

HIGHER CTX-M, TEM, AND SHV EXTENDED-SPECTRUM BETA-LACTAMASE PLASMID GENE COMBINATION FREQUENCY IN ESBL PRODUCING *KLEBSIELLA PNEUMONIAE* COMPARED WITH ESBL PRODUCING *ESCHERICHIA COLI*

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Abstract

Introduction: Extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae confer antibiotic resistance to broad-spectrum penicillins, cephalosporins, using ESBL genes CTX-M, TEM, SHV, which are encoded in bacterial plasmid genome.

Methods: We compared plasmid CTX-M, TEM, and SHV ESBL gene combinations in ESBL producing *Escherichia coli* and *Klebsiella pneumoniae*. Altogether, 136 ESBL producing Enterobacteriaceae isolation cases were analyzed.

Results: The ESBL producing Enterobacteriaceae, mostly *K. pneumoniae* (n = 66; 48.53%), *E. coli* (n = 36; 26.47%), were isolated from 52 (38.2%) female and 84 (61.8%) male patients from Riga East Clinical University Hospital. Overall, 132 isolates (97.06%) registered positive for the plasmid CTX-M gene, 97 isolates (71.32%) for the plasmid TEM gene, and 87 isolates (63.97%) for the plasmid SHV gene. Patients with the three ESBL plasmid gene (CTX-M+ TEM+ SHV+) combination were significantly older (67.11 ± 14.16 years) than patients with other gene combinations (59.63 ± 18.14 years; $p = 0.047$). Plasmid SHV gene frequency was higher in *K. pneumoniae* ($p < 0.001$). The *K. pneumoniae* mostly presented with the three plasmid ESBL gene (CTX-M+ TEM+ SHV+) combination, whereas *E. coli* presented with other gene combinations ($p = 0.014$).

Conclusions: *Klebsiella pneumoniae*, more often, presented with the plasmid SHV ESBL gene and the three ESBL gene (CTX-M+ TEM+ SHV+) combination, compared to *E. coli*. Older patients with ESBL producing Enterobacteriaceae infection, more often, presented with the three ESBL gene (CTX-M+ TEM+ SHV+) combination, compared to younger patients with the infection.

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Introduction

Since the first outbreak in 1983, reports of extended-spectrum beta-lactamase (ESBL) producing Enterobacteriaceae have increased due to extensive antibiotic consumption and widespread gene mutation, conferring resistance to extended spectrum penicillins and cephalosporins (Coque, Baquero, & Canton, 2008; Alekshun & Levy, 2007).

Extended-spectrum beta-lactamase are mostly produced by *Escherichia coli* and *Klebsiella pneumoniae* (Shaikh, Fatima, Shakil, Rizvi, & Kamal, 2015; Lee, Bae, & Lee, 2012; Cantón et al., 2008) and their production is determined by the presence of three extended-spectrum beta-lactamase

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genes: CTX-M, TEM, and SHV (Poirel, Naas, & Nordmann, 2008) in the bacterial plasmid genome, each determining a specific resistance towards different antimicrobials (Shaikh et al., 2015; Lee et al., 2012; The New β -Lactamases - NEJM, 2015).

The ESBL producing microorganisms predominate in cases of urinary tract infections, ventilator-associated pneumonias, wound infections, intra-abdominal infections, and a wide variety of other nosocomial and community acquired infections (Spadafino, Cohen, Liu, & Larson, 2014; Takaba et al., 2014; Tacconelli et al., 2014).

Adequate empiric antibacterial therapy in ESBL producing microorganism infection cases should be started early, preferably within 72 hours of confirmation of ESBL producing bacteria (Tumbarello et al., 2007), and should be based on local antibacterial resistance patterns. This is often challenging due to high antimicrobial resistance and lack of local resistance pattern data (The New β -Lactamases - NEJM, 2015 ; Peralta et al., 2012).

Plasmid CTX-M, TEM, and SHV genes, and their combinations, which determine antimicrobial resistance and clinical course severity, vary widely depending on the geographic location, hospital, ward, patient group, or even type of infection (Alekhshun & Levy, 2007; Shaikh et al., 2015; Lee et al., 2012; Cantón et al., 2008). Therefore, it is essential to determine the local ESBL producing bacteria strains and plasmid extended-spectrum beta-lactamase gene frequency in order to establish local resistance patterns and start an effective empiric antibacterial treatment (Tacconelli et al., 2014).

Aim of the Study

The aim of the study is to compare plasmid CTX-M, TEM, and SHV extended-spectrum beta-lactamase gene presence and combinations in ESBL producing *E. coli* and *K. pneumoniae* strains.

Methods

Setting and Study Design

A cross-sectional single-center study was conducted in Riga East Clinical University Hospital (RECUH), Latvia, the largest medical center (a 2270-bed center) in the Baltic countries.

Patient Selection and Sample Size

All consecutive ESBL producing Enterobacteriaceae cases were selected over a 6-month period (during 2013–2014). A total of 136 ESBL producing Enterobacteriaceae isolation cases, containing plasmid genes CTX-M, TEM, and SHV, were included in the study, regardless of patient age, gender, and clinical severity.

Demographic Data

Demographic data were gathered from medical records on “day” patients that were discharged, and this included information about the patient’s age and gender.

Bacteriological Analysis

Bacteriological analysis was performed according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines (version 1.0, December 2013) for detection of resistance mechanisms and specific resistances of clinical or epidemiological importance or both (EUCAST: Resistance mechanisms, 2015). This analysis provided information about the tested biomaterial, including wound biomaterial, urine, bronchoalveolar fluid, abdominal cavity biomaterial, blood, abscess, cerebrospinal fluid, and sputum, as well as ESBL producing Enterobacteriaceae strains that were isolated during the study.

Genetic Analysis

Genetic analysis, analyzing only the acquired antibiotic resistance, provided information about the three most common bacterial plasmid extended-spectrum beta-lactamase genes: CTX-M, TEM, and SHV. Bacteria selected from ESBL producing colonies were grown in 2 ml Lysogeny broth (LB) media at 37 °C overnight. Plasmid DNA were isolated by manufacturer protocol using E.Z.N.A. plasmid Mini Kit I (Omega Bio-Tek, USA). The presence of plasmid extended-spectrum beta-lactamase genes was confirmed by polymerase chain reaction (PCR), using gene specific primers as described in the literature (Edelstein, Pimkin, Palagin, Edelstein, & Stratchounski, 2003; Wu et al., 2001). These PCR products were analyzed using 2% agarose gel.

Statistical Analysis

An original study protocol was developed with 106 parameters, including demographic, bacteriological, and genetic parameter groups and completed for each ESBL producing Enterobacteriaceae isolation case; and used for database development using Microsoft Office Excel 2013 software. Statistical analysis was conducted with IBM SPSS 22.0 software using Spearman correlation coefficient, Mann-Whitney U test, and Pearson's Chi-square test. All p-values were two-tailed and considered significant at 0.05 level.

Results

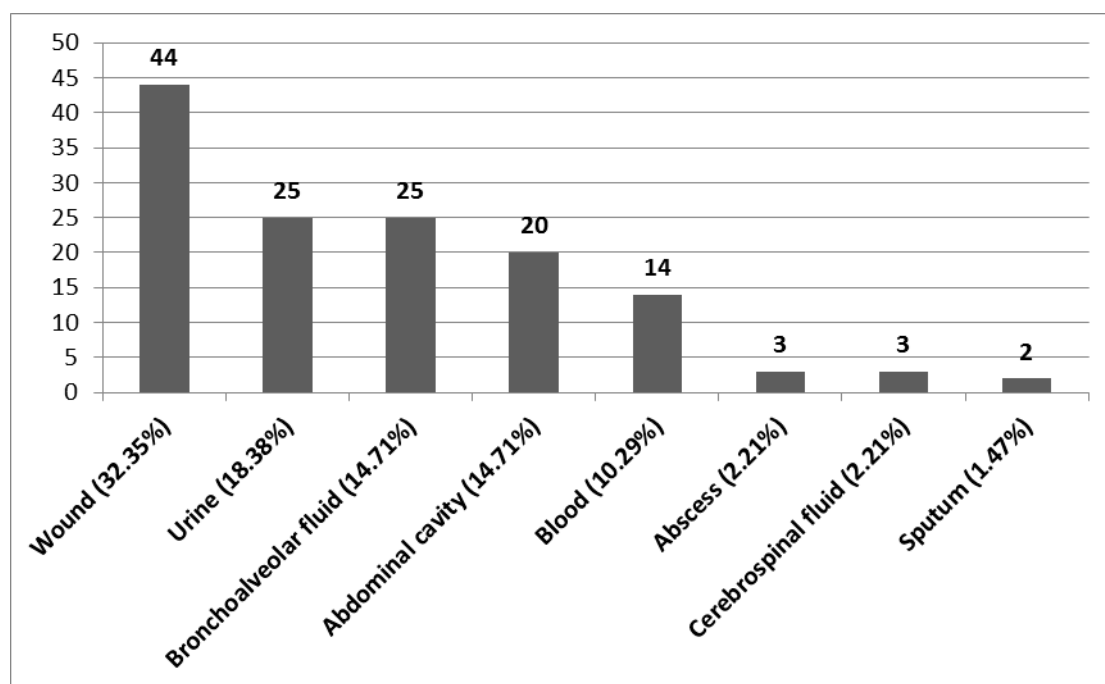
During the study, ESBL producing Enterobacteriaceae were isolated from 52 (38.2%) female and 84 (61.8%) male patients in the age range 22 – 89 years; the mean age was 61.35 ± 16.92 (CI 95%; 57.76 – 64.78) years.

Bacteriological Data

Most commonly, ESBL producing Enterobacteriaceae were isolated from wound (n = 44, 32.35%), urine (n = 25, 18.38%), bronchoalveolar fluid (n = 25, 18.38%), and abdominal cavity (n = 20, 14.71%) biomaterial. (Figure 1)

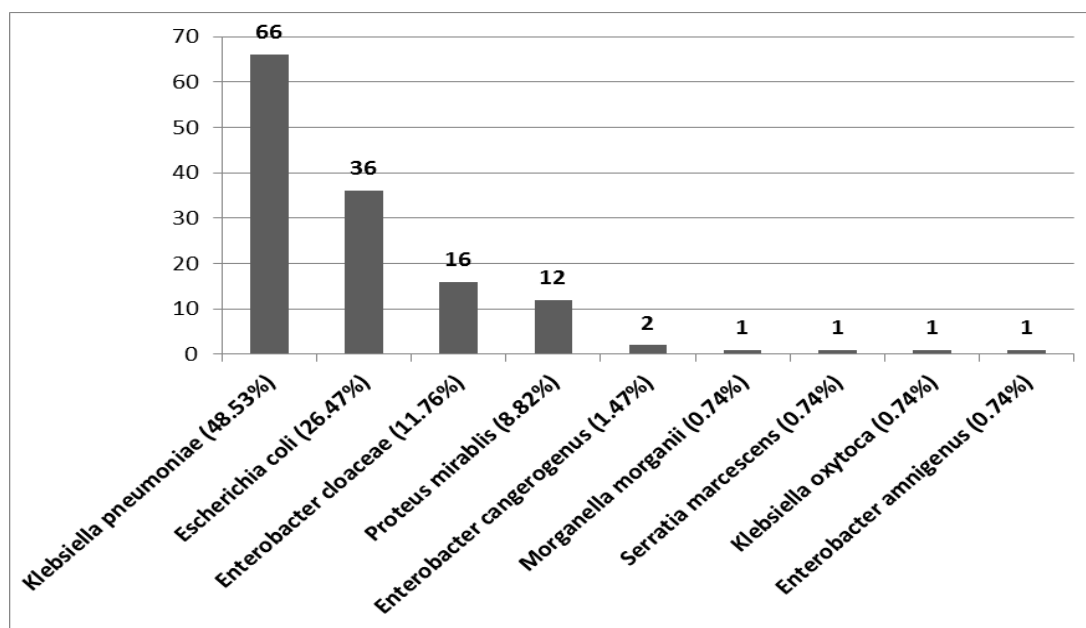
In most cases, ESBL producing *K. pneumoniae* (n = 66, 48.53%), *E. coli* (n = 36, 26.47%), *Enterobacter cloacae* (n = 16, 11.76%), and *Proteus mirabilis* (n = 12, 8.82%) were isolated during the study (Figure 2).

Figure 1: Biomaterial for extended-spectrum beta-lactamase producing Enterobacteriaceae isolation, n (%)



Source: Author

Figure 2: Extended-spectrum beta-lactamase producing Enterobacteriaceae isolated in the study, n (%)



Source: Author

Genetic Profile

There were 132 ESBL producing Enterobacteriaceae isolates (97.06%) that registered positive for the plasmid CTX-M gene, 97 isolates (71.32%) positive for the plasmid TEM gene, and 87 isolates (63.97%) positive for the plasmid SHV gene.

In most cases (n = 62, 45.59%), all three plasmid extended-spectrum beta-lactamase genes: CTX-M, TEM, and SHV, were detected in ESBL producing Enterobacteriaceae plasmid genome at the same time (Table 1).

Table 1: Results of extended-spectrum beta-lactamase producing Enterobacteriaceae extended-spectrum beta-lactamase plasmid gene detection, n (%)

Gene combinations	N total (136)	%
CTX-M+ TEM+ SHV+	62	45.59
CTX-M+ TEM+ SHV-	31	22.79
CTX-M+ TEM- SHV+	23	16.91
CTX-M+ TEM- SHV-	16	11.77
CTX-M- TEM+ SHV+	2	1.47
CTX-M- TEM+ SHV-	2	1.47

Source: Author

Demographic Data Correlations

No statistically significant differences were found between male and female patient groups concerning bacterial and genetic factors (Table 2).

Patients with plasmid CTX-M+ TEM+ SHV+ gene combination were significantly older (67.11 ± 14.16 years; CI 95%, 63.31–70.76 years) than patients with other plasmid gene combinations (59.63 ± 18.14 years; CI 95%, 55.61–63.70 years), including CTX-M+ TEM+ SHV-; CTX-M+ TEM- SHV+; CTX-M- TEM+ SHV+; CTX-M+ TEM- SHV-; and CTX- TEM+ SHV- plasmid gene combinations (p = 0.047). No other significant differences were found between the three plasmid extended-spectrum

beta-lactamase genes and their combinations concerning patient age or gender. Also, no statistically significant differences were found between *K. pneumoniae* and *E. coli* patient groups in terms of age.

Bacteriological Data Correlations

Statistically significant differences were found between *K. pneumoniae* and *E. coli* groups in terms of plasmid SHV gene frequency, which was higher in the *K. pneumoniae* group, compared to the *E. coli* group ($p < 0.001$). Statistically significant differences were also found in plasmid gene combinations, where *K. pneumoniae* mostly presented in the three plasmid CTX-M+ TEM+ SHV+ extended-spectrum beta-lactamase gene combination, whereas *E. coli* was evident in other plasmid gene combinations, including CTX-M+ TEM+ SHV-; CTX-M+ TEM- SHV+; CTX-M- TEM+ SHV+; CTX-M+ TEM- SHV-; and CTX- TEM+ SHV- plasmid gene combinations ($p = 0.014$; Table 3).

Table 2: Bacteriological and genetic data (plasmid genes) comparison in male and female patients, n (%)

	Male patients (n=84, 61.8%)	Female patients (n=52, 38.2%)	p-value
<i>K. pneumoniae</i>, n (%)	43 (51.2%)	23 (33.2%)	0.674
<i>E. coli</i>, n (%)	21 (25%)	15 (28.8%)	0.621
Other <i>Enterobacteriaceae</i>^a, n (%)	20 (23.8%)	14 (26.9%)	0.684
CTX-M+, n (%)	81 (96.4%)	51 (98.1%)	0.580
TEM+, n (%)	57 (67.9%)	40 (76.9%)	0.256
SHV+, n (%)	55 (65.5%)	32 (61.5%)	0.642
CTX-M+ TEM+ SHV+, n (%)	39 (46.6%)	23 (44.2%)	0.860
Other plasmid gene combinations^b, n (%)	45 (53.6%)	29 (55.8%)	0.860

^a *E. cloacae*, *P. mirabilis*, *E. cancerogenus*, *M. morgani*, *S. marcescens*, *K. oxytoca*, *E. amnigenus*

^b CTX-M+ TEM+ SHV-; CTX-M+ TEM- SHV+; CTX-M- TEM+ SHV+; CTX-M+ TEM- SHV-; and CTX-TEM+ SHV-

Percentage stated within the gender group

Source: Author

Table 3: Genetic data comparison (plasmid genes) in different extended-spectrum beta-lactamase producing *Enterobacteriaceae*, n (%)

	<i>K. pneumoniae</i> (n=66, 48.53%)	<i>E. coli</i> (n=36, 26.47%)	p-value^a	Other <i>Enterobacteriaceae</i>^b (n=34, 25%)
CTX-M+, n (%)	65 (98.5%)	36 (100%)	0.458	31 (91.2%)
TEM+, n (%)	46 (69.7%)	26 (72.2%)	0.789	25 (73.5%)
SHV+, n (%)	52 (78.8%)	15 (41.7%)	<0.001	20 (58.8%)
CTX-M+ TEM+ SHV+, n (%)	37 (56.1%)	11 (30.6%)	0.014	14 (41.2%)
Other plasmid gene combinations^c, n (%)	29 (43.9%)	25 (69.4%)	0.014	20 (58.8%)

^a p values calculated comparing *K. pneumoniae* and *E. coli* isolates

^b *E. cloacae*, *P. mirabilis*, *E. cancerogenus*, *M. morgani*, *S. marcescens*, *K. oxytoca*, *E. amnigenus*.

^c CTX-M+ TEM+ SHV-; CTX-M+ TEM- SHV+; CTX-M- TEM+ SHV+; CTX-M+ TEM- SHV-; and CTX-TEM+ SHV-

Percentage stated within the bacteria group.

Source: Author

Discussion

Patients were approximately the same gender and age as described in other studies concerning ESBL producing Enterobacteriaceae infection with male patients around 60 years of age (Kassakian & Mermel, 2014; Mehrgan & Rahbar, 2008).

Klebsiella pneumoniae was the most frequently isolated ESBL producing bacteria in this study, whereas *E. coli* was the most commonly isolated ESBL producing bacteria in other literature sources (Sasirekha, 2013; Moor et al., 2008; Kargar, Kargar, Jahromi, Najafi, & Ghorbani-Dalini, 2014). These differences may be explained by the various biomaterials used in the different studies. Wound biomaterial was the most common source of biomaterial for ESBL producing Enterobacteriaceae isolation in this study, while urine was the most commonly used biomaterial in other studies (Peralta et al., 2012; Kassakian & Mermel, 2014; Moor et al., 2008; Ruiz de Alegría et al., 2011; Kolar et al., 2006). The frequency of other biomaterial for ESBL producing bacteria detection, including abdominal cavity and abscess biomaterial, bronchoalveolar fluid, blood, and cerebrospinal fluid was approximately the same as that described in other literature sources (Peralta et al., 2012; Kassakian & Mermel, 2014; Ruiz de Alegría et al., 2011; Kolar et al., 2006; Mengistu et al., 2013). The frequency of other Enterobacteriaceae found in this study, including *P. mirabilis*, *S. marcescens*, and *K. oxytoca*, was similar to that in other studies (Sasirekha, 2013). The frequency of *Enterobacter* spp., including *E. cloacae*, *E. cancerogenus*, and *E. amnigenus*, was slightly higher in this study, compared to other literature sources (Sasirekha, 2013; Moor et al., 2008; Kargar et al., 2014).

The three most frequently found plasmid extended-spectrum beta-lactamase gene classes (CTX-M, TEM, and SHV) were analyzed in this study without further plasmid gene subgroup analysis. Plasmid CTX-M, TEM, and SHV extended-spectrum beta-lactamase gene frequency was higher in this study than in other literature sources, namely: 1) in this study plasmid CTX-M was 97.06% compared to 73.8 – 85.4% elsewhere; 2) plasmid TEM was 71.32% compared to elsewhere 42.4 – 62.3%; and 3) plasmid SHV was 63.97% compared to the wide range of 7.7 – 62.3% in the literature (Hayakawa et al., 2013; Lin, Hsu, Chen, Huang, & Lo, 2010; Manoharan, Premalatha, Chatterjee, & Mathai, 2011). In addition, plasmid gene combination frequency was higher, compared to other similar studies: CTX-M+ TEM+ SHV+ plasmid gene combination (45.59% vs 25.64%); and CTX-M+ TEM+ SHV- plasmid gene combination (68.38% vs 26.5–35.5%; Hayakawa et al., 2013; Manoharan et al., 2011). Considerably higher plasmid gene frequency was found in this study compared to the literature for CTX-M+ TEM- SHV+ plasmid gene combination (63.5% vs 4.7–5.98% in literature), as well as CTX-M- TEM+ SHV+ gene combination (47.06% vs 3.42% in literature; Hayakawa et al., 2013; Manoharan et al., 2011). This study's finding of higher bacterial plasmid gene frequency could be explained by the use of different material, i.e., the higher plasmid gene frequency in wound infections compared to urinary tract infections and bacteria strains; higher plasmid gene frequency in ESBL producing *K. pneumoniae* compared to ESBL producing *E. coli*; as well as geographical differences in plasmid gene expression among different populations worldwide (Alekshun & Levy, 2007; Shaikh et al., 2015; Lee et al., 2012; Cantón et al., 2008). There is a considerable geographical difference in ESBL producing microorganisms and plasmid gene frequency in European countries. These differences also exist within countries and vary significantly between hospitals (Paterson & Bonomo, 2005). Similar data, confirming higher bacterial plasmid gene frequency in Latvia were found in another study by Pai et al. (see Lillo et al., 2014).

Only some of these studies analyzed the differences between male and female patients with ESBL producing Enterobacteriaceae infection, and of these, findings indicated that patients with *E. coli* and plasmid CTX-M gene presence were more likely to be male patients around the age of 68.2 years (Hayakawa et al., 2013). Prior to this, plasmid gene combination association with demographic factors had not been described in the literature.

Other studies have demonstrated higher plasmid SHV gene frequency in the *K. pneumoniae* group, compared to the *E. coli* group (Manoharan et al., 2011), as well as slightly higher plasmid CTX-M gene frequency in the *E. coli* group and slightly higher plasmid TEM gene frequency in the *K. pneumoniae* group (Kargar et al., 2014; Lin, Hsu, Chen, Huang, & Lo, 2010).

Until now, plasmid gene combination associated with a certain ESBL producing Enterobacteriaceae strain had not been described in the literature. However, one study had suggested that the three

plasmid extended-spectrum beta-lactamase gene (CTX-M+ TEM+ SHV+) combination was more frequently found in *K. pneumoniae* whereas other plasmid gene combinations were related to *E. coli*. Nevertheless, statistical significance was not proven by the authors of this study (Manoharan et al., 2011).

The three plasmid extended-spectrum beta-lactamase gene (CTX-M+ TEM+ SHV+) combination has been associated, in various literature sources, with higher antimicrobial resistance, determining resistance to ampicillin, ciprofloxacin, ceftriaxone, cefotaxime, cefexime, aztreonam, and erythromycin (Shaikh et al., 2015; Talukdar et al., 2013). Considering the three plasmid extended-spectrum beta-lactamase gene (CTX-M+ TEM+ SHV+) combination in this study occurred more frequently in *K. pneumoniae* and in older patients, as well as the geographical differences found associated with ESBL producing microorganism, plasmid gene frequency, and local resistance patterns, the authors suggest that older patients, who suffer from wound infections caused by ESBL producing *K. pneumoniae* may benefit from empirical therapy with broad spectrum antibacterial therapy.

Conclusion

Based on this study's findings the following is concluded:

1. Geographical differences concerning Enterobacteriaceae producing strains and plasmid gene frequency exist with higher *K. pneumoniae* and bacterial plasmid gene frequency in Latvia compared to other European countries.
2. *Klebsiella pneumoniae* is more often present with plasmid SHV+ extended-spectrum beta-lactamase gene and the three plasmid extended-spectrum beta-lactamase gene (CTX-M+ TEM+ SHV+) combination, as compared to *E. coli*.
3. Older patients with ESBL producing Enterobacteriaceae infection, more often, present with the three plasmid extended-spectrum beta-lactamase gene (CTX-M+ TEM+ SHV+) combination, compared to younger patients with this infection.

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