present in most isolates. In its absence, *K. pneumoniae* is less able to cause UTI, septicemia and pneumonia. The *wab*G gene, on the other hand, is present in almost all strains, according to most studies in 88–100% of isolates [7].

Fimbriae play a key role in bacterial adhesion to mucosal surfaces, an important step in the development of infection. The best-known and most common are fimbriae types 1 and 3. They play a special role in the biofilm formation of urinary catheters, leading to UTI [4, 11]. The virulence of *K. pneumoniae* strains equipped with fimbriae is thought to be primarily due to the bacteria's binding to cells of the urinary, respiratory and intestinal tracts [1, 11].

Siderophores are small molecules with low molecular weight and high affinity, secreted by some bacteria to obtain iron. This is because iron is required by bacteria for various metabolic pathways and the supply of free iron in the host for bacteria is too low because it is bound to other molecules such as transferrin [1]. Thus, in order to obtain iron, *K. pneumoniae* secretes siderophores, which have a higher affinity for iron than transport proteins and have the ability to obtain iron from both iron-binding proteins and the environment. *Klebsiella pneumoniae* produces several types of siderophores. The most commonly secreted is enterobactin, in addition to yersiniabactin, salmochelin and aerobactin. Different siderophores are associated with different clinical forms of infection [9].

Klebsiella pneumoniae's iron acquisition is mediated by the *kfu* iron transport system, which shows strong expression in hypervirulent strains and is more common in strains that cause liver abscesses complicated by meningitis or intraocular inflammation [12]. *Klebsiella pneumoniae* also exhibits hemolytic activity. The hemolysin it produces, HlyA, is a toxin classified as a thiol-activated cytolysin. It belongs to RTX proteins – exotoxins that bind calcium ions. The attachment of these ions allows binding not only to erythrocytes but also to other cells. The RTX family has the ability to create pores in host cells, thus leading to lysis of epithelial cells. This allows the bacterium to obtain components necessary for survival and growth, such as iron. The HlyA protein is encoded by the *hly*A gene [3].

Klebsiella pneumoniae is one of the bacteria experiencing a significant increase in resistance to most antibiotics [13, 14]. Resistance to antibacterial drugs is usually associated with the spread of plasmids and the acquisition of resistance genes. Several factors contribute to the expansion of antibiotic resistance, primarily encompassing inappropriate and excessive antibiotic use and the absence of new antibacterial agents. *Klebsiella pneumoniae* exhibits multiple mechanisms of antibiotic resistance, with resistance to β-lactams having the greatest impact on treatment [14].

The most common mechanism involves the production of extended-spectrum β-lactamases (ESBL) [15, 16]. Genes encoding these enzymes are often present on plasmids, and in some cases on transposons, allowing for transfer between bacteria of different species [16]. Within the ESBL family, the SHV, TEM and CTX enzymes, clinically significant and prevalent in *K. pneumoniae*, constitute the core group [17]. The OXA group of enzymes derives from the OXA-10 enzyme, which can hydrolyze cefotaxime, ceftriaxone and aztreonam. It is found mainly in *Pseudomonas aeruginosa* but has been identified in various other Gram-negative bacteria. Unlike SHV or TEM, these enzymes can hydrolyze oxacillin and are mainly responsible for ampicillin resistance. They are weakly inhibited by clavulanic acid and lack activity against newer-generation cephalosporins [15].

The most concerning resistance mechanism involves the production of carbapenem-hydrolyzing enzymes. The carbapenemases produced by *K. pneumoniae* are primarily group A enzymes – *Klebsiella pneumoniae* carbapenemase (KPC), group B New Delhi metallo-β-lactamase (NDM) and group D-derived OXA-48 carbapenemases. These carbapenemases confer resistance to most antibiotics and allow the bacteria to spread worldwide, which poses a significant threat due to limited treatment options [18, 19].

Klebsiella pneumoniae infections present a substantial challenge for today's public health sector. As asymptomatic carriers become increasingly widespread and eradicating these bacteria from the hospital environment proves exceptionally difficult, we are confronted with strains resistant to almost all available drugs, as well as with increasingly higher virulence potential.

The aim of the study was to determine drug susceptibility and detect selected virulence genes among clinical multidrug-resistant strains of *K. pneumoniae.*

MATERIALS AND METHODS

Identification and determination of drug susceptibility

A total of 134 *K. pneumoniae* strains, isolated during routine microbiological diagnostics at the Microbiology Laboratory of the Independent Public Clinical Hospital No. 2 of Pomeranian Medical University in Szczecin (SPSK-2) between March 2019 and February 2020, were used in the study. These strains were obtained from various clinical materials, including urine, blood, bronchoalveolar lavage, wound and peritoneal fluid. The strains were preserved at freezer temperature in tryptose-soy liquid medium (TSB; Becton Dickinson, Poland), supplemented with anhydrous glycerin (Chempur, Poland).

The study was conducted at the Department of Microbiology, Immunology and Laboratory Medicine of the Pomeranian Medical University in Szczecin. They did not require approval from the Bioethics Committee.

Strains were identified through biochemical tests using the Vitek2Compact instrument (bioMérieux, Poland). Mueller-Hinton agar (MHA; bioMérieux, Poland) was employed for drug susceptibility assessments, and a bacterial suspension of appropriate density was applied to the medium, with the corresponding antibiotic-saturated disco (Becton Dickinson, Poland) applied to adhere completely to the agar surface (Tab. 1). After incubation, the boundary of the zone of inhibition was read and interpreted following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations [20].

TABLE 1. List of antibacterial agents used in the study

Antibacterial agents	mg
Amoxicillin/clavulanic acid	20/10
Piperacillin/tazobactam	100/10
Cefotaxime	30
Ceftazidime	30
Cefepime	30
Gentamicin	10
Amikacin	30
Imipenem	10
Meropenem	10
Ertapenem	10
Ciprofloxacin	5
Norfloxacin	10
Trimethoprim/sulfamethoxazole	1.25/23.75

Detection of extended-spectrum β-lactamases

The Double Disc Synergy Test (DDST) was used to detect β-lactamase production using cefotaxime, ceftazidime (third-generation cephalosporins), along with an amoxicillin-clavulanate disc [20].

Detection of carbapenemases

To detect the production of carbapenemases, a chromogenic medium (ChromidCarba, bioMérieux, France) was used, on which the carbapenemase-producing strains grow green colonies, interpreted as a positive result.

Detection of genes associated with virulence and antibiotic resistance

Genes encoding bacterial virulence factors and antibiotic resistance were detected using the polymerase chain reaction (PCR) method. Genomic DNA isolation was carried out using the Gene MATRIX Bacterial & Yeast Genomic DNA Purification Kit column kit (EURx, Poland). Plasmid DNA isolation was performed using the Gene MATRIX Plasmid Miniprep DNA Purification Kit (EURx, Poland). The PCR reaction was conducted using the gene-specific primer sequences shown in Tables 2 and 3.

TABLE 2. Primer sequences of genes encoding virulence factors

Target gene	Primer sequences (5'-3')	Size of PCR product (bp)	Reference
uge	GATCATCCGGTCTCCCTGTA	534	[21]
	TCTTCACGCCTTCCTTCACT		
wabG	CGGACTGGCAGATCCATATC	683	[22]
	ACCATCGGCCATTTGATAGA		
fimH	ATGAACGCCTGGTCCTTTGC	688	[23]
	GCTGAACGCCTATCCCCTGC		
kfu	GAAGTGACGCTGTTTCTGGC	797	[23]
	TTTCGTGTGGCCAGTGACTC		
iroN	AAGTCAAAGCAGGGGTTGCCCG	665	[23]
	GACGCCGACATTAAGACGCA		
hlyA	AACAAGGATAAGCACTGTTCTGGCT	1177	[23]
	ACCATATAAGCGGTCATTCCCGTCA		
rmpA	ACTGGGCTACCTCTGCTTCA	535	[22]
	CTTGCATGAGCCATCTTTCA		
magA	GGTGCTCTTTACATCATTGC	1280	[22]
	GCAATGGCCATTTGCGTTAG		

PCR – polymerase chain reaction

TABLE 3. Primer sequences of genes encoding antibiotic resistance

Target gene	Primer sequences (5'-3')	Size of PCR product (bp)	Reference
bla_{SHV}	ATGCGTTATATTCGCCTGTG	747	[24]
	TGCTTTGTTATTCGGGCCAA		
bla_{TEM}	TCGCCGCATACACTATTCTCAGAATGA	445	[24]
	ACGCTCACCGGCTCCAGATTTAT		
bla_{CTX-M}	ATGTGCAGYACCAGTAARGTKATGGC	553	[24]
	TGGGTRAARTARGTSACCAGAAYCAGCGG		
bla_{OXA-1}	ACACAATACATATCAACTTCGC	814	[25]
	AGTGTGTGTTTAGAATGGTGATC		
bla_{KPC}	ATG TCA CTG TAT CGC CGT CT	882	[26]
	TTT TCA GAG CCT TAC TGC CC		
bla_{NDM}	GGT TTG GCG ATC TGG TTT TC	612	[27]
	CGG AAT GGC TCA TCA CGA TC		
bla_{IMP}	GGA ATA GAG TGC CTT AAY TCT C	232	[28]
	GGT TTA AYA AAA CAA CCA CC		
bla_{VIM}	GAT GGT GTT TGG TCG CAT A	390	[28]
	CGA ATG CGC AGC ACC AG		

PCR – polymerase chain reaction

The amplification reaction, consisting of 30 cycles, was carried out in an Applied BiosystemsVeriti 96 WellThermalCycler (Applied Biosystems, USA) according to the following parameters: initial activation at 95°C for 15 min, followed by 30 cycles at 94°C for 30 s; annealing at 60°C for 90 s, followed by extension at 72°C for 60 s and a final extension at 72°C for 10 min. Electrophoresis was performed using a 1.5% agarose gel (DNA Gdansk, Poland) mixed with ethidium bromide (Sigma-Aldrich, Germany) at a concentration of 0.5 µg/mL. Positive controls were standard strains suitable for specific genes. Electrophoresis was performed at 80 V for 80 min. The result was read under UV light using a GelDoc-It2 Imager system (Upland, CA, USA).

RESULTS

Klebsiella pneumoniae isolation sources

The highest percentage of strains was isolated from urine (46%), followed by blood (25%). Twenty-two percent of the isolates came from bronchoalveolar lavage and 6% were from wound swabs. The smallest percentage, only 1%, was from the peritoneal cavity fluid. Among all *K. pneumoniae* isolates, more than half were from male patients (Fig. 1).

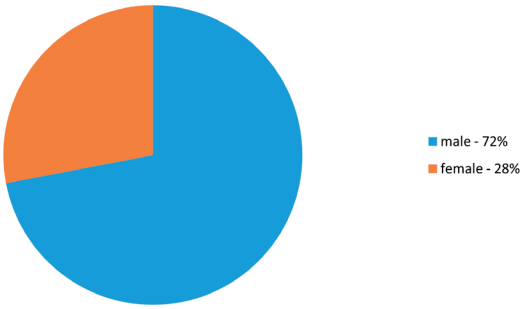

FIGURE 1. Occurrence of *Klebsiella pneumoniae* in the studied population of women and men

Drug resistance of *Klebsiella pneumoniae*

Irrespective of the type of clinical material from which *K. pneumoniae* was isolated, they showed fairly high resistance to many of the antibiotics tested. Phenotypically, ESBL-type resistance was confirmed in 76 strains (57%).

KpESBL strains displayed resistance to penicillins in combination with β-lactamase inhibitors and to cephalosporins. There was also a high percentage of strains resistant to fluoroquinolones – 76.3% to ciprofloxacin and 74% to norfloxacin. For trimethoprim/sulfamethoxazole, 68.4% of strains showed resistance. Among aminoglycosides, a higher percentage of strains were sensitive to amikacin (79%) than to gentamicin (68.4%). All strains were susceptible to meropenem and ertapenem, with only 2.6% of resistant strains noted for imipenem (Tab. 4).

The production of carbapenemases was phenotypically confirmed in 58 strains (43%).

Carbapenemase-producing *K. pneumoniae* exhibited resistance to all antibiotics used in the study, with the exception of amikacin (32% sensitive) and gentamicin (26% sensitive) – Table 5.

TABLE 4. Drug resistance of KpESBL strains

Antibiotic	Strains n (%)
Amoxicillin with clavulanic acid	76 (100.0)
Piperacilin/tazobactam	76 (100.0)
Cefotaxime	76 (100.0)
Ceftazidime	76 (100.0)
Cefepime	76 (100.0)
Gentamicin	24 (31.6)
Amikacin	16 (21.0)
Ciprofloxacin	58(76.3)
Norfloxacin	56 (74.0)
Imipenem	2 (2.6)
Meropenem	–
Ertapenem	–
Trimethoprim/sulfamethoxazole	52 (68.4)

TABLE 5. Drug resistance of carbapenemase-producing *Klebsiella pneumoniae* strains

Antibiotic	Strains n (%)
Amoxicillin with clavulanic acid	58 (100.0)
Piperacilin/tazobactam	58 (100.0)
Cefotaxime	58 (100.0)
Ceftazidime	58 (100.0)
Cefepime	58 (100.0)
Ciprofloxacin	58 (100.0)
Norfloxacin	58 (100.0)
Imipenem	58 (100.0)
Meropenem	58 (100.0)
Ertapenem	58 (100.0)
Trimethoprim/sulfamethoxazole	58 (100.0)
Gentamicin	43 (74.0)
Amikacin	39 (67.2)

Occurrence of genes encoding resistance mechanisms

Among the 4 genes encoding the ESBL mechanism, the most common among the studied strains were genes encoding enzymes of the TEM family (74/76). The bla_{SHV} and bla_{OXA-1} genes were detected in 72 strains, while the least frequent was the bla_{CTX-M} gene present in 68 strains. The most numerous group consisted of strains in which the presence of all 4 genes encoding the ESBL mechanism was detected (61/76). Three genes were confirmed in 12 isolates. The presence of 2 genes was detected in 3 strains, while the presence of a single gene was not reported (Tab. 6).

TABLE 6. Distribution of genes: bla_{TEM}, bla_{OXA-1}, bla_{SHV}, bla_{CTX-M} among *Klebsiella pneumoniae*

Distribution of genes *bla*	Gene	Number of strains	Percent
Presence of 2 genes	bla_{SHV} + bla_{TEM}	1	4
	bla_{TEM} + bla_{OXA-1}	2	
Presence of 3 genes	bla_{SHV} + bla_{TEM} + bla_{OXA-1}	5	15.8
	bla_{SHV} + bla_{CTX-M} + bla_{OXA-1}	2	
	bla_{SHV} + bla_{TEM} + bla_{CTX-M}	3	
	bla_{TEM} + bla_{CTX-M} + bla_{OXA-1}	2	
Presence of 4 genes	bla_{SHV} + bla_{TEM} + bla_{CTX-M} + bla_{OXA-1}	61	80.2
	Total	76	100

In all strains phenotypically confirmed to produce carbapenemases, a gene encoding an NDM-type carbapenemase was detected.

Occurrence of genes encoding virulence factors

Regardless of the mechanism of resistance, the most common genes encoding bacterial virulence factors were the *fim*H gene, which encodes fimbriae and mediates bacterial adhesion, and the *wab*G gene, which is involved in the production of LPS that protects bacteria from the action of complement. Both genes were detected in all isolates. The next most frequently detected gene was the *uge* gene, present in 93.4% of the KpESBL strains and in 89.6% NDM-producing *K. pneumoniae*, which is also responsible for the production of LPS. Genes conditioning iron acquisition – *kfu* and *iro*N – were detected in 90.8 % and 86.2% of KpESBL isolates, and 76.3% and 84.5% in NDM-producing strains, respectively. The gene *hly*A, which conditions the production of the toxin, was present in only 17.1% of KpESBL and 13.8% of NDM-producing isolates. One of the 2 genes responsible for mucus production, characteristic of hypervirulent strains, i.e. *mag*A, was detected only in KpESBL strains (2.6%), while the *rmp*A gene was not detected in any of the strains (Tab. 7). The results were not statistically significant.

TABLE 7. The presence of virulence genes among ESBL- and NDM-producing *Klebsiella pneumoniae*

Gene	KpESBL n (%)	**NDM-producing** *K. pneumoniae* **n (%)**
***fim*H**	76 (100)	58 (100)
***wab*G**	76 (100)	58 (100)
uge	73 (93.4)	52 (89.6)
kfu	69 (90.8)	50 (86.2)
***iro*N**	58 (76.3)	49 (84.5)
***hly*A**	13 (17.1)	8 (13.8)
magA	2 (2.6%)	–
***rmp*A**	–	–

ESBL – extended-spectrum β-lactamase; KpESBL – *K. pneumoniae* producing ESBL; NDM – New Delhi metallo-β-lactamase

DISCUSSION

Klebsiella pneumoniae is known to be an opportunistic pathogen, particularly problematic for hospitalized and immunocompromised patients. Its ability to spread in healthcare environments and develop resistance to various antibiotic classes renders infections caused by these bacteria increasingly challenging due to limited treatment options [29].

In our study, the majority of strains were isolated from urine (46%) and blood (25%). In contrast, 22% of the isolates came from bronchoalveolar lavage. A similarly high percentage of strains isolated from urine and blood (39%) was reported by Müller-Schulte et al. [30]. In the work of Wang et al., the percentage of isolates from the urine was comparable at 38.8%, while the strains isolated from the respiratory tract oscillated at 28.25% [31]. In contrast, Mrowiec et al. found the highest number of isolates in the respiratory tract (45.3%), followed by urine (26.7%) and then blood (11.6%) [32]. In our study, we observed

that 6% of the strains came from wound swabs, which is consistent with the study by Lev et al. where the percentage was also low at 5% [33].

It is a well-known fact that one of the risk factors for UTI is female gender. This was confirmed, among other things, by a study by Dehshiri et al. in which the highest number of *K. pneumoniae* isolated from urine came from women (69.7%) [34]. Although the highest number of strains were isolated from urine in our study, the majority of patients with UTI were men (60%). This is probably related to the fact that they were mostly catheterized patients hospitalized in the intensive care unit.

Regardless of the clinical material, the strains exhibited high resistance to many antibiotics. Most *K. pneumoniae* strains showed ESBL-type resistance, rendering them completely resistant to β-lactamase inhibitor penicillins and third- and fourth-generation cephalosporins. Similar high rates of resistance were reported by Mahmoudi et al., with resistance rates of 97% for cefotaxime and 83% for cefepime [35]. A study conducted in Polish hospitals in 2019 also reported resistance to cefotaxime and ceftazidime, with 96.4% resistance to cefepime [32]. In contrast, Wang et al. reported lower resistance rates to ceftazidime (60.5%) and cefepime (36.3%) in ESBL-producing *K. pneumoniae* [31].

Sugumar et al. found that all isolates in their study were resistant to the combinations of β-lactams and inhibitors [36]. Mrowiec et al. reported that most isolates in their study were resistant to fluoroquinolones, with 71% resistance to ciprofloxacin and 73% to norfloxacin [32]. These results are in line with our study, where resistance rates to these antibiotics were 76.3% and 74%, respectively. Lev et al. even reported a higher resistance rate to ciprofloxacin at 87% [33]. Additionally, most *K. pneumoniae* strains in our study exhibited resistance to cotrimoxazole (68%). As many as 68.4% of strains were sensitive to gentamicin and amikacin. These results are consistent with the findings of Mączyńska et al. who also reported over half of the strains being resistant to cotrimoxazole (79%) and higher resistance to gentamicin (98%) than amikacin (2%) in aminoglycoside antibiotics [37]. Wang et al. showed an even higher resistance rate to cotrimoxazole at 80.6% and resistance rates of 64.2% for gentamicin and 10.5% for amikacin in ESBL-producing *K. pneumoniae* [31]. Carbapenems are often the last resort for treating infections caused by multidrug-resistant strains. In our study, all KpESBL strains were fully sensitive to 2 carbapenem antibiotics, meropenem and ertapenem. Only 2.6% of strains showed resistance to imipenem. Similar findings were presented by Abayneh et al., where both ESBL-producing and non-ESBL-producing *K. pneumoniae* strains showed complete susceptibility to carbapenems [38]. Bora et al. also reported that imipenem, meropenem and ertapenem were the most effective antimicrobial drugs, even against ESBL-positive strains [39].

Among carbapenemase-producing strains, we observed complete resistance to β-lactamase inhibitor penicillins, cephalosporins, fluoroquinolones, trimethoprim-sulfamethoxazole and carbapenems. For aminoglycosides, resistance was noted at 74% for gentamicin and 67.2% for amikacin. Such high resistance to most antibiotics among carbapenemase-positive *K. pneumoniae* strains is consistent with reports from various authors worldwide [40, 41, 42]. However, it is common to find strains that, although in a small percentage, still exhibit sensitivity to aminoglycoside antibiotics [41].

According to reports, the most prevalent enzymes, both in Poland and globally, among *K. pneumoniae* strains are β-lactamases from the CTX-M family, which constitute the predominant group of ESBLs. Zeynudin et al. identified this gene in 95.8% of the studied isolates [43]. In contrast, Bora et al. [39] and Ferreira et al. [17] found that the most common enzymes in the ESBL group belonged to the TEM family, being present in 77.58% and 100% of strains, respectively. Similarly, in our study, it was observed that the strains most frequently carried the bla_{TEM} gene (96%). The gene encoding enzymes belonging to the OXA-1 family were present in only 38% of isolates in Lev et al. analysis [33]. In the same work, the bla_{SHV} gene was most frequently observed in 91% of isolates. However, in our study, the percentage of strains producing SHV and OXA-1 family enzymes was identical at 94.7%. In contrast to most reports, $bla_{\text{CTX-M}}$ was the least frequent gene, present in 89.5% of the studied isolates. The presence of 2 out of the 4 tested genes encoding ESBL-type enzyme production was confirmed in 4% of strains. The presence of 3 genes was found in 15.8% of isolates, while the highest percentage of *K. pneumoniae* strains (80.2%) possessed all 4 genes simultaneously. No single gene was found in any of the isolates tested. These results seem to align with many literature reports, where genes encoding the production of enzymes belonging to 2, 3 or more ESBL families were found among *K. pneumoniae* strains. Bora et al. also observed isolates of *K. pneumoniae*, producing several variants of enzymes belonging to different ESBL families [39]. In contrast, the results compiled by Lev et al. showed that the highest percentage (37%) of tested strains had a combination of 3 ESBL genes, while strains with 4 genes were less frequent (21%). Two types of genes were detected in 20% of isolates, while 16% had only 1 gene [33].

A gene encoding NDM-type enzymes was detected in all strains phenotypically confirmed to produce carbapenemases. The emergence of NDM-type metallo-β-carbapenemases, encoded by the $bla_{\text{NDM-1}}$ gene, has become a serious threat to medicine. This gene is carried on large plasmids that often carry other alleles encoding antibiotic resistance. The result of this mutation has been the creation of enzymes that confer resistance to most available antibiotics [44]. This underscores the significant role played by plasmids in the spread of NDM resistance among microorganisms. Currently, this drug resistance is most frequently detected in *K. pneumoniae* and *Acinetobacter* spp. The rapid spread and the lack of effective therapy have led to the designation of bacteria carrying NDM as „superbugs" [45].

In addition to its ability to develop resistance to numerous antibiotics, *K. pneumoniae* possesses various mechanisms that enhance colonization and evasion of the host immune response. Several virulence factors have been identified as crucial in the

development of infection [7]. In our study, the most common genes encoding virulence factors were *fim*H and *wab*G, both of which were detected in all strains studied, irrespective of the antibiotic resistance mechanism. A high percentage of isolates from urine carrying the *fim*H gene was observed in the study by Remya et al. who pegged it at 89% [46]. El Fertas-Aissani et al. also observed that among *K. pneumoniae* test populations, the most common virulence gene was *fim*H, whose presence was confirmed in all strains [47]. The *fim*H gene, responsible for the production of the FimH subunit, which is part of type 1 fimbriae, is expressed in the urinary tract. This type of adhesin is involved in adhesion to the epithelium, especially lining the bladder, contributing to the development of UTI.

The *wab*G gene is involved in the production of LPS and, as confirmed by the study of Candan and Aksöz among others, it is very common – in 88% of isolates from various clinical specimens [48]. In contrast, in the work of Lev et al., the frequency of this gene was even higher, at 94%. The same work also observed that the second most common gene right after *wab*G by was the *uge* gene, present in 81% of isolates [33]. Among other things, the *uge* gene is involved in the synthesis of the polysaccharide envelope, which protects the pathogen from the host humoral response, but can also stimulate cytokine production, activating the cellular response. In our study, the percentage of strains equipped with the *uge* gene was also high, at 93.4% among KpESBL and 89.6% among *K. pneumoniae* NDM-positive group. It happens that the gene is present at a lower frequency, as evidenced by the work of Remya et al. [46] and Zhang et al. in [49] which the percentages of isolates possessing the *uge* gene were 48.6% and 56.5%, respectively.

A less common gene is the *kfu* gene, which is responsible for iron acquisition by bacteria, as demonstrated, among others, by a study by Remya et al. in which the presence of this gene was confirmed in 27.8% of isolates [46]. In turn, Rastegar et al. in 2019 proved that the presence of the *kfu* gene in clinical strains was 23.4%, while a higher percentage (45.5%) was observed among hypervirulent strains [50]. These results support the fact that there is an undeniable link between *kfu* gene expression and infections caused by the most dangerous hypervirulent strains [6]. In our study, the percentage of strains carrying the *kfu* gene was high, at 90.8% in the KpESBL group and 86.2% in the *K. pneumoniae* NDM-positive group. A similar percentage of tested isolates (76.3% and 84.5%, respectively) possessed the *iro*N gene, also related to iron metabolism, which in the work of Candan and Aksöz was observed in a much smaller number of strains, only 3.7% [48].

The gene responsible for the synthesis of the toxin, i.e. *hly*A, is a virulence factor determining the high pathogenicity of *K. pneumoniae*. This gene is also commonly found in uropathogenic strains of *Escherichia coli* [51]. In our study, we showed the presence of *hly*A in 17.1% of KpESBL and 13.8% of *K. pneumoniae* NDM-positive strains, which may indicate the spread of this gene between different species belonging to *Enterobacteriaceae*. However, it is not characteristic of most *K. pneumoniae* strains, as shown, among other things, by the results of a study by Kuş et al., in which none of the isolates of *K. pneumoniae* produced this toxin [52].

Further genes specific for hypervirulent strains, related to the envelope polysaccharide and affecting the ability to produce mucus – *mag*A and *rmp*A – are very rare or not present at all in classical *K. pneumoniae* strains. This is evidenced, for example, by a study by Rastegar et al., in which both of the aforementioned genes were not observed in any of the clinical *K. pneumoniae* strains. In contrast, the *rmp*A gene was detected in 18.2% of hypervirulent isolates, while the *mag*A gene was present in 13.6% of strains [50].

Klebsiella pneumoniae strains equipped with K1 and K2-type envelopes are believed to be among the most virulent serotypes in humans and are closely associated with severe infections [46]. Most hypervirulent *K. pneumoniae* belong to these 2 serotypes [53]. The *mag*A characteristic gene is mainly for the K1 serotype. In our study, we observed only 2.6% of KpESBL strains carrying the *mag*A gene, while the mucosal phenotype regulator *rmp*A was not detected in any of the isolates. These results correlate with observations by Remya et al., in which the presence of the *mag*A gene was detected in 0.2% of isolates, while, as in our study, the *rmp*A gene was not reported in any strain [46].

In conclusion, the conducted studies confirm the risk of infections with *K. pneumoniae* etiology due to multidrug resistance and the virulence of these microorganisms. The molecular biology methods used have allowed for a deeper characterization of the studied strains, confirming the presence of genes encoding the most common resistance (ESBL) but also the type of carbapenemase (NDM) produced. Additionally, the virulence profile was characterized by detecting genes that directly impact the infection process.

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