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Clonal dissemination of highly virulent ESBLs-producing Escherichia coli strains isolated from urine of nonhospitalized patients in Zagreb region

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Abstract

Recent data suggest that extended-spectrum beta-lactamases (ESBLs)-producing Escherichia coli is an emergent cause of urinary tract infections in nonhospitalized patients in different countries. The aim of this study was to characterize the ESBLs-producing E. coli strains isolated from the urine of outpatients in the Zagreb region. During the five-month study period a total of 2, 451 E. coli strains were isolated from urine of nonhospitalized patients with significant bacteriuria.. A total of 39 ESBLs-producing E. coli strains (1.59%) were collected and characterised.

Key words: Community-acquired urinary tract infections, extended-spectrum beta-lactamases (ESBLs)-producing Escherichia coli, Clonal dissemination

1. Introduction

Until recently, most infections caused by extended-spectrum beta-lactamases (ESBLs)-producing Escherichia coli strains had been described as nosocomially acquired. However, recent data indicate that urinary tract infections caused by ESBLs-producing E. coli may be an emerging problem in outpatient settings in various parts of the world. Possible community-acquisition of ESBLs-producing E. coli was first reported in 1998 from Ireland when a nalidixic acid resistant E. coli producing an ESBL was isolated from urine of an eldery patient who did not have a recent history of hospitalization [1]. Since than, ESBLs-producing E. coli have been recognized increasingly in the community [2-9].

The fact that community-acquired ESBLs-producing E. coli strains often exhibit co-resistance to trimethoprim-sulfamethoxazole, tetracycline, gentamicin and ciprofloxacin is a cause of additional concern [8, 9]. A heightened awareness of these organisms by clinicians and enhanced testing by laboratories, including molecular surveillance studies, is required to reduce treatment failures and to prevent the spread of these emerging pathogens [9].

The aim of this study was to characterize the ESBLs-producing E. coli strains isolated from the urine of non-hospitalized patients in the Zagreb region based on their susceptibility to antimicrobial agents and their virulence characteristics, and to analyze molecular relatedness between the strains by pulsed-field gel electrophoresis (PFGE).

2. Materials and methods

Bacterial strains: origin and identification

During the five-month study period (January to May 2004) a total of 2, 451 E. coli strains were isolated from urine of non-hospitalized patients with significant bacteriuria in the Department of Microbiology at the Zagreb Institute of Public Health, Zagreb.The Zagreb Institute of Public Health is the largest regional institute of public health in Croatia, which collects samples for microbiological analysis from over 800 general practitioners A total of 39 ESBLs-producing E. coli strains (1.59%) were collected. Based on the diagnosis established by general practitioners, most strains were isolated from the urine of non-hospitalized patients with acute cystitis, and only four strains were isolated from urine of nonhospitalized patients with acute pyelonepritis. Only one isolate per patient was included. The 8 male and 31 female patients ranged in age from 1 to 79 years (median age 39 years). No data regarding patients prior hospitalization or predisposition to urinary tract infection were available. Patients were not institutionalized and lived in different parts of the region. The E. coli isolates were identified by standard biochemical procedures and stored in deep-agar tubes at 4ºC (1.5% nutrient agar, Difco Lab., Detroit, Mich., USA) for further characterization studies.

Antibiotic susceptibility testing

Antibiotic susceptibility testing was performed on Mueller-Hinton agar (Oxoid Ltd., Hampshire, UK) medium by a standard disk diffusion method with a panel of 17 antimicrobial drugs according to the recommendations of the National Committee for Clinical Laboratory Standards [10, 11]. The tablet disks contained the following antimicrobial agents: ampicillin, amoxicillin-clavulanic acid, piperacillin, piperacillin-tazobactam, cephalexin, cefuroxime, ceftazidime, ceftibuten, ceftriaxone, aztreonam, imipenem, gentamicin, netilmicin, amikacin, trimethoprim-sulfamethoxazole,

ciprofloxacin and nitrofurantoin. Strains with intermediate zones were considered resistant.

ESBL tests

ESBL production was detected by double disk diffusion technique. In this test a plate was inoculated as for a standard disk diffusion test. Disks containing aztreonam and expanded-spectrum cephalosporins were than placed 30 mm from an amoxicillin-clavulanate disk prior to incubation. After overnight incubation at 37ºC the production of ESBLs was detected by the presence of characteristic distortions of the inhibition zones indicative of clavulanate potentiation of the activity of the test drug [12]. The broth-dilution minimal inhibitory concentration (MIC) reduction method was used as confirmatory test (>3-dilution reduction in the MIC of ceftazidime in the presence of clavulanic acid) [13].

Serogroup determination

All E. coli isolates were serotyped using 17 different O-antisera (Institute of Immunology, Zagreb, Croatia), These O types (O1, O2, O4, O5, O6, O7, O8, O9, O11, O15, O17, O18, O20, O25, O50, O62 and O75) were selected because of their frequent occurrence as urinary pathogens. Serotyping was performed on glass slides and confirmed using a mechanized microtechnique [14].

Hemolytic activity

The production of α hemolysin was tested on human blood agar plates. The bacteria growing on TSA were stabbed with a sterile straight wire into 5% human blood agar. After 18 to 24 h of incubation at 37ºC, the clearing zone was observed.

Adhesins determination

The expression of adhesins was defined by hemagglutination and inhibition of hemagglutination in microtiter plates, as previously described [15, 16]. Briefly,

hemagglutination (HA) was performed using human erythrocytes and sheep, ox and guinea pig erythrocytes. Inhibition of HA was performed with P_1 antigen-containing pigeon egg white and with D-mannose (Sigma Chemical Co., St. Louis, USA), as previously described [15, 17]. Isolates were considered to express P-fimbriae if HA was positive with human erythrocytes and inhibition of HA was positive with pigeon egg white, which was confirmed by agglutination of receptor-coated latex beads. The type 1 fimbriae were considered to be expressed if HA was positive with guinea pig erythrocytes. D-mannose always inhibited HA of guinea pig erythrocytes (mannose sensitive, MS HA), but it never inhibited HA of human, ox or sheep erythrocytes (mannose resistant, MR HA). The strain with MR HA ability, and without detected P-specificity, was considered to express X adhesin.

DNA macrorestriction and PFGE

Genomic DNA was prepared by a protocol devised from different methods published elsewhere [18, 19, 20]. Cleavage of the agarose-embedded DNA was achieved with 0.2 U/µl XbaI (Invitrogen) according to instructions of the manufacturer. PFGE was performed in the CHEF DRII System (Bio-Rad, Richmond, CA, USA) under the following conditions: 0.5 TBE, 1% agarose, 12ºC, 6V/cm. Run times and pulse times were 5-50s for 22h with linear ramping. The gels were stained with ethidium bromide (1 µg/ml) and photographed under UV light. The PFGE patterns were compared initially by visual comparison according to the guidelines of Tenover et al. [21]. Patterns were considered indistinguishable if every band was shared, closely related if they differed from one another by only three or fewer clearly visible bands, and different if they differed by seven or more bands. PFGE patterns were also analyzed with the GelCompar II computer software (Applied Maths, Sint-Martns-Latem, Belgium). Cluster analysis of

the Dice similarity coefficients based on the unweighted pair group method using aritmetic averages was done to generate a dendrogram describing the relationship among E. coli pulsotypes. Isolates were considered to be identical if they showed 100% similarity and were considered clonally related if they showed greater than 80% similarity (comparable to the three or fewer fragment difference already noted).

Statistical methods

Proportions were compared by the χ^2-test and by Fisher's exact test when the number in any of the 2x2 table was ≤ 5. A p-value <0.01 was considered statistically significant.

3. Results

Characteristics of the community-acquired ESBLs-producing strains in the Zagreb region

The characteristics of 39 community-acquired ESBLs-producing E. coli strains isolated from urine of non-hospitalized patients are presented in Table 1. Among 35 ESBLs-producing strains tested, co-resistance to various antimicrobial agents was observed, such as resistance to gentamicin, netilmicin, amikacin, piperacillin-tazobactam, trimethoprim-sulfamethoxazole, nitrofurantoin, and ciprofloxacin. Co-resistance to gentamicin was the most frequently observed, followed by resistance to amikacin, netilmicin and trimethoprim-sulfamethoxazole. The most frequently detecteded serogroup was O4, detected in 26 out of 39 investigated strains. High virulence capacity of the strains based on adhesins expression and hemolytic activity was observed, and 28 strains produced α hemolysin, 27 strains expressed P-fimbriae, while 30 strains expressed type 1 fimbriae.

Results of molecular characterization of the strains

Molecular characterization of 39 ESBLs-producing strains performed by PFGE revealed genetic relatedness between 25 strains (>80% similarity). After the extraction of genomic

DNA and digestion with XbaI restriction enzyme, the PFGE fingerprints exposed the existence of two clusters (Figure 1), which were closely related, resulting in one to three band differences (the Dice similarity coefficient, Dsc. ≥ 83.49%). The first cluster was composed of 11 strains signated as subclone I (≥85.48% of similarity) and the second cluster was composed of 14 strains signated as subclone II (≥87.99% of similarity). The rest of 14 ESBLs-producing strains tested were not clonally related (Dsc. <80%) (Table 1, Figure 1).

Comparison of the clonally related and nonclonal group of strains

O4 serogroup, hemolysin production and expression of type 1 and P fimbriae were all significantly more often identified among strains which were clonally related ($p<0.01$, Table 2). Based on the detection of adhesins and hemolysin production, a high virulence capacity of clonally related strains was detected. All clonal strains expressed type 1 and P fimbriae, and all except one produced α hemolysin (Table 1).

The pattern of antimicrobial sensitivity/resistance of the clonally related group of ESBLs-producing strains and nonclonal ESBLs-producing strains was similar and both showed high in vitro co-resistance to gentamicin ($p>0.05$) and amikacin ($p>0.01$). Statistically significant difference was observed between those two groups of strains in resistance to nitrofurantoin ($p<0.01$) with higher frequency of resistance in nonclonal group of strains, while the higher frequency of resistance to trimethoprim-sulfamethoxazole observed in the same group was not statistically significant ($p>0.05$)(Table 2).

4. Discussion

The present study demonstrated clonal dissemination of highly virulent ESBLs-producing E. coli strains isolated from the urine of non-hospitalized patients in a large, well defined

region in Croatia. Zagreb region has 1,200,000 inhabitants and in addition to Zagreb, eight smaller towns are included in the region.

The recent observations of the emergence of ESBLs-producing E. coli strains in community settings mirror the epidemiology of meticillin-resistance in Staphylococcus aureus [22-24]. In both cases, the resistance mechanisms were first reported in nosocomial pathogens, but this has been followed by the appearance of different clones in the community. Analogous to community-acquired MRSA strains, the ESBLs-producing E.coli strains that have become established in the community settings might have virulence determinants that confer a competitive advantage and make them particulary well equipped to succeed as community-based pathogens. The present study showed that clonally related ESBLs-producing E. coli strains isolated from the urine of non-hospitalized patients in Zagreb region were highly virulent, i.e. co-expressed type 1 and P-fimbriae, and produced hemolysin. Those virulence factors enable them to cause uncomplicated urinary tract infection in non-hospitalized patients.

Although urinary tract infection is not usually thought of as a disease associated with community-wide outbreaks, certain multidrug-resistant, uropathogenic lineages of E. coli have exhibited epidemic behavior [25]. E. coli O15:K52:H1 caused an outbreak of community-acquired cystitis, pyelonephritis, and septicemia in South London, England in 1986 to 1987, when strains of this serotype expressed P fimbriae, produced aerobactin, and displayed an unusual multiple antimicrobial resistance phenotype [25]. The subsequent recognition of O15:K52:H1 as the second most common serotype among E. coli bacteriemia isolated at a Copenhagen hospital (originating from urinary tract), together with the observation that Copenhagen isolates exhibited the same virulence factors as the South London outbreak strains, provided further evidence of the pathogenic potential of E. coli O15:K52:H1 and suggested that this serotype might constitute a

widespread virulent clone [26]. This was confirmed by the findings from Spain, which indicated that E. coli O15:K52:H1 constitutes a broadly distributed and clinically significant uropathogenic clone with fluid antimicrobial resistance capabilities, and is an endemic cause of urinary tract infection in Barcelona [27]. In 2001 Manges and coworkers reported that a single clonal group accounted for nearly half of community-acquired urinary tract infections in women caused by E. coli strains with resistance to trimethoprim-sulfamethoxazole in three geographically diverse communities in the United States of America [28]. Subsequently, those strains were designated as a clonal group A (CGA) and it was found that they exhibit a robust virulence profile suggesting enhanced extraintestinal virulence [29, 30]. Johnson and coworkers concluded that this combination of resistance and virulence may account for CGA's recent emergence as a broadly disseminated epidemic clone [29]. In the present study, the observed high virulence of clonally related ESBLs-producing E. coli strains isolated from urine of non-hospitalized patients in the Zagreb region causes concern and requires additional surveillance of the clone spread in the community.

Recent studies indicated community-associated emergence of clonally related CTX-M beta-lactamase-producing E. coli strains in various parts of the world including Europe [3, 30-32,]. The majority of ESBLs identified in clinical isolates to date, have been SHV or TEM types, which have evolved from narrow-spectrum beta-lactamases such as TEM-1, -2 and SHV-1 [33]. The CTX-M enzymes have originated from Kluyvera spp., and recently gained prominence in Enterobacteriaceae with reports from Europe, Africa, Asia, South America and North America [9]. Further molecular characterization of the clonally related community-acquired E. coli strains detected in Zagreb region and detection of the beta-lactamases type is needed.

A number of risk factors have been linked with the acquisition of community-acquired infections involving ESBLs-positive isolates. Previous hospitalization or antibiotic therapy within the previous three months, old age (>60 years), male gender, confinement to bed with debilitation and urinary catheterization were detected as risk factors [34, 35]. In the present study, females had significantly higher rates of acquisition of the strains and the median age of the patients was only 39 years. Limitations of the study include the paucity of clinical and epidemiological data, therefore no conclusions about risk factors, spread or origin of the strains can not be made.

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Table 1. Characteristics of 39 ESBLs-producing E. coli strains.

Isolate No.	Diagnosis [a]	Resistotype [b]	Serogroup [c]	Adhesins [d]	Hemolysin	PFGE profile [e]
21	AC	/	O4	P+type 1 fimbriae	+	I A
15	AC	Gm, NET, AK	O4	P+type 1 fimbriae	+	I B
14	AC	Gm, AK	O4	P+type 1 fimbriae	+	I C
28	AC	Gm, AK	O4	P+type 1 fimbriae	+	I C
18	AC	Gm, AK	O4	P+type 1 fimbriae	+	I D
20	AC	Gm, NET, AK	O4	P+type 1 fimbriae	+	I E
4	AC	/	O4	P+type 1 fimbriae	+	I F
13	AC	Gm, NET, AK, PTZ	O4	P+type 1 fimbriae	+	I G
7	AC	/	O4	P+type 1 fimbriae	+	I H
5	AC	/	O4	P+type 1 fimbriae	+	I I
12	AC	Gm, NET, AK	O4	P+type 1 fimbriae	+	I J
24	AC	Gm, AK	O4	P+type 1 fimbriae	+	II A
22	AC	Gm ,AK	O4	P+type 1 fimbriae	+	II A
19	AC	Gm, AK	O4	P+type 1 fimbriae	+	II A
35	AP	Gm, AK	O4	P+type 1 fimbriae	-	II A
8	AC	Gm, AK	O4	P+type 1 fimbriae	+	II A
9	AC	Gm, AK, SXT	O4	P+type 1 fimbriae	+	II B
16	AP	Gm, AK	O4	P+type 1 fimbriae	+	II C
29	AC	Gm, NET, AK	O4	P+type 1 fimbriae	+	II C
39	AC	Gm, AK	NT	P+type 1 fimbriae	+	II D
10	AC	Gm, AK, SXT	O4	P+type 1 fimbriae	+	II E
23	AC	Gm, AK	O4	P+type 1 fimbriae	+	II F
17	AC	Gm, AK	O4	P+type 1 fimbriae	+	II G
27	AC	Gm, AK	O4	P+type 1 fimbriae	+	II H
11	AC	Gm, SXT	O4	P+type 1 fimbriae	+	II I
36	AP	Gm, NET, AK	O6	P+type 1 fimbriae	+	NC
25	AC	Gm, SXT, NF, CIP	O6	HA=0	-	NC
2	AC	SXT	NT	HA=0	-	NC
33	AC	Gm, SXT, NF, CIP	O6	HA=0	-	NC
30	AC	Gm, NET, AK	O15	HA=0	-	NC
32	AC	Gm, NET, AK	O6	type 1 fimbriae	+	NC
6	AC	Gm, NET, AK	O6	type 1 fimbriae	+	NC
37	AC	NF, CIP	O4	type 1 fimbriae	-	NC
34	AC	Gm	NT	HA=0	-	NC
26	AC	AMC, SXT, CIP	O1	HA=0	-	NC
31	AC	Gm, NET, NF	O4	HA=0	-	NC
38	AP	Gm, AK, SXT	NT	HA=0	-	NC
1	AC	NF	NT	P fimbriae	+	NC
3	AC	SXT, NF	NT	X+type 1 fimbriae	-	NC

[a]Acute cystitis, AC, acute pyelonephritis AP; [b]Gentamicin, Gm; netilmicin, NET; amikacin, AK; amoxicillin-clavulanate, AMC; piperacillin-tazobactam, PTZ; trimethoprim-sulfamethoxazole, SXT; nitrofurantoin, NF; ciprofloxacin, CIP; [c]Nontypeable strain, NT; [d]Nonagglutinating strain, HA=0; [e]Subclone I (A-J): Dice similarity coefficient (Dsc.) 85.48%, subclone II (A-I) Dsc. 87.99%, nonclonal strains, NC.

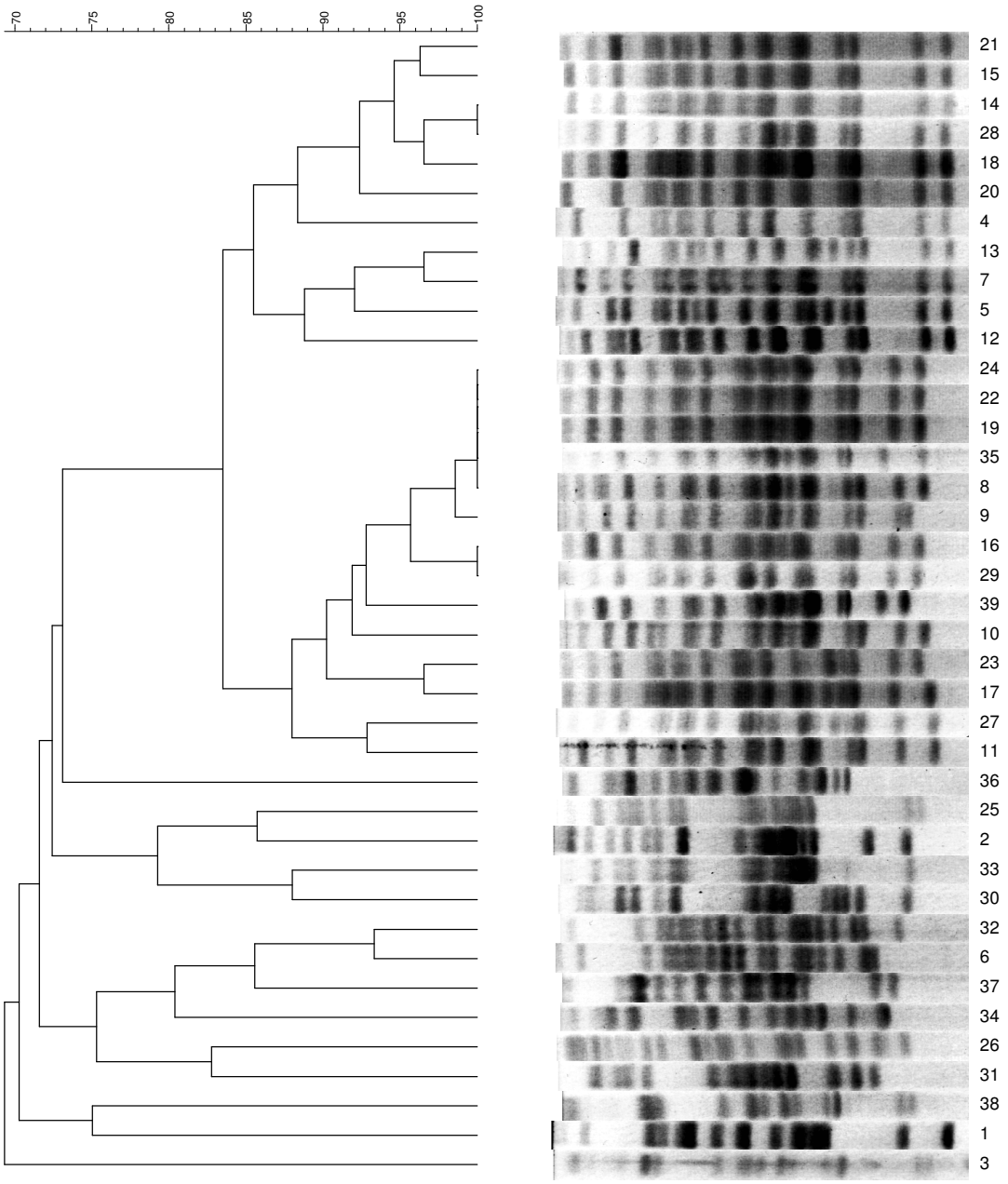

Figure 1. Dendrogram and PFGE fingerprints of 39 ESBL-producing E.coli isolates after digestion with XbaI restriction enzyme. The Dice similarity coefficient (Dsc.) for strains 21, 15, 14, 28, 18, 20, 4, 13, 7, 5, 12, 24, 22, 19, 35, 8, 9, 16, 29, 39, 10, 23, 17, 27 and 11 ≥ 83.49%. Other strains are not clonally related (Dsc.<80%).

Table 2. Phenotypical differences between clonally related and nonclonal strains of ESBLs-producing E.coli.

Strain properties	CR [a] No./n	NC [b] No./n	p-value
O4 serogroup	24/25	2/14	2.818×10^{-7}
Hemolysin production	24/25	4/14	1.515×10^{-5}
P-fimbriae	25/25	2/14	2.350×10^{-6}
Type 1 fimbriae	25/25	5/14	9.447×10^{-6}
Nitrofurantoin resistance	0/25	6/14	9.204×10^{-4}
Trimethoprim-sulfamethoxazole resistance	3/25	6/14	0.9948
Amikacin resistance	20/25	5/14	0.0156
Gentamicin resistance	21/25	9/14	0.3146

[a] Clonally related strains, CR

[b] Nonclonal strains, NC