# Characterization of the extended-spectrum β-lactamases and determination of the virulence factors of uropathogenic Escherichia coli strains isolated from children

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**CHARACTERIZATION OF THE EXTENDED-SPECTRUM β-LACTAMASES AND DETERMINATION OF THE VIRULENCE FACTORS OF UROPATHOGENIC *ESCHERICHIA COLI* STRAINS ISOLATED FROM CHILDREN**

ABSTRACT

**Background and aim**

The aim of the study was to characterize ESBL-producing uropathogenic *Escherichia coli* (UPEC) strains isolated in children. That included the investigation of virulence factors and the analysis of the types of β-lactamases at the molecular genetic level.

**Material and methods**

During the two-year study period, 77 ESBL-producing *E. coli* strains were recovered from urine samples of febrile children with significant bacteriuria hospitalized at one Croatian hospital. Susceptibility of isolates to bactericidal serum activity was tested by Shiller and Hatch method, while adhesin expression was determined by agglutination methods. Characterization of ESBLs was performed by PCR with specific primers for ESBLs and by sequencing of $bla_{\text{ESBL}}$ genes. Genotyping of the *E. coli* isolates was performed by pulsed-field gel electrophoresis (PFGE).

**Results**

Twenty-seven (35.1%) and 50 (64.9%) ESBL-producing UPEC strains were isolated in neonates and infants, respectively. Of 70 strains investigated for the presence of virulence factors, adhesins were detected in 48.6% strains (8.6% in the neonate and 40% in the infants group) giving a statistically significant difference in adhesin expression between the two groups ($p<0.01$). Hemolysin was produced by 84.3%, whereas 70% of strains were serum-resistant. The $bla_{\text{TEM}}$ gene was detected in 22 (28%) and $bla_{\text{SHV}}$ gene in 57 strains (74%), whereas $bla_{\text{CTX-M}}$ gene was detected in only two isolates (2.5%). In ten isolates, $bla_{\text{TEM}}$ and $bla_{\text{SHV}}$ were simultaneously detected. Sequencing of $bla_{\text{SHV}}$ genes revealed that SHV-5 β-lactamase was by far the most prevalent and was found in 51 strains (66%). The strains were clonally related as demonstrated by PFGE and assigned into ten clusters.

**Conclusions**

Infection control measures should be employed and the consumption of expanded-spectrum cephalosporins in the hospital should be restricted.

KEY WORDS: *Escherichia coli*, virulence, urinary tract infections, children, extended-spectrum β-lactamases

## INTRODUCTION

Extended-spectrum β-lactamases (ESBL) producing bacteria have been increasingly reported in pediatric patients. Production of ESBLs is the major mechanism of resistance to oxymino-cephalosporins and aztreonam in Gram-negative bacteria [1-2]. ESBLs are predominantly derivatives of plasmid-mediated TEM or SHV β-lactamases and arise through mutations that alter the configuration of the active site, thereby expanding the hydrolytic spectrum of the enzyme [2]. Both TEM and SHV ESBLs are distributed worldwide with lots of variants described (data are available at http://www.lahey.org/studies/), and are mostly found in *Enterobacteriaceae* species like *Klebsiella pneumoniae* and *Escherichia coli*. Recently a new family of ESBLs with predominant activity against cefotaxime (CTX-M β-lactamases) has been reported [3]. In contrast to TEM or SHV-ESBLs CTX-M β-lactamases are native ESBLs and are derived from the chromosomal β-lactamases of the genus Kluyvera [4-5]. Over 50 CTX-M enzymes have been described so far, which can be grouped into five main subgroups according to amino acid sequence identity (CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25) [6]. Most CTX- M β-lactamases hydrolyze cefotaxime better than ceftazidime, but some of them such as CTX-M-15 hydrolyze also ceftazidime efficiently [6]. In some countries CTX-M β-lactamases are the most prevalent types of ESBLs, for instance in Russia [7], Greece [8], Spain [9], Switzerland [6], Japan [10], Taiwan [11], China [12] and Argentina [13]. ESBL producing *Enterobacteriaceae* are described with increasing frequency as causative agents of urinary tract infections (UTIs) [14-17]. *E. coli* is the most frequent cause of UTIs, and the probability of UTI depends on the virulence of the infecting bacteria and the susceptibility of the host. Factors described as virulence factors for uropathogenic *E. coli* (UPEC) are adherence ability, hemolysin, aerobactin and cytotoxic necrotizing factor type 1 production, resistance to the bactericidal action of normal human serum and to phagocytosis [18].

The aim of present study was to characterize ESBL-producing UPEC isolated in children over a 2-year period at the Split University Hospital, Croatia, and to determine the possible differences in the characteristics of strains isolated in neonates and older children. The virulence factors of the strains were determined, and the prevalence of different types of ESBLs among those strains was investigated. ESBLs were characterized genetically and clonal relatedness of the strains was assessed thereby allowing the molecular epidemiology of the ESBL-producing *E. coli* strains.

## MATERIAL AND METHODS

### Patients

From January 2002 until December 2003 urine samples were collected from all children with symptoms of urinary tract infection or febrile neonates and infants with suspected UTI. Out of 530 children with significant bacteriuria and *E. coli* in pure culture (>$10^5$CFU/ml) and pyuria (>5 white blood cells/high power field), in 77 ESBL-positive *E. coli* was detected. There were two groups of children with ESBL-positive strain: 26 neonates (up to one month old) hospitalized at neonatology unit and 51 older children (infants from one month to one year old) hospitalized at paediatric unit. All urine samples in neonates and infants were obtained by urine bag collection, only urine samples in older children, in which ESBL strains were not detected, were collected by mid stream urine.

### Bacteria

The ESBL-positive *E. coli* isolates were collected prospectively from febrile neonates and infants with significant bacteriuria with *E. coli* in pure culture and pyuria. The species were identified by conventional biochemical testing. All 77 uropathogenic ESBL-producing *E. coli* isolates were further analyzed in this study.

### Bacterial adhesins determination

The expression of adhesins was defined by hemagglutination and inhibition of hemagglutination in microtiter plates, as previously described [18]. 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 [19]. 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 inhibits HA of guinea pig erythrocytes (mannose sensitive, MS HA), but it never does with human, ox or sheep erythrocytes (mannose resistant, MR HA). An isolate displaying MR HA was considered to express X adhesin when P-specificity was not detected.

**Determination of hemolytic activity**

The production of α hemolysin was tested on human blood agar plate and was considered positive when bacteria were stabbed with a sterile straight wire into 5% human blood agar, and after 18 to 24 h of incubation at 37 ºC, a clearing zone was observed.

**Sera and serum sensitivity assay**

Blood was obtained by venipuncture from three healthy volunteers and was allowed to clot at room temperature for 30 min and overnight at 4$^{o}$C. After centrifugation at 1000 g for 15 min at 4$^{o}$C, serum was removed and pooled. A portion of the pooled serum was decomplemented by heating at 56$^{o}$C for 30 min and used as test controls. Bacterial susceptibility to serum killing was measured by assessing regrowth after incubation in normal human serum according to Schiller and Hatch method [20].

**Susceptibility to antibiotics**

Antibiotic susceptibilities were determined by broth microdilution method according to the most recent CLSI criteria [21]. The concentrations of antibiotics which inhibited 50 and 90% of the strains ($MIC_{50}$ and $MIC_{90}$) and percentage of resistant strains were calculated. method. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 used as quality control strains [22].

**Detection of extended-spectrum β-lactamases**

ESBL production was determined by double-disk synergy test (DDST) [23] and confirmed by at least threefold reduction in ceftazidime (MIC) by clavulanate. For DDST, an overnight broth culture of test strain was diluted in saline, adjusted to McFarland standard suspension 0.5 and inoculated onto MH agar. Disk containing amoxycillin/clavulanate (co-amoxiclav) (20/10 µg) was placed in the middle of the plate and surrounded by disks containing

ceftazidime, cefotaxime, ceftriaxone and aztreonam (30 µg) at distance of 2.5 cm (edge to edge). Plates were incubated overnight at 37ºC. Distorsion of the inhibition zones around cephalosporine disks towards co-amoxiclav disk was indicative of ESBL production. *K. pneumoniae* ATCC 70068 used as a positive control strain.

**Transfer of resistance determinants**

Conjugation experiments were set up employing *E. coli* A15 R⁻strain which is free of plasmids and resistant to rifampicin [24]. Overnight BHI (Brain-Heart Infusion, Difco Lab., Detroit, MI, USA) broth cultures of donor *E. coli* strains and E. coli recipient strain were mixed in the ratio 1:2 in 5 ml BHI broth and incubated 18 h at 37°C without shaking. 100 µl of the mating mixture was seeded on the MH plates containing ceftazidime (1 mg/L), rifampicin (256 mg/L) and combined plates containing ceftazidime+rifampicin. Transconjugants were selected on the combined plates containing ceftazidime (1 mg/L) and rifampicin (256 mg/L). The frequency of conjugation was expressed relatively to the number of donor cells.

**Characterization of extended-spectrum β-lactamases**

The presence of $bla_{TEM}$, $bla_{SHV}$, $bla_{CTX-M}$ genes and $bla_{PER-1}$ genes was determined for *E. coli* strains by polymerase chain reaction (PCR) using primers and conditions as described previously [25-28]. In brief, bacterial DNA was extracted by boiling and applied to PCR-amplification using Taq polymerase (Invitrogen, Germany) under the following conditions: 94°C for 5 min, 35 cycles consisting of 95°C for 30s, 55°C for 30s, and 72°C for 50s each, followed by a final extension at 72°C for 8 min. Primers used in this studies are shown in Table 1 [25-28]. The PCR products were detected by agarose gel electrophoresis after staining with ethidium bromide. PCR products obtained with primers specific for SHV β-lactamases were subjected to PCR *Nhe* test [25] to distinguish between SHV-1 and SHV-ESBL. Strains positive for CTX-M β-lactamases were tested further with specific primers for

groups 1, 2, 8 9 and 25 as described previously in order to amplify the whole coding sequence [29]. Amplicons were then column purified using Nucleo Spin Extract II (Machery-Nagel, Germany). Purified PCR products were sent to Medigenomix (Martinsried, Germany) for sequencing using an automated ABI3700 sequencer and the respective forward and reverse primers of the PCR amplification.. Designation of *bla* genes based on identified mutations was done according to the summary provided by the website of the Lahey Clinic (http://www.lahey.org/studies/). Lysates from reference strains producing TEM-1, TEM-2, SHV-1, SHV-2, SHV-3, SHV-4, SHV-5, PER-1 and CTX-M-15 were used as positive controls for PCR.

**Characterization of plasmids**

Plasmids were extracted with Qiagen Plasmid Mini kit (QIAGEN Hamburg, Germany) according to manufacturer's recommendations, from 17 strains which produced transconjugants in the mating experiments, run in 0.7% agarose gel, and stained with ethidium bromide. *E. coli* NTCC 50192 yielding four bands of know sizes of 148, 64, 36 and 7 kb was used as positive control. Plasmids were digested with *EcoR*1 restriction endonuclease.

**Molecular typing by pulsed-field gel electrophoresis (PFGE) of bacterial DNA**

PFGE of *Xba*-digested genomic DNA was performed with a CHEF-DRIII system (Bio-Rad, Hercules, CA, USA) as described previously [30]. The images were processed using the Gel-Compare software, and a dendrogram was computed after band intensity correlation using global alignment with 2% optimization and UPGMA (unweighted pair-group method using arithmetical averages) clustering [31]. Cluster analysis of the Dice similarity coefficients based on the UPGMA using arithmetic averages was done to generate a dendrogram describing the relationship among *E. coli* genotypes. Isolates were considered to be identical if they showed 100% similarity and were considered clonally related if they showed greater than 80% similarity

**Statistical methods**

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

**RESULTS**

**Prevalence of uropathogenic ESBL isolates**

From January 2002 until December 2003, 661 *E. coli* isolates were recovered from children treated in pediatric units (pediatrics, neonatology) at the Split University Hospital. A total of 530 (80.18%) were isolated from urine in significant number ($\geq 10^5$ bacteria per ml) from febrile children with pyuria.

The proportion of ESBL-positive *E. coli* found in pediatric/neonate units during the 2-year period was 17% (114/661). The prevalence of uropathogenic *E. coli* producing ESBLs in febrile children with significant bacteriuria and pyuria was 14.5% (77/530*).* These 77 uropathogenic ESBL-producing strains were subjected to further analysis. During the study period there were 453 ESBL-negative *E. coli* strains isolated from children with significant bacteriuria and pyuria.

*Clinical data*

Twenty six patients were hospitalized in neonatology unit (children younger than one month) and 51 in paediatic unit (children older than one month). Gender data were available for 54 children. Female/male ratio was 2:1. A total of 27 (35.1%) and 50 (64.9%) of ESBL-producing *E. coli* strains were isolated from urine of 77 neonates and infants with diagnosed urinary tract infection, respectively. Since the children with ESBL-positive strains were all neonates or infants it was difficult to obtain information on symptoms of urinary tract infections because in this age the children cannot verbalize their symptoms.

Of the 77 patients providing strains for this study complete clinical data were available for 35 patients. Clinical data are summarized in Table 2. There were several risk factors associated with UTIs. Eleven children were diagnosed with malformations of the urinary tract, seven females and four males (five with reflux, two with *ureter stenosis*, two with *pyelon et ureter duplex*, one with *ren arcuatus* and one with other malformation of the kidney). Additional two were low-birth-weight infants, while one was premature-delivered. For other 24 patients no risk factor for urinary tract infection was determined, as shown in Table 2. In all except 8 cases the UTIs were nosocomially acquired. The patients were treated with antibiotics. Fourteen patients received cefuroxime alone or combined with another beta-lactam. Sixteen patients were treated with ampicillin combined with netilmicin, gentamicin, ceftazidime or meropenem. Two patients received only meropenem, two ceftriaxone with vankomicin and one only sulphamethoxazole. The patients responded to the therapy. Only one patient with *hydronephrosis* caused by ureter stenosis ended up with nephrectomy, but the operation was not indicated because of urinary tract infection. In five patients ESBL positive *E. coli* persisted after completition of therapy. One of them received sulphamethoxazole/trimethoprim, one cefuroxime and the other three had combined therapy which included ampicillin combined with netilmicin and either ceftazidime or co-amoxiclav.

**Characterization of virulence factors**

Of 70 strains investigated for the presence of virulence factors adhesins were detected in 34 (48.6%) strains, six in neonate and 28 in infants group, and difference in adhesin expression between the two groups was statistically significant ($\chi^2$=9.20, p<0.01). P-fimbriae were detected in 14 strains, and only two of them were neonatal urine samples. Hemolysin was produced by 59 (84.3%) strains (24 in neonates and 35 in older children), whereas 49 (70%) strains were resistant to serum bactericidal activity (18 in neonate and 31 in infants group); the difference in hemolysin production and serum sensitivity of the strains between the two

groups was not statistically significant ($p>0.05$, $\chi^2=1.16$ and 0.03, respectively). Out of 14 P-fimbriated strains, the *bla*$_{\text{TEM-1}}$ gene was detected in 10 strains, while strains without this *bla* gene expressed this adhesin only rarely ($\chi^2=19.68$, $p<0.01$).

**Antibiotic susceptibilities**

According to the most recent CLSI criteria of bacterial drug susceptibility 92% of the isolates were resistant to gentamicin and, 74% to netilmicin. No resistance was observed to ciprofloxacin and carbapenems (Table 3). The susceptibility to β-lactam antibiotics except of carbapenems is not mentioned because according to the CLSI they are not recommended for the therapy. All except two strains (36, 86) displayed the so called "CAZ phenotype" meaning that the resistance was more pronounced to ceftazidime and aztreonam than to cefotaxime and ceftriaxone.

**Conjugation**

Twenty- three out of 77 strains transferred ceftazidime resistance to *E. coli* recipient. Resistance to tetracycline was co-transferred alongside with ceftazidime resistance from 18 and to aminoglycosides from 16 strains. Resistance to cloramphenicol and cotrimoxazole was co-transferred from only one strain.

**Characterization of ESBLs**

All ESBL-producing *E. coli* strains were tested for the presence of *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{PER}}$ genes by PCR. Twenty two isolates (28%) yielded an amplicon with the TEM primers, 57 isolates (74%) with the SHV primers but only two isolates (2.5%) with the CTX primers. The latter two possessed an IS26 insertion sequence. In ten isolates, *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ were simultaneously detected. No *bla*$_{\text{PER-1}}$ genes were detected by PCR. In six isolates no *bla*-encoding genes were identified with the PCR assays applied. There was no statistically significant difference in *bla* gene detection between the neonate and older children groups ($p>0.05$).

SHV-type ESBL were identified by sequencing as SHV-5 in 51 isolates (66%), SHV-2 in three isolates (4%), SHV-2a in two strains (2.6%), and SHV-12 in one isolate (1.3%). Sequencing of TEM-type ESBLs yielded TEM-1 in 17 isolates (22%), TEM-2 in three isolates (4%), and TEM-116 and TEM-E2 in one isolate each (1.3%). In one isolate, TEM-1 and TEM-2 were simultaneously present. CTX-M-type ESBLs corresponded to CTX-M-3 β-lactamase in both cases.

**Characterization of plasmids**

SHV-5 β-lactamase has been encoded on different large plasmids. The strains contained one to ten plasmid bands. Transonjugants displayed similar plasmid profiles as their respective donors. Ten different restriction profiles were observed.

**Genotyping by PFGE**

According to their genetic relatedness they were assigned into ten clusters (I to X) as shown in Figure 1. Several outbreaks with ESBL producing *E. coli* strains were identified in the study period. The first outbreak was caused by a clone referred to as clone I-1 represented by isolates 1, 5 and 6, the second one by a clone represented by isolates 9 and 10; both clones contained TEM-1 producing isolates. Further outbreaks were caused by clones I-3 (isolates 76, 81, 83, I-4 (isolates 68, 69), II-1 (isolates 40, 41, 42, 43, 89), II-2 (isolates 38, 44), II-3 (isolates 92, 93, 95), II-4 (isolates 65, 67), II-5 (isolates 61,62), III-1 (isolates 30, 33, 34, 35, 53), III-2 (isolates 26, 27), III-3 (isolates 58, 60), III-4 (isolates 55,56) IV-1 (isolates 19, 20), IV-2 (isolates 23, 24), IV-3 (isolates 14, 15), IV-4 (isolates 17,18), and finally VI-1 (isolates 70, 72). These outbreaks involved SHV-5 β-lactamase producing isolates except isolates of subcluster IV-3 which lost their resistance. The other isolates could not be assigned to genetic clones.

## DISCUSSION

The aim of this investigation was a comprehensive characterization of ESBL-producing UPEC strains detected with increasing frequency in neonate and pediatric units over 2-year period in a Croatian university hospital. There are many reports on ESBLs in *Enterobacteriaceae*, but this study differs from previous because it describes molecular epidemiology of ESBL-producing uropathogenic *E. coli* isolates in children in relatedness to the virulence characteristics of the strains.

The first goal of the study was to characterize the uropathogenic ESBLs at the molecular genetic level and to identify possible breakouts. The proportion of ESBL found in pediatric/neonate units during the 2-year period was 17% which is much higher than in South America (1.7%) [5] and Poland (13%) [32], but lower than in Korea (25%) [33]. The overall proportion of ESBL producing *E. coli* in all hospital units from both adults and children during the study period was 4.51% (143/3164) [34]. It is higher than in Italy (1.2%) and most other European countries [35-36]. but lower than in China (34%) [37], South America (25%) [38] and Turkey (9%) [39]. Much higher proportion of ESBLs in the study population compared to the overall proportion in the hospital could be explained by the spread of clonally related strains only in the pediatric hospital units.

SHV-5 was found to be the predominant type of extended-spectrum β-lactamase. Some isolates harboured also and additional TEM-1 β-lactamase. SHV-5 β-lactamase is widespread in Middle and East Europe and has been previously described in Austria [40], Germany [41], Hungary [42], Poland [43], Greece [44], Bosnia and Herzegovina [16], Mexico [45] and many other countries in the world. The resistance phenotype of the isolates is consistent with SHV-5 β-lactamase (high level ceftazidime resistance in most strains). The presence of additional TEM-1 β-lactamase in some isolates could be responsible for resistance to co-amoxiclav (amoxicillin/clavulanate). The finding that SHV-5 β-lactamase was the most

prevalent in our collection of uropathogenic *E. coli* isolates is in concordance with previous investigations of ESBL in Croatia [46-47]. The identification of SHV-2 and SHV-5 β-lactamases enables us to speculate on the evolutionary sequence of SHV type ESBLs; from SHV-2 to SHV-5, according to the mutation process. Since there is only one amino acid substitution(Gly240→Lys) it can be hypothesized that in this hospital the mutation process took place in *E. coli* from SHV-2 to SHV-5 and from SHV-2a to SHV-12. The similar phenomenon was previously described in Mexican pediatric hospital in *K. pneumoniae* strain [45]. CTX-M β-lactamases were identified in only two of 77 strains and were both of the same type (CTX-M-3). The presence of IS26 insertion sequence upstreams of the gene facilitates the expression of $bla_{\text{CTX-M}}$ gene and is important for the mobilization of $bla_{\text{CTX-M}}$ genes. Thus, the by far most prevalent ESBL found in *E. coli* isolates in Croatia was SHV-5, which is in contrast with reports from many other countries in which CTX-M β-lactamases appear to be the most frequent type [6-13]. Recent studies found very high prevalence of CTX-M β-lactamases in Croatia which reached 60% in hospital isolates of *K. pneumoniae* [48] and 4.9% in community samples [49]. However, there are no published data on the prevalence of the CTX-M β-lactamases in the hospital where the study was conducted. The resistance phenotype of two CTX-M positive strains was consistent with CTX-M β-lactamases. The MICs of ceftazidime were below the susceptibility breakpoint in contrast to cefotaxime and ceftriaxone MICs which were high (>512 mg/L). Contrarily, to previous reports [7] the both strains were susceptible to β-lactam/inhibitor combinations. There were no significant difference in the prevalence of SHV, TEM and CTX-M- ESBLs in the neonate and infants group. TEM-E2 β-lactamase, a ceftazidimase found in one of our strains was previously reported from UK [50]. Strains containing only TEM-1 or TEM-2 β-lactamase most probably have some other resistance mechanism to expanded-spectrum cephalosporins such as production of rare type ESBLs for instance IBC or VEB β-lactamase, loss of porins or

production of plasmid-mediated AmpC β-lactamases. Since those strains were susceptible to cefoxitin and to inhibition by clavulanate it is not likely to expect that they harbour plasmid–mediated AmpC β-lactamases. However, the clarification of these resistance mechanisms requires further testing, but this was beyond the scope of this study.

According to the results of the study, SHV-5 producing UPEC strains were frequently clonally related and associated with breakouts in contrast to strains producing SHV-2, SHV-2a, SHV-12 and CTX-M-3 β-lactamases, which were usually single isolates without tendency to spread throughout the hospital wards. The fact that the strains were clonally related and show similar resistance phenotypes points out that there is endemic and epidemic spread of SHV-5 producing *E. coli* in University hospital Split probably due to the insufficient infection control measures. Clustering of the *E. coli* isolates sustains the hypothesis of either patient to patient transmission of strains or a common source acquisition. The fact that genotypically related strains persisted over years raises the possibility that these strains may have persisted unnoticed in the pediatric units on the hospital, which served as a source of patient contamination. Our data document the emergence of *E. coli* strains producing SHV-5 β-lactamase arising from the horizontal transfer of $bla_{\text{SHV-5}}$ genes and clonal dissemination of the strains. All strains were obtained from pediatric units and neonatal wards indicating a localized dissemination within the hospital and pointing to a potential source of spread of an SHV-5 encoding plasmid in the hospital. Spread of SHV-5 producing *E. coli* strains throughout the hospital units could be due to the selection pressure of cefazidime which is widely prescribed in our hospital and favours survival of the strains possessing mutation at the Ambler position 240 responsible for ceftazidime and aztreonam resistance. Hospital hygiene measures should be employed and the consumption of expanded-spectrum cephalosporins in the Split University Hospital should be restricted in order to reduce the spread of ESBL producing *E. coli* isolates throughout pediatric units. Since plasmids encoding ESBLs also

contain resistance genes for aminoglycosides it is possible that consumption of these antibiotics could also exert the selection pressure which favours the spread of plasmids with ESBL genes.

Carbapenems are the antibiotics of choice for the treatment of infections caused by the SHV-5 producing *E. coli* strains isolated from children. Despite there is no fluoroquinolone resistance observed ciprofloxacin could not be considered as treatment choice in children age group. All strains were susceptible to combination of ceftazidime and clavulanate but in general β-lactam combinations with inhibitors are not recommended for the therapy of infections caused by ESBL producing bacteria due to pronounced inoculum effect and possibility of developing mutants hyperpoducing ESBL during therapy [51]. Cefoxitin exhibited good in vitro antibacterial activity against our ESBL producing *E. coli* but it is not recommended for the therapy of UTIs because it does not achieve high concentrations in urine. The fact that all strains are fully susceptible to ciprofloxacin is probably due to the policy of not prescribing fluoroquinolones in children because of their side effects on bone growth.

The second goal of the study was the analysis of the virulence of ESBL strains isolated in neonates and older children. The strains predominantly produced hemolysin and were resistant to serum bactericidal activity, and the distribution of those virulence determinants did not differ significantly between the two studied groups of children. However, adhesin expression was detected in less than 50% of the strains and was especially rare in the strains isolated from neonatal urine samples. Among 34 adhesin-positive strains, six in neonate and 28 in older children group, only 14 strains were P-fimbriated, almost exclusively detected in the infants group and in strains in which $bla_{\text{TEM-1}}$ gene was detected. This is in concordance with the fact that in older children and adults, most UTIs are thought to occur by the ascending route, and bacterial adherence ability is very important first step in the pathogenesis [18]. By adhering to the urinary tract epithelium bacteria resist the urine flow enabling them

to persist and invade the tissues. Less frequently, blood-borne infection of the kidney occurs. It is well known that serum-resistance plays a key role in *E.* coli survival in blood. In neonates it is more likely that hematogenous invasion of the kidney can cause UTI, which could be the explanation for the high frequency of serum-resistant strains isolated in neonatal group but can not explain the high frequency of serum-resistant strains isolated in older children. The ESBL strains which were spread in the Split University Hospital in studied period were predominantly SHV and hemolysin producers and were resistant to serum bactericidal activity. The observed associations between the detection of *bla* genes and expression of certain virulence characteristics need additional studies. In the ten years since the study was done, infection control practices have changed and the antimicrobial prescribing practices have actually altered. As a consequence of that, the proportion of ESBL producing *E. coli* in paediatric and neonatology unit has decreased decreased from 14% in 2003 to 6% in 2011.

Conclusions based on the results of the study was that hygienic regulation, reduction of antibiotic consumption and surveillance networks should be recommended in order to limit the spread of ESBL positive *E. coli* in the hospital departments involved.

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**COMPETING INTERESTS**

None to declare

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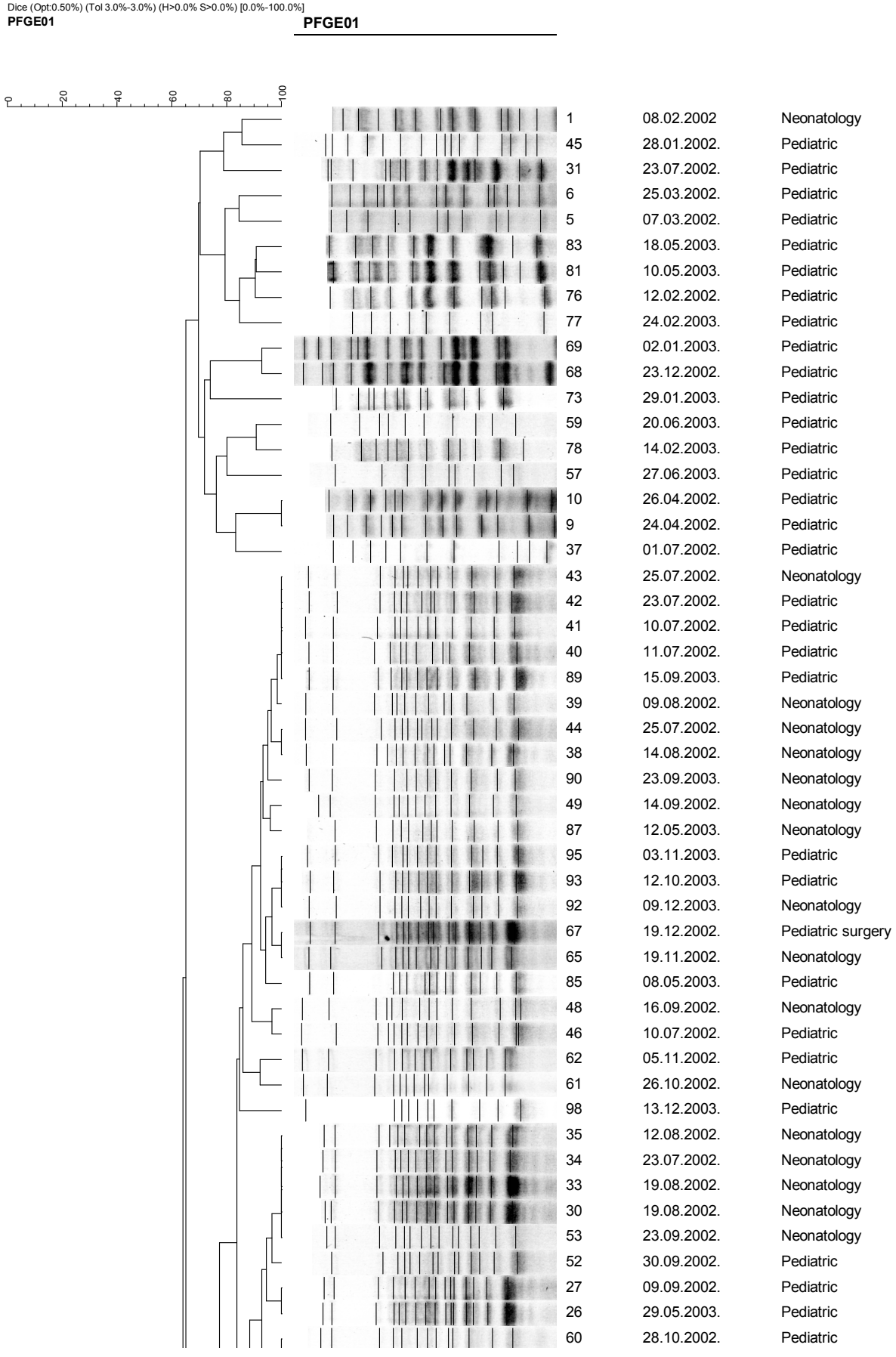
52.

**Table 1**.Primers used in this study

| *Bla* gene | Primer | Sequence | Annealing temp. | Amplicon size (bp) | Reference |
|---|---|---|---|---|---|
| SHV | MN-¸1<br>MN-2 | 5'- CGC- CGG- GTT- ATT –CTT- ATT- TGT CGC-3'<br>5'- TCT -TTC- CGA-TGC- CGC- CGC- CAG- TCA-3' | 58° | 1016 | 25 |
| TEM | OT-3<br>OT-4 | 5'-ATG-AGT-ATT- CAA-CAT-TTC-CG-3'<br>5'-CCA-ATG-CTT-AAT-CAG-TGA-GG-3' | 58° | 850 | 26 |
| CTX-M | MA-1<br>MA-2: | 5'-SCS-ATG-TGC-AGY-ACC-AGT-AA-3'<br>5'-CGC-CRA-TAT-GRT-TGG-TGG-TG-3' | 55° | 554 | 27 |
| CTX-M- 1 | M1-F<br>M1-R | 5'-AAA-AAT-CAC-TGC-GCC-AGT--TC-3'<br>5'-TTG-GTG-ACG-ATT-TTA-GCC-GC-3' | 52° | 415 | 29 |
| CTX-M- 2 | M2-F<br>M2-R | 5'-CGA-CGC-TAC-CCC-TGC-TAT-T--3'<br>5'-CCA-GCG-TCA-GAT-TTT-TCA-GG-3' | 52° | 552 | 29 |
| CTX-M-. 8 | M8-F<br>M8-R | 5'-TCG-CGT-TAA-GCG-GAT-GAT-GC<br>5'-AAC-CCA-CGA-TGT-GGG-TAG-C-3' | 52°<br>52° | 666 | 29 |
| CTX-M- 9 | M9-F<br>M9-R | 5'-CAA-AGA-GAG-TGC-AAC-GGA-TG<br>5'ATT-GGA-AAG-CGT-TCA-TCA-CC | 52° | 205 | 29 |
| CTX-M -25 | M25-F<br>M25-R | 5'-GCA-CGA-TGA-CAT-TCG-GG-3'<br>5'-AAC-CCA-CGA-TGT-GGG-TAG-C-3' | 52° | 325 | 29 |
| PER | PER-EXT-1-F<br>PER-EXT-1-R | 5' GGG- ACA -R TC-SKA-TGA-ATG-TCA<br>5' GGG- YSG- CTT-AGA- TAG- TGC –TGA-T | 52° | 966 | 28 |
| IS26 | IS26-F<br>IS26-R | 5'-GCG-GTA-AAT-CGT-GGA-GTG-AT-3'<br>5'-ATT-CGG-CAA-GTT-TTT-GCT-GT-3 | 52° | 400 | 27 |

**Table 3.** Cumulative MIC values and percentage of resistant isolates.

| Antibiotic and CLSI breakpoint | MIC range | $MIC_{50}$ | $MIC_{90}$ | % resistance |
|---|---|---|---|---|
| Amoxycillin (32) | 128-≥1024 | ≥1024 | ≥1024 | 100 (77/77) |
| Amoxycillin/clavulanate (32/16) | 4-64 | 8 | 32 | 12. 98 (10/77) |
| Cefazoline (32) | 16-≥1024 | 256 | ≥1024 | 97.40 (75/77) |
| Cefuroxime (32) | 8-≥1024 | 64 | ≥1024 | 80.51 (62/77) |
| Ceftazidime (32) | 4-≥1024 | 256 | ≥1024 | 89.61 (69/77) |
| Ceftazidime/clavulanate | 0.06-4 | 0.5 | 2 | 0 (0/77) |
| Cefotaxime (64) | 1-256 | 16 | 64 | 22.07 (17/77) |
| Ceftriaxone (64) | 2-≥1024 | 64 | 128 | 55.84 (43/77) |
| Ceftibuten (32) | 0.12-256 | 4 | 64 | 20.07 (16/77) |
| Cefepime (32) | 0.06-512 | 16 | 64 | 37.66 (29/77) |
| Cefoxitin (32) | 2-8 | 2 | 8 | 0 (0/77) |
| Imipenem (16) | <0.008-0.5 | 0.25 | 0.25 | 0 (0/77) |
| Meropenem (16) | 0.016-0.06 | 0.03 | 0.03 | 0 (0/77) |
| Gentamicin (16) | 2-512 | 64 | 256 | 92.20 (71/77) |
| Netilmicin (32) | 0.5-256 | 32 | 128 | 74.02 (57/77) |
| Ciprofloxacin (8) | ≤0.008-2 | ≤0.008 | 0.03 | 0 (0/77) |


Dice (Opt:0.50%) (Tol 3.0%-3.0%) (H>0.0% S>0.0%) [0.0%-100.0%]
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PFGE01
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1 08.02.2002 Neonatology
45 28.01.2002. Pediatric
31 23.07.2002. Pediatric
6 25.03.2002. Pediatric
5 07.03.2002. Pediatric
83 18.05.2003. Pediatric
81 10.05.2003. Pediatric
76 12.02.2002. Pediatric
77 24.02.2003. Pediatric
69 02.01.2003. Pediatric
68 23.12.2002. Pediatric
73 29.01.2003. Pediatric
59 20.06.2003. Pediatric
78 14.02.2003. Pediatric
57 27.06.2003. Pediatric
10 26.04.2002. Pediatric
9 24.04.2002. Pediatric
37 01.07.2002. Pediatric
43 25.07.2002. Neonatology
42 23.07.2002. Pediatric
41 10.07.2002. Pediatric
40 11.07.2002. Pediatric
89 15.09.2003. Pediatric
39 09.08.2002. Neonatology
44 25.07.2002. Neonatology
38 14.08.2002. Neonatology
90 23.09.2003. Neonatology
49 14.09.2002. Neonatology
87 12.05.2003. Neonatology
95 03.11.2003. Pediatric
93 12.10.2003. Pediatric
92 09.12.2003. Neonatology
67 19.12.2002. Pediatric surgery
65 19.11.2002. Neonatology
85 08.05.2003. Pediatric
48 16.09.2002. Neonatology
46 10.07.2002. Pediatric
62 05.11.2002. Pediatric
61 26.10.2002. Neonatology
98 13.12.2003. Pediatric
35 12.08.2002. Neonatology
34 23.07.2002. Neonatology
33 19.08.2002. Neonatology
30 19.08.2002. Neonatology
53 23.09.2002. Neonatology
52 30.09.2002. Pediatric
27 09.09.2002. Pediatric
26 29.05.2003. Pediatric
60 28.10.2002. Pediatric

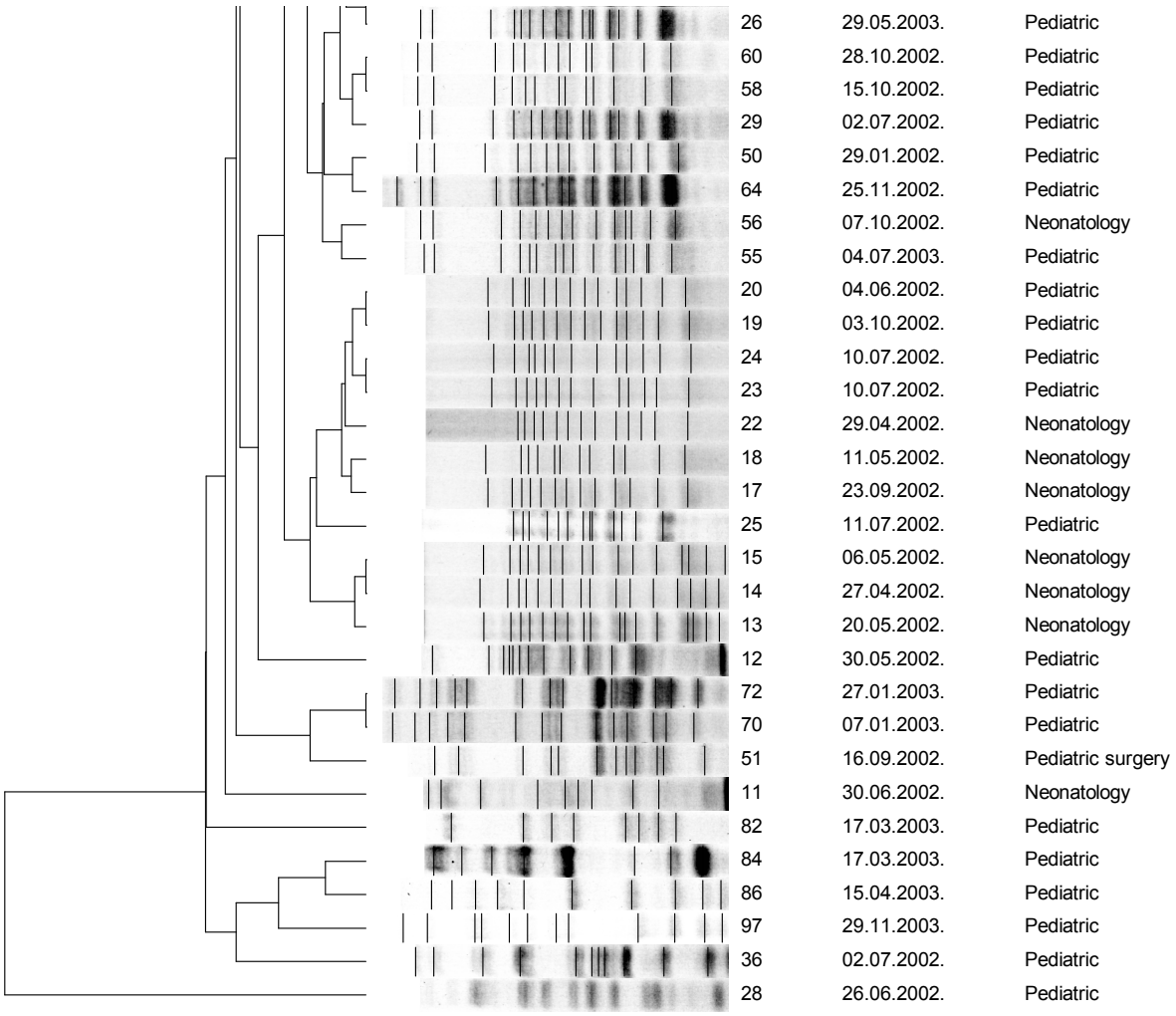


**Figure 1.**

Pulsed field gel electrophoresis of genomic DNA. Date of isolation and hospital ward are shown. Isolates were considered to be identical if they showed 100% similarity and were considered clonally related if they showed greater than 80% similarity (differend in less than three bands).