Studies on molecular epidemiology of ESβL-producing *Klebsiella pneumoniae* isolated from patients hospitalized in a specialist hospital in southern Poland

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Abstract

Introduction. Extended-spectrum β-lactamase (ESβL)-producing *Klebsiella pneumoniae* is currently one of the most common causes of nosocomial infections worldwide. The study aimed to characterize antibiotic resistance profile, the prevalence of selected genes encoding ESβLs, virulence, and the genetic relationship in 139 *K. pneumoniae* isolates identified in John Paul II Specialist Hospital in Southern Poland, collected in 2016.

Materials/Methods. Bacterial identification and the preliminary antibiotic susceptibilities was performed using the VITEK® 2 Compact automated system. Genes encoding ESβLs were amplified by CTX-Mplex PCR and PCR reactions. The presence of nine genes encoding virulence factors was studied by multiplex PCR. Clonality was investigated by PFGE after digestion with *SpeI* endonuclease.

Results. *K. pneumoniae* were mostly recovered from the respiratory tract (40.3%), urine (32.4%), wound swabs (19.4%) and blood (5%). In summary, 82.7% of strains were classified as multidrug resistant (MDR). All isolates were confirmed as ESβL producers and carried *bla*_{CTX-M-type} (85.6%), *bla*_{SHV} (82%), *bla*_{TEM} (77.7%), *bla*_{CTX-M-9} (75.6%) and *bla*_{CTX-M-1} (1.4%) in various combinations. Moreover, triple *bla* genes were observed in 72% of isolates. The most common virulence-associated genes found among the isolates were *entB* (91.4%), *ybtS* (55.4%), *iutA* (55.4%), *magA* (53.2%), *kfu* (14.4%), *K2* (11.5%), *mrkD* (10.1%), *rmpA* (7.9%) and *aII*S (5%). The PFGE analysis identified 4 major clusters (A–D) comprising 61% of the entire collection.

Conclusions. Our results indicate that the presence of a wide variety of MDR *K. pneumoniae* harbor ESβLs and virulence genes. Studies on molecular epidemiology of ESβL-producing *K. pneumoniae* isolates are needed, particularly for epidemiological surveillance in the hospital environment.

Keywords

ESβL-producing *K. pneumoniae* • MDR • molecular epidemiology • PFGE • virulence-associated genes

Received: 02.12.2020, Accepted: 09.08.2021

INTRODUCTION

The effective worldwide spread of extended-spectrum β-lactamase (ESβL)-producing *Enterobacteriaceae*, particularly *Klebsiella pneumoniae*, remains a critical concern for therapies against multidrug-resistant (MDR) bacteria [1]. *K. pneumoniae* is an opportunistic ESKAPE pathogen responsible for a wide spectrum of infections, including bacteremia, pneumonia, meningitis, purulent abscesses, urinary tract infections, and wound infections, at diverse sites [2]. Additionally, *K. pneumoniae* is known for its high prevalence and variety of antimicrobial resistance (AMR) genes [3]. These microorganisms are intrinsically resistant to ampicillin due to the presence of the SHV-1

penicillinase in their chromosomes. Resistance to additional drugs sporadically occurs through chromosomal mutations; however, most AMR in this species results from acquisition of these genes via HGT (horizontal gene transfer) mainly via large conjugative plasmids [3]. The most common mechanism of resistance to oxymino-cephalosporins observed in *K. pneumoniae* is the production of ESβLs (extended-spectrum β-lactamases), followed by AmpC-like cephalosporinases, and KPC (*Klebsiella pneumoniae* carbapenemase) [4].

ESβLs are β-lactamases able of conferring bacterial resistance to the penicillins; first-, second-, third-generation cephalosporins; and aztreonam (but not the cephamycins or carbapenems) by hydrolysis of these antibiotics, which are inhibited by β-lactamase inhibitors such as clavulanic

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acid [5]. In recent years, most ES β L-producing organisms have also been found to be resistant to other major classes of antibiotics, such as aminoglycosides, fluoroquinolones, and sulfonamides, owing to the coexistence of associated resistant genes encoded in and transmitted by plasmids [6].

ES β L-producing bacteria were first detected in Europe in the 1980s. Although the initial reports were from Germany and England, the vast majority of reports in the first decade after the discovery of ES β Ls were from France [5]. According to the SENTRY Antimicrobial Surveillance Program (1997 to 2016), the prevalence rate of ES β L-producing *Enterobacteriaceae* in 4 main geographic areas is high, although the rates vary substantially in different regions: Europe (19.5%), North America (12.3%), Latin America (11%), and Asia-Pacific (10.2%) [7]. The study conducted in 16 European countries over 20 years reported that ES β L screen-positive *Enterobacteriaceae* showed an increasing trend from 10.3% in 1997–2000 to 24% in 2013–2016, and the percentage of ES β L screen-positive *K. pneumoniae* isolates increased from 7.1% to 19.4% [7]. In this global survey, MDR *K. pneumoniae* rates decreased from 18.9% in 2013 to 15.8% in 2016; however, in many European countries these rates ranged from 16.9% to 55.7% in 2016 [7]. Meanwhile, the proportions of MDR *K. pneumoniae* in the study by Chong *et al.* in 2018 were 15%–60% [1]. By contrast, already in 2016, Morris *et al.* published data from the European Antimicrobial Surveillance Network which indicated that across Europe the percentages of invasive *K. pneumoniae* resistant to third-generation cephalosporins ranged from 0% to 70.1%, with ES β L producers accounting for between 85% and 100% of all strains reported [8].

The global problem of the presence of ES β L-producing *K. pneumoniae* is treated with great care; but still, the epidemiological situation in individual countries varies. In Poland, it is diverse, as it ranges from sporadic cases to dangerous epidemic outbreaks. Due to the small number of scientific reports on the spread of pathogenic *K. pneumoniae* strains in southern Poland, there is a need to learn more about the epidemiological situation in highly specialized hospitals in this area. This need served as a launching pad for carrying out this study.

Our work aimed to characterize a collection the clinical isolates of *K. pneumoniae* with an emphasis on the antibiotic resistance profile, identification of ES β L genes, virulence-associated gene detection, and the genetic relationship, thereby helping guide the monitoring of the status, prevalence, and intra-hospital spread of such pathogenic strains.

MATERIAL AND METHODS

Bacterial strains

A total of 139 *K. pneumoniae* isolates were collected from adult patients hospitalized at the John Paul II (JPII) Specialist Hospital in Krakow (Poland). The study period ran from January 1 to December 31, 2016. Most of the patients came from five different hospital wards: Cardiology and Cardiac Surgery with Cardiac Rehabilitation and Prevention (CARDIO); Pulmonology and Chest Medicine (PULMO); Neurology (NEURO); The Ward of Pediatric Infectious Diseases and Hepatology (INFECT DIS); and The Center for Diagnosis, Prevention and Telemedicine (DIAGNOST). *K. pneumoniae* isolates were mainly obtained from the respiratory tract (40.3%, *n*=56) and included: bronchial aspirates (62.5%, *n*=35); bronchoalveolar lavage, BAL (16.1%, *n*=9); sputum (10.7%, *n*=6); microball (3.6%, *n*=2); sore swabs (3.6%, *n*=2); pleural fluid (1.8%, *n*=1); and vac swab (1.8%, *n*=1). Isolates were also derived from: urine (32.4%, *n*=45); wound swabs (19.4%, *n*=27); blood (5%, *n*=7); fecal samples (1.4%, *n*=2); catheter JJ (0.8%, *n*=1); and urethral swab (0.8%, *n*=1).

Data collection

The demographic information (sample type, ward) of the patients and ES β L-producing isolate information (number, infectious site, and antibiotic susceptibility profile) were collected from the microbiological laboratory in the hospital.

Species identification, antimicrobial susceptibility testing, and phenotype definitions

The initial species identification, performed by the laboratory staff as part of routine diagnostics, was based on the assays of biochemical profiles of the isolates using the VITEK® 2 Compact system with GN ID card (bioMérieux, France). In vitro susceptibility of *K. pneumoniae* to 15 antibacterial agents including penicillins (amoxicillin/clavulanic acid, piperacillin/tazobactam), cephalosporins (ceftazidime, cefotaxime, cefepime), carbapenems (imipenem, meropenem), monobactams (aztreonam), aminoglycosides (amikacin, gentamicin), fluoroquinolones (ciprofloxacin, norfloxacin), and miscellaneous agents (colistin, nitrofurantoin, trimethoprim/sulfamethoxazole) was analyzed by the VITEK® 2 Compact system with AST-N332 cards according to the EUCAST recommendations and interpreted according to the EUCAST breakpoints [9]. The reference strain was *Escherichia coli* ATCC® 25922™. ES β L production was confirmed by the standard double disc synergy test (DDST) [10]. Multidrug-resistant (MDR) isolates were defined as non-susceptible to at least one agent in three or more antibiotic categories in accordance with the international protocol proposed by Magiorakos *et al.* [11].

ES β L gene identification

Confirmed ES β L-producing strains were screened for *bla*_{SHV} and *bla*_{TEM} by polymerase chain reaction (PCR) assays using primers and protocols as described previously [12]. A multiplex PCR assay (CTX-Mplex PCR) was used to detect *bla*_{CTX-M-type}, *bla*_{CTX-M-1}, and *bla*_{CTX-M-9} genes, as described by Xu et al. [13].

Virulence-associated gene detection

Multiplex PCR assays as published by Compain et al. were used to detect the presence of nine virulence-associated genes, including those encoding for regulators of capsular serotype K1 and hypermucoviscosity phenotype (*magA*), capsular serotype K2 and hypermucoviscosity phenotype (K2), mucoid phenotype A (*rmpA*), type 3 adhesins (*mrkD*), allantoin metabolism (*allS*), iron transport and phosphotransferase function (*kfu*), enterobactin (*entB*), yersiniabactin (*ybtS*) and aerobactin siderophore system (*iutA*) [2].

Pulsed field gel electrophoresis (PFGE)

The genetic relatedness of the ES β L-producing *K. pneumoniae* isolates was examined by pulsed field gel electrophoresis (PFGE) following the extraction of genomic DNA and digestion with *SpeI* as the restriction enzyme and under the following experimental conditions: 0.5 s–20 s pulses for 18 h, 14°C, 6V/cm² [14]. *K. pneumoniae* ATCC® BAA-1705™ was used as the reference strain. The electrophoresis was run on the CHEF DR®II system (Bio-Rad Laboratories, CA, USA). Images were captured on the Gel Doc 2000 system (Bio-Rad). The PFGE patterns were analyzed using GelCompar II and BioNumerics software packages (version 6.5, Applied Maths, Belgium). Similarity analysis was performed by Dice coefficients with customized tolerance of 1%. Additionally, PFGE types and subtypes were discerned visually according to the criteria by Tenover et al. [15].

Statistical analysis

All statistical analyses were performed using R (version 3.5.3; The R Foundation for Statistical Computing, Austria). Statistical significance was assessed via the Fisher's exact test for categorical variables in order to compare the resistance to selected antibiotics and the presence of virulence factors among the four main PFGE clonal types identified (A–D). The correlation analysis between virulence genes and resistance genes in the studied *K. pneumoniae* isolates was performed by calculating the Pearson's linear correlation coefficient (r_{xy}). P-values of <0.05 were considered statistically significant.

RESULTS

Antibiotic resistance patterns

All examined *K. pneumoniae* (100%, n=139) were resistant to amoxicillin with clavulanic acid, ceftazidime, cefotaxime, cefepime, and aztreonam. These isolates also showed different degrees of resistance to other antibiotics, such as norfloxacin (99.3%, n=138); ciprofloxacin (90.6%, n=126); trimethoprim/sulfamethoxazole (88.5%, n=123); piperacillin/tazobactam (54.7%, n=76); gentamicin (68.3%, n=42); and amikacin (0.7%, n=1) [Fig. 1]. In summary, 82.7% (n=115) of the strains were classified as MDR.

ES β Ls

The isolates were phenotypically confirmed as ES β L producers and carried *bla*_{CTX-M-type} (85.6%, n=112); followed by *bla*_{SHV} (82%, n=114) and *bla*_{TEM} (77.7%, n=108). The strains produced mostly ES β L-types CTX-M-9 (75.6%, n=105) and less frequently CTX-M-1 (1.4%, n=2). Our analysis of the co-occurrence of the detected ES β L-encoding genes among the strains tested has revealed that the most common phenomenon is the co-existence of three genes observed in 72% (n=100) of the isolates, including *bla*_{CTX-M-9}/*bla*_{SHV}/*bla*_{TEM} (65.5%, n=91); *bla*_{CTX-M-type}/*bla*_{SHV}/*bla*_{TEM} (5.8%, n=8) and *bla*_{CTX-M-1}/*bla*_{SHV}/*bla*_{TEM} (0.7%, n=1). We have even observed that 5% (n=7) of strains harbored *bla*_{CTX-M-9}/*bla*_{SHV}; 2.9% (n=4) of strains harbored *bla*_{CTX-M-9}/*bla*_{TEM}; 2.2% (n=3) of strains harbored *bla*_{SHV}/*bla*_{TEM}, and 1.4% (n=2) of strains harbored *bla*_{CTX-M-type}/*bla*_{SHV}. Nevertheless, considering 6.5% (n=9) of isolates having only a single β -lactamase gene, we noted a predominance of *bla*_{CTX-M-9} (2.2%, n=3).

Virulence

Multiplex polymerase chain reaction analysis demonstrated that the *kfu* and *mrkD* genes, encoding iron transport and phosphotransferase function and type 3 fimbrial adhesins, were present in 14.4% (n=20) and 10.1% (n=14) of isolates, respectively. The regulators of the mucoid phenotype A (*rmpA*) gene were detected in 7.9% (n=11) of isolates. Seven isolates (5%) were positive for *allS* gene encoding allantoin metabolism. Additionally, the enterobactin (*entB*) gene was found in 91.4% (n=127), the yersiniabactin (*ybtS*) gene in 55.4% (n=77), and the aerobactin siderophore system (*iutA*) gene in 55.4% (n=77) of isolates. Seventy-four isolates (53.2%) presented the capsular serotype K1 (*magA* gene), and the capsular K2 (*K2* gene) was found in 11.5% (n=16) of isolates. Thirty-nine combinations of genes encoding selected virulence factors were observed, with clear dominance of: *ybtS/entB/iutA* in 16.5% (n=23) of isolates, *ybtS/entB/magA* in 14.4% (n=20), and *ybtS/entB/iutA/magA* in 8.6% (n=12).

Figure 1: Antimicrobial resistance patterns of ES β L-producing *K. pneumoniae* isolates.

Correlation between virulence genes and resistance genes

Twenty-five different combinations were observed based on the coexistence of virulence genes with resistance genes [Fig. 3]. The largest group of isolates ($n=52$, 37.4%) were strains with three genes encoding virulence factors and four resistance genes in different lists: $bla_{SHV}/bla_{TEM}/bla_{CTX-M-type}/bla_{CTX-M-9}+ybtS/entB/iutA$ ($n=17$, 32.7%); $bla_{SHV}/bla_{TEM}/bla_{CTX-M-type}/bla_{CTX-M-9}+ybtS/entB/magA$ ($n=51$, 28.8%); $bla_{SHV}/bla_{TEM}/bla_{CTX-M-type}/bla_{CTX-M-9}+entB/iutA/magA$ ($n=10$, 19.2%); $bla_{SHV}/bla_{TEM}/bla_{CTX-M-type}/bla_{CTX-M-9}+entB/iutA/magA$ ($n=10$, 19.2%); $bla_{SHV}/bla_{TEM}/bla_{CTX-M-type}/bla_{CTX-M-9}+mrkD/entB/iutA$ ($n=6$, 11.5%); $bla_{SHV}/bla_{TEM}/bla_{CTX-M-type}/bla_{CTX-M-9}+entB/kfu/magA$ ($n=3$, 5.8%); $bla_{SHV}/bla_{TEM}/bla_{CTX-M-type}/bla_{CTX-M-9}+ybtS/entB/magA$ ($n=1$, 1.9%). The obtained Pearson's coefficient ($r_{xy}=0.27$, $P=0.001$) indicates a weak correlation ($0.1 \leq r_{xy} < 0.3$) between the coexistence of virulence genes and resistance genes in the studied *K. pneumoniae* isolates.

PFGE

PFGE identified 41 different profiles which fell into 4 major clusters (A–D) in 85 isolates (comprising 61% of the entire collection), based on a similarity of $\geq 98\%$ (cut-off) [Fig. 2]. The largest cluster, A, accounted for 28.1% ($n=39$) of all isolates and contained strains from four hospital wards, including 19 (48.7%) isolates from CARDIO, 15 (38.5%) from PULMO, 4 (10.2%) isolates from NEURO, and 1 (2.6%) isolate from the DIAGNOST ward. The next clusters were B (16.5%, $n=23$) with 14 (60.9%) isolates from CARDIO, 6 (26%) isolates from PULMO, 2 (8.7%) isolates from NEURO, and 1 (4.3%) isolate from the DIAGNOST ward; cluster C (8.6%, $n=12$) with 7 (58.3%) isolates from CARDIO, 3 (25%) isolates from PULMO, 1 (8.3%) isolate from INFECT DIS, and 1 (8.3%) isolate from the DIAGNOST ward; cluster D (7.2%, $n=10$) with 7 (70%) isolates from CARDIO, 2 (50%) isolates from PULMO, and 1 (10%) isolates from INFECT DIS. Clone A was mostly isolates extracted from bronchial aspirates (46%), clones B and C were mostly isolates from urine (34.8% and 41.7%, respectively), clone D was mostly isolates from urine (50%). Isolates classified into clonal type A demonstrated a significantly higher sensitivity to trimethoprim/sulfamethoxazole than isolates from the other three main PFGE clonal types (B–D) ($p=0.06$). Isolates classified into clonal type C demonstrated a significantly lower resistance to amikacin and piperacillin/tazobactam than isolates from the remaining main clonal types A, B, D ($p<0.001$). Isolates classified into clonal type D showed a significantly higher sensitivity to gentamicin than isolates from the remaining clonal types A, B, and C ($p<0.001$). As for other antibiotics, the results were not statistically significant. Among the main clonal types A–D, three subgroups were distinguished, which differed as regards the presence of

bla_{SHV} statistically significantly ($p<0.001$), starting from the highest detectability: D (93.75%), A (82.05%), B and C (79.17% and 77.78%, respectively). No statistically significant differences were found between the affiliation of the strains to main clonal types (A–D) and virulence, the type of the ward, or the kind of clinical material they were isolated from. The remaining strains, 16% ($n=22$), were clustered into 7 minor clonal lineages: G ($n=3$), H ($n=3$), I ($n=2$), J ($n=2$), K ($n=2$), L ($n=2$), M ($n=2$), and 32 singletons.

DISCUSSION

ES β L-producing *Enterobacteriaceae* are among the most common pathogens involved in nosocomial infections worldwide, with species other than *Escherichia coli* (especially *K. pneumoniae*) being increasingly reported in recent years [16].

In *K. pneumoniae* rods, ES β Ls are still some of the most important mechanisms of drug resistance clinically and epidemiologically, due to their potential for rapid spread, as the genes encoding them are usually transferred on mobile genetic elements such as plasmids. Moreover, the widespread use of antibiotics has led to the emergence of MDR strains which accumulate mechanisms conditioning resistance to various groups of antibiotics. Currently, such strains are a global problem [3, 6, 7, 17]. Studies on molecular epidemiology of ES β L-producing *K. pneumoniae* isolates are needed, particularly under multi-level epidemiological monitoring, which is considered essential to understanding trans-hierarchical evolutionary changes in antibiotic resistant populations, and ultimately to establish appropriate control interventions [16].

This study investigates drug resistance profiles, the presence of genes encoding selected virulence factors, and genetic diversity of the ES β L-producing *K. pneumoniae* strain collection. Considering the latest research, this examination is part of a general trend of research on the molecular epidemiology of the species *K. pneumoniae*, and the obtained strain characterization results are similar to the results presented by other Polish authors [4, 18, 19, 20].

In their analysis of hospital-acquired infections caused by ES β L-producing *K. pneumoniae*, Castanheira et al. demonstrated that these rods were mainly the cause of wound infections (20.8%), respiratory tract infections (16.3%), urinary tract infections (13%), and generalized infections (11.9%) [7]. Therefore, respiratory secretions, urine, wound swabs, and blood are the main materials from which these pathogens were isolated [7]. In our study, the greatest number of *K. pneumoniae* isolates (40.3%) came from materials from the respiratory tract, urine (32.4%), wound swabs (19.4%), and blood (5%). Similarly, Wang et al. described

Figure 2. Dendrogram illustrating the PFGE patterns of 139 ESBL-producing *K. pneumoniae* isolates after restriction with *SpeI* enzyme. PFGE settings: Similarity coefficient: Dice, Optimization: 1%, Tolerance: 1%, Clustering method: UPGMA.

Figure 3. Correlation between virulence genes and resistance genes in ESβL-producing *K. pneumoniae* isolates. Pearson's correlation coefficient $r_{xy}=0.27$, $P=0.001$.

most *K. pneumoniae* isolates that were cultured from urine (38.8%), materials from the respiratory tract (28.25%), followed by wound swabs (11.04%) and blood (7.22%) [6]. Correspondingly, most *K. pneumoniae* isolates characterized by Ranjbar et al. came from urine (63.75%), blood (37.5%), sputum (35.21%) and wounds (33.33%) [21].

The results of drug susceptibility testing as regards the *K. pneumoniae* collection under study demonstrated a resistance profile to β-lactam antibiotics that is typical of ESβL producers. All isolates were found to be resistant to several marker oxyimino-β-lactams of the ESβL mechanism: for instance, ceftazidime, cefotaxime, cefepime and aztreonam.

It should be underlined that our investigations revealed a high percentage (54.7%) of strains resistant to piperacillin/tazobactam. Similar results were obtained by other Polish researchers, Mrowiec et al., who recorded 53.5% of *K. pneumoniae* isolates resistant to this antibiotic in the adult population [18]. On the other hand, an investigation carried out by Korean researchers, Lee et al., found a lower percentage (20.9%) of *K. pneumoniae* strains which were resistant to piperacillin/tazobactam [22]. In his 1995 study, Livermore proved that ESβL-producing strains frequently turn out to be resistant in vitro to combinations of β-lactams with inhibitors, despite the fact that ESβLs, by definition, are inhibited by β-lactamase inhibitors (clavulanic acid, sulbactam, or tazobactam) [23]. Furthermore, literature data indicate that the development of resistance to piperacillin/tazobactam in clinical *K. pneumoniae* isolates may be the result of several different resistance mechanisms, including the hyperproduction of constitutive chromosomal

β-lactamases SHV-1 or the hyperproduction of TEM-1 β-lactamase due to the presence of either strong promoters or multiple bla_{TEM-1} copies [22]. In our study, among all isolates resistant to piperacillin/tazobactam, as many as 93.4% of the strains demonstrated the presence of several (2–3) variants of ESβL enzymes. Hence, the observed growing resistance to piperacillin/tazobactam in *K. pneumoniae* is caused by the co-occurrence of different variants of ESβL enzymes in individual strains.

In this study, examinations conducted using PCR confirmed the presence of genes encoding ESβL in all the studied *K. pneumoniae* isolates; in as many as 72% of the strains, genes belonging to three different families were detected: CTX-M-type (85.6%), SHV (82%), and TEM (77.7%). Within the cefotaxime group, however, a clear dominance of the CTX-M-9 enzyme group was demonstrated at 75.6%, while the presence of CTX-M-1 was confirmed in only two (1.4%) strains. The study showed a high percentage of isolates (65.5%) that were characterized by the coexistence of three genes: $bla_{CTX-M-9}/bla_{SHV}/bla_{TEM-1}$. We have even observed that 5% of strains harbored $bla_{CTX-M-9}$ and bla_{SHV} , 2.9% of strains harbored $bla_{CTX-M-9}$ and bla_{TEM-1} , and 2.2% of strains harbored bla_{SHV} and bla_{TEM-1} . Contrarily, in 2019, a similar Polish research into *K. pneumoniae* isolates carried out by Mrowiec et al. showed that 91.2% of strains carried $bla_{CTX-M-1}$, 63.5% carried bla_{TEM-1} , and 11.8% carried bla_{SHV} ; 7% of strains had all 3 genes, 67.4% had $bla_{CTX-M-1}$ and bla_{TEM-1} , 1.2% had bla_{TEM-1} and bla_{SHV} , and 1.2% had $bla_{CTX-M-1}$ and bla_{SHV} [18]. However, the *K. pneumoniae* isolates coming from 2011 and described by Walter de Walthoffen et al. in Poland demonstrated the presence of bla_{CTX-M} at 94%, bla_{TEM} at 94%, and bla_{SHV} at 52.8%, while at the same time, 41.6% of strains had all 3 genes, 86.1% had bla_{TEM} and bla_{CTX-M} , 50% had bla_{SHV} and bla_{CTX-M} , and 44.4% had bla_{TEM} and bla_{SHV} [24]. The studies quoted above show that a spread of many different variants of ESβL enzymes is observed in Poland. In Portugal, studies carried out by Rodrigues et al. confirm the fact that, among *K. pneumoniae* strains, there is coexistence of β-lactamases belonging to CTX-M-15, SHV and TEM, detected at 47%, 45%, and 9%, respectively [16]. Yet, as early as in 2003 in North America, Paterson et al. documented the occurrence of *K. pneumoniae* with multiple ESβL types in different regions of the world, among others, Argentina, Australia, South Africa, Turkey, and United States [25].

A review of the literature indicates that in *K. pneumoniae* genes encoding plasmid-transmitted ESβL β-lactamases often coexist with other plasmid-encoded resistance mechanisms, including to fluoroquinolones, aminoglycosides, co-trimoxazole, tetracyclines, and chloramphenicol [26]. The characterization of the collection of ESβL-producing *K. pneumoniae* strains that was carried out confirmed the high percentage of isolates resistant to antibiotics from therapeutic

groups other than penicillins, such as fluoroquinolones (ciprofloxacin, 90.6%), aminoglycosides (amikacin, 0.7%) and trimethoprim/sulfamethoxazole (88.5%). Studies carried out in Poland by Ojdana et al. found a high percentage of *K. pneumoniae* strains resistant to trimethoprim/sulfamethoxazole (100%), amikacin (58.3%), and 16.7% of isolates resistant to ciprofloxacin [19]. In Brazil, Wang et al. recorded 10.5%, 59.8%, and 80.6% of strains resistant to, respectively, amikacin, ciprofloxacin, and trimethoprim/sulfamethoxazole [6]. The differences observed in the above-mentioned investigations prove the fact that drug resistance profiles in individual ES β L-producing *K. pneumoniae* strains vary and can be characteristic of a given region, hospital, or unit. In this study, 82.7% of the examined *K. pneumoniae* isolates were classified into the category of MDR strains, which is similar to the results of the Brazilian researchers, who recorded 84% of strains from this species with the MDR phenotype [26].

In the course of analysis of the most common genes encoding virulence factors in the studied pool of *K. pneumoniae* isolates, 55.4%, 10.1%, and 7.9% demonstrated the presence of genes *iutA*, *mrkD*, and *rmpA*, respectively. Contrarily, in the examination by Compain et al., the *mrkD* gene was detected in 100% of *K. pneumoniae* isolates, while the presence of the *iutA* gene was not found [2]. Also Ranjbar et al. demonstrated the presence of the *mrkD* gene in 84.34% and the *rmpA* gene in 13.91% of the studied *K. pneumoniae* isolates [21]. On the other hand, Ferreira et al. recorded *iutA* in 40% and *mrkD* in 96% of isolates, but they did not detect the presence of the *rmpA* gene [26]. Compain et al. showed the presence of the *rmpA* gene in 7.1% of isolates, which is similar to the proportion in this study (7.9%). The remaining virulence-associated genes found among the isolates were *entB* (91.4%), *ybtS* (55.4%), *kfu* (14.4%), *K2* (11.5%), *allS* (5%). The French researchers also detected these virulence genes: *entB* (100%), *kfu* (35.7%), *ybtS* (21.4%), and *allS* (7.1%) [2], and the researchers from Brazil, respectively: *entB* (100%), and *ybtS* (60%) [26]. The *magA* gene was detected in 53.2% of the strains, contrary to Brisse et al. in France and Lascols et al. in Canada, who did not demonstrate the presence of the *magA* gene in any of the *K. pneumoniae* isolates studied [27, 28].

As for the *K. pneumoniae* strains under study, it was observed according to the data in the literature [8, 20], that the majority of the isolates (61%) were of clonal origin. The assessment of the genetic similarity of the studied *K. pneumoniae* rods was done with the use of PFGE. According to Paterson and Bonomo, PFGE is the most frequently used molecular reference method to genotype ES β L-producing *Enterobacteriaceae* in the world [5]. It is confirmed by

numerous international, as well as Polish, publications concerning molecular analysis of the *K. pneumoniae* population: in France, Ireland, Brazil, China, and Poland [8, 14, 20, 26, 27]. Our work showed good typeability, reproducibility of *SpeI* for *K. pneumoniae* subtyping, and revealed high genetic diversity among the studied isolates. Analysis of the *SpeI*-PFGE genetic profiles of the strains demonstrated that their genetic similarity ranged from 66.6% to 100%. The highest degree of similarity (100%) was found between isolates classified into four PFGE clonal types (A–D) and seven minor clonal lineages (G–M). These isolates came from various clinical materials from patients hospitalized in different hospital wards. The obtained results of genotyping indicate that *K. pneumoniae* epidemic clones spread between different hospital departments. Similarly, Morris et al. demonstrated clonal organization of the *K. pneumoniae* strain collection: among 137 isolates, they identified 111 various PFGE profiles, which fell into 16 major clusters (A–P) in 112 isolates based on similarity of >85%, with each cluster comprising 2–43 isolates [8]. The clonal genetic structure in *K. pneumoniae* was also confirmed by Lascols et al. in their work published in 2013, which identified 3 major clonal groups among the isolates under study with more than 10 isolates per cluster, 10 minor clusters with less than 10 isolates per cluster, and 13 singletons [28]. In our study, most isolates (28%) were classified into clonal group A (cluster A), and in the research by Morris et al., the percentage of isolates classified into the largest clonal group (cluster A) was very similar and amounted to 31% (Morris et al. 2016). Yet, Lascols et al. described the largest clonal group (cluster C) comprising 53% of *K. pneumoniae* isolates [28].

The epidemiological situation of a hospital or a given ward may be influenced by many factors; among them, the specificity of the facility, the profile of patients that are treated there, the antibiotics employed, hygienic and sanitary measures undertaken or the persistence of certain pathogenic microorganisms.

To conclude, due to worldwide reports of a significant increase in resistance of *K. pneumoniae* to the antibacterial drugs used and the observed regional changes in the scale of this phenomenon, it is necessary to find the ES β L+ phenotype in all *K. pneumoniae* isolates as part of routine microbiological diagnostics. In addition, it should be remembered that when choosing empirical therapy for serious infections with an etiology of *K. pneumoniae* ES β L+, it is extremely important to take into account the local epidemiological characteristics. The application of molecular techniques to identify multidrug-resistant strains can contribute to improved surveillance and rapid detection of epidemic clones, and as a consequence, to a reduction in their spread in the hospital environment.

Acknowledgments

We would like to thank the staff and employees of the microbiological laboratory at John Paul II Hospital in Krakow for their participation in the study. A special thank you to Catherine Gąsior-Kulasiak for English language editing.

Authors' Contribution

D.O.: research concept and design; supervising the project; carrying out the experiments; data analysis and interpretation; writing – original draft preparation; writing – review and editing; visualization; literature review; final proofreading and approval of the version for publication; funding acquisition; **A.O.J.:** acquisition of data; final proofreading and approval of the version for publication; **A.D.:** carrying out the experiments; final proofreading and approval of the version for publication; **M.B.:** acquisition of data; final proofreading and approval of the version for publication; **M.B.W.:** research concept and design; content-related supervision over research; final proofreading and approval of the version for publication.

Funding

This research was financially supported by the Jagiellonian University Medical College (Grant/K/ZDS/007829).

Conflict of Interest

The authors have no potential conflicts of interest to declare.

Ethics Approval

The study was approved by the Bioethics Committee of the Jagiellonian University in Krakow, Poland (KBET/1072.6120.264.2019).

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