THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

Epidemiology of faecal carriage of extended-spectrum beta-lactamase producers in healthy individuals and in different patient populations

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bp	base pair
DNA	Deoxyribonucleic acid
ERIC	Enterobacterial repetitive intergenic consensus
ESBL	Extended spectrum beta-lactamase
ICU	Intensive care unit
LTC	Long-term care
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis

1. Introduction

Improper antimicrobial use is considered as one of the main reasons for selection and transmission of resistant bacteria (Hawkey, 2008). Beta-lactams are the most widely used antibiotic classes; however, the spiral of appearance of resistance and subsequent development of newer drugs is a returning characteristic throughout their history. Beta-lactamase production is the most important defense mechanism against beta-lactam antibiotics among members of Enterobacteriaceae family; a major change in resistance was the appearance of extended-spectrum beta-lactamases (ESBLs), which provide resistance to penicillins and cefalosporins. Most of ESBL enzymes are encoded by *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and *bla*$_{\text{CTX-M}}$ gene families (see below). Those Enterobacteriaceae that produce ESBLs have become a major problem worldwide, especially since the occurrence and spread of the beta-lactamase genes belong to *bla*$_{\text{CTX-M}}$ family. Many of these genes, in contrast to the *bla*$_{\text{TEM}}$ and *bla*$_{\text{SHV}}$ groups, are linked to epidemic clones (Damjanova et al., 2008) and some of them, e.g. the *bla*$_{\text{CTX-M-15}}$–producing *Escherichia coli* O25b:ST131 and *Klebsiella pneumonia* ST11, ST15 and ST147 clonal complexes, have become pandemic both in the nosocomial and community settings as well as in long-term care (LTC) facilities (Canton and Coque, 2006; Damjanova et al., 2008; Woodford et al., 2004). The switch from *K. pneumoniae* to *E. coli* as the major ESBL producer species is also linked to the dissemination of *bla*$_{\text{CTX-M}}$ genes (Livermore et al., 2007).

The gastrointestinal tracts of humans and even animals are obviously a major reservoir of ESBL producer species (Ewers et al., 2010; Woerther et al., 2013) and also serve as a site for the horizontal spread of resistance genes (Salyers et al., 2004). Carriage is related to hospitalization and antibiotic consumption (Luvsansharav et al., 2012; Tian et al., 2008), the significance of carriers was highlighted both in hospital and community settings (Miro et al., 2005). The duration of asymptomatic carriage can be very long, up to several years in some

cases (Tham et al., 2010), and international travel contributes to efficient spreading (van der Bij and Pitout, 2012). It has been shown that travelling to countries in which the rate of ESBL-producers is high, is a risk factor for colonization with ESBL-producers (Lausch et al., 2013; Tangden et al., 2010; von Wintersdorff et al., 2014). These data show that asymptomatic carriers play an increasingly important role in the epidemiology of ESBL producers. It is, therefore, important to monitor the changes in the prevalence, distribution and dynamics of ESBL producers not only in infected patients, but in asymptomatic carriers as well.

The aim of this thesis is to investigate the prevalence of faecal colonization with ESBL producers among healthy individuals, people with a hospitalization history applying for LTC facilities, pediatric and adult inpatients of wards or intensive care units (ICUs), as well as among outpatients and screened medical students and comparing the characteristics of ESBL producers in these populations.

2. Review of the literature

2.1. Enterobacteriaceae

Enterobacteriaceae is a large family of Gram-negative bacteria which includes genera and species causing well-defined diseases as well as nosocomial infections. These bacteria are natural inhabitants of the intestinal flora; however, some of them are human intestinal and extraintestinal pathogens. Enterobacteriaceae can also be found in the environment, soil, plants and water. *E. coli* and *K. pneumoniae* are the most frequently observed members of Enterobacteriaceae in human clinical samples, and may cause common infections such as pneumonia, urinary tract infections and bloodstream infections (Abbot, 2011; Nataro et al., 2011). *E. coli* strains in the gut are mostly non-pathogenic commensals; however, certain strains may carry a combination of virulence genes (Table 1) which enable them to cause intestinal infections (e.g. diarrhoea or haemorrhagic colitis), or to cause extra-intestinal infections (e.g. neonatal meningitis, surgical site infection, nosocomial septicaemia and urinary tract infections) (Masters et al., 2011). According to the distribution of some target genes and multi-locus sequence typing methods, most *E. coli* isolates can be grouped into eight phylogenetic groups: A, B1, B2, C, D, E, F and clade I (Clermont et al., 2013). Group A, B1, C and E comprise mostly commensal strains (Lee et al., 2010) while group B2, D and F are mainly associated with virulent extraintestinal strains (Kudinha et al., 2013; Lee et al., 2010; Piatti et al., 2008). Pathogenic *E. coli* strains (Table 1) can be grouped into intestinal and extraintestinal pathogenic *E. coli*, on the basis of their virulence factors and clinical symptoms (Kaper et al., 2004). Intestinal pathogenic strains can be further classified according to pathogenic features into enteropathogenic, enterohemorrhagic, enterotoxigenic, enteroinvasive, enteroaggregative and diffusely adherent *E. coli* (Nataro and Kaper, 1998). Some of the virulence genes are shared by more than one *E. coli* pathotypes.

E.coli pathotype	virulence genes					
	Adhesin	Toxin	Invasin	Capsule synthesis	Siderophore	Others
Extraintestinal pathogenic *E. coli*	*papC, fimH, sfaS, sfa/focD*	*cnf1, cvaC*	*ibeA*	*kpsMTII, kpsMTIII*	*iutA, fyuA*	PAI, *traT, csgA*
Enteropathogenic *E. coli*	*eaeA*	*exhAa*				
Enterohemorrhagic *E. coli*	*iha, eaeA, saa*	*exhAa, stx2, stx1*				*chuA*
Enterotoxigenic *E. coli*		*LT1*, LT2				
Enteroinvasive *E. coli*			*ipaH*			
enteroaggregative *E. coli*		*east1*				
Diffusely adherent *E. coli*	*aah (orfA)*					

Table 1. Categories of some virulence genes based on their function and *E. coli* pathotypes carrying these genes.

Similarly to *E. coli, K. pneumoniae* isolates are also found in the human gastrointestinal tract. Most *K. pneumoniae* are associated with pneumonia in immunocompromised hosts, but are also able to cause urinary tract infections, abdominal infections and septicemia (Podschun and Ullmann, 1998). Furthermore, nosocomial outbreaks of *K. pneumoniae* are common due to their ability of rapid dissemination among hospitalized patients, particularly in neonatal units (Bojer et al., 2010; Damjanova et al., 2007).

Five major virulence factor groups of *K. pneumoniae* have been found up to now to contribute to the pathogenesis, capsular serotype, lipopolysaccharide, hypermucoviscosity phenotype, siderophores, and pili (Podschun and Ullmann, 1998).

2.2. Antibiotic resistance

Antibiotics are one of the wonder discoveries of the 20th century in medical history. However, the successful use of any antibiotics is compromised by the potential rise of antibiotic resistance concomitant with their use. The prevalence of antibiotic resistance is a growing problem all over the world, and has become a major threat to public health.

Antibacterial resistance may be intrinsic (natural) or acquired. Intrinsic resistance is an inherent attribute that protects the organism, such as the outer membrane of Gram-negative bacteria, which is impermeable to many molecules /for example, *E. coli* has innate resistance to vancomycin, because it is too large to pass through porin channels of its cell wall (Chen et al., 2009)/. Furthermore, bacteria have the remarkable ability of environmental adaptation by changing their genome through mutations or by horizontal gene transfer, or by differential gene expression (Harbottle et al., 2006; Rowe-Magnus and Mazel, 1999). The extensive and inappropriate use of antibiotics in humans and animals, has led to selective pressure on bacteria, promoting acquisition of resistance determinants. Acquired resistance is a change in genetic composition of a microorganism, therefore an antimicrobial agent that was initially effective against the organism is no longer effective (Chen et al., 2009)

Resistance may be acquired in three ways: a) mutations in chromosomal genes leading to altered antibacterial targets or transcriptional changes, e.g. mutation in chromosomal topoisomerase/gyrase genes resulting in fluoroquinolone resistance (Martinez et al., 1998), b) acquisition of new genes by horizontal transfer gene, e.g. plasmid mediated acquisition of beta-lactamase encoding genes, c) mutations in previously acquired genes, e.g. mutation in the beta-lactamase genes $bla_{\text{TEM-1}}$ and $bla_{\text{SHV-1}}$, resulting in production of enzymes with a broader spectrum (Jacoby and Medeiros, 1991). There are several biochemical mechanisms of resistance according to the modes of action which can be divided into subgroups as follows: a) alteration of the chemical structure of the drug (degradation, enzymatic modification) b) decreased drug concentration (reduced membrane permeability, active efflux) c) alterations related to the target (hyper-production of the target, new target, new pathway bypassing the target and target protection).

Resistance to beta-lactams may be due to beta-lactamase production, alteration of the target site such as penicillin-binding proteins, alternative cell wall synthesis pathways (new penicillin-binding protein), change in permeability and active efflux (Gold and Moellering, 1996; Pitout et al., 1997).

2.3. Beta-lactamases and their classification

The major defense mechanism that Gram-negative bacteria have against beta-lactam antibiotics is beta-lactamase production. The mechanism of the resistance is inactivation of the beta-lactam drugs by hydrolysis of the beta-lactam ring. The first beta-lactamase was identified in *E. coli*, before penicillin entered into clinical use in 1940 by Abraham and Chain (Abraham and Chain, 1988; Turner, 2005).

Beta-lactamases can be classified according to two general schemes; the Ambler molecular classification scheme and the Bush-Jacoby-Medeiros functional classification scheme (Ambler, 1980; Bush, 1989a, b; Bush et al., 1995). According to the Ambler scheme, beta-

lactamases are divided into four major classes (A to D). The basis of this classification scheme rests upon protein homology and not phenotypic characteristics. Class A, C and D are phylogenetically different serine beta-lactamases and class B is the class of metallo-beta-lactamases (Ambler, 1980). The Bush-Jacoby-Medeiros scheme classifies these enzymes according to functional similarity. This classification system is based on substrate profile and lactamase inhibitor susceptibility (Table 2).

Functional group	Molecular class	Distinctive substrate(s)	Inhibited by CA	Inhibited by EDTA	Defining characteristic(s)	Representative enzyme(s)
1	C	Cephalosporins	No	No	Greater hydrolysis of cephalosporins than benzylpenicillin; hydrolyzes cephamycins	*E. coli* AmpC, P99, ACT-1, CMY-2, FOX-1, MIR-1
1e	C	Cephalosporins	No	No	Increased hydrolysis of ceftazidime and often other oxyimino-β-lactams	GC1, CMY-37
2a	A	Penicillins	Yes	No	Greater hydrolysis of benzylpenicillins than caphalosporins	PC1
2b	A	Penicillins, early cephalosporins	Yes	No	Similar hydrolysis of benzylpenicillin and cephalosporins	TEM-1, TEM-2, SHV-1
2be	A	Extended-spectrum cephalosporins, monobactams	Yes	No	Increased hydrolysis of oxyimino-β-lactams (cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam)	TEM-3, SHV-2, CTX-M-15, PER-1, VEB-1
2br	A	Penicillins	No	No	Resistance to clavulanic acid, sulbactam, and tazobactam	TEM-30, SHV-10
2ber	A	Extended-spectrum cephalosporins, monobactams	No	No	Increased hydrolysis of oxyimino-β-lactams combined with resistance to clavulanic acid, sulbactam, and tazobactam	TEM-50
2c	A	Carbenicillin	Yes	No	Increased hydrolysis of carbenicillin	PSE-1, CARB-3
2ce	A	Carbenicillin, cefepime	Yes	No	Increased hydrolysis of carbenicillin, cefepime and cepirome	RTG-4
2d	D	Cloxacillin	Variable	No	Increased hydrolysis of cloxacillin or oxacillin	OXA-1, OXA-10
2de	D	Extended-spectrum cephalosporins	Variable	No	Hydrolyzes cloxacillin or oxacillin and oxyimino-β-lactams	OXA-11, OXA-15
2df	D	Carbapenems	Variable	No	Hydrolyzes cloxacillin or oxacillin and carbapenems	OXA-23, OXA-48
2e	A	Extended-spectrum cephalosporins	Yes	No	Hydrolyzes cephalosporins. Inhibited by clavulanic acid but not aztreonam	CepA
2f	A	Carbapenems	Variable	No	Increased hydrolysis of carbapenems, oxyimino-β-lactams, cephamycins	KPC-2, IMI-1, SME-1
3a	B (B1)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	IMP-1, VIM-1, CcrA, IND-1
	B (B3)	Carbapenems	No	Yes	Broad-spectrum hydrolysis including carbapenems but not monobactams	L1, CAU-1, GOB-1, FEZ-1
3b	B (B2)	Carbapenems	No	Yes	Preferential hydrolysis of carbapenems	CphA, Sfh-1

Table 2. Classification scheme for bacterial beta-lactamase (Bush and Jacoby, 2010). CA, clavulanic acid.

Beta-lactamases are plasmid- or chromosomally encoded enzymes; the genes encoding these enzymes were originally found on the bacterial chromosome (Bradford, 2001; Livermore, 1995). The first plasmid-encoded beta-lactamase, TEM-1, was described in early 1960s from a Greek patient named Temoniera, the enzyme was named after the patient (Datta and Kontomichalou, 1965). It was isolated from a single strain of *E. coli* and showed resistance mainly to aminopenicillins. Within a few years, the TEM-1 beta-lactamase spread worldwide and is now found in different members of the Enterobacteriaceae family, as well as in *Haemophilus influenzae, Neisseria gonorrhoeae* and other Gram-negative pathogens (Liu et al., 1998). Another enzyme group with the same resistance profile, SHV-1, was detected among *K. pneumoniae* and also in other members of the family Enterobacteriaceae (Heritage et al., 1999). Both genes coding for TEM-1 and SHV-1 , carried by conjugative transposons and plasmids, spread rapidly and became ubiquitous in Gram-negative bacteria (Philippon et al., 1989), and TEM-1-producing *K. pneumoniae* became endemic in many hospitals (Medeiros, 1997). Over the last 20 years, many beta-lactam antibiotics have been developed to overcome the action of beta-lactamases. However, with each new class of antibiotics which has been used to treat patients, new beta-lactamases emerged to cause resistance. The selective pressure exerted by overuse of new antibiotics has been associated with the emergence of new variants of beta-lactamases. One of these new drug classes was (third generation) oxyimino-cephalosporins, which were widely used for the treatment of serious infections due to Gram-negative bacteria in 1980s (Liu et al., 1998). In 1983, the first plasmid-encoded beta-lactamase capable of hydrolyzing the extended-spectrum oxyimino-cephalosporins was found in a strain of *K. ozaenae* in Germany, a mutated form of the existing SHV-1 enzyme (named SHV-2) (Knothe et al., 1983). The first TEM-derived beta-lactamase conferring resistance to cefotaxime, TEM-3 (initially named CTX-1), was reported in *K. pneumoniae* from France a few years later (Brun-Buisson et al., 1987; Sirot et al.,

1987), and to distinguish these enzymes from broad-spectrum beta-lactamases the term extended-spectrum beta-lactamase was introduced by Philippon in 1989 (Philippon et al., 1989; Sirot et al., 1987). In 1989, a non-TEM/SHV-producing *E. coli* isolate resistant to cefotaxime was recognized in Munich and was designated CTX-M due to its predominant activity against cefotaxime rather than ceftazidime (Bauernfeind et al., 1990).

2.4. ESBLs

ESBLs may be classified based on their functional characteristics or their primary structure (Ambler, 1980; Bush et al., 1995). ESBLs were first defined as plasmid-mediated beta-lactamases which provide resistance to extended-spectrum cephalosporins and monobactams, and are inhibited *in vitro* by beta-lactamase inhibitors. In 2009 Giske et al. proposed a new classification scheme (from the clinical prospective) with a simplified definition of ESBLs (Giske et al., 2009). According to this classification, ESBLs are divided into three main groups; $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$. $ESBL_A$ represents the classical ESBLs (functional group 2be) and are further divided into three main subgroups including, TEM, SHV and CTX-M. (In the thesis ESBL signifies $ESBL_A$). $ESBL_M$ are miscellaneous ESBLs that provide resistance to penicillins, cephalosporins and sometimes also carbapenems and are inhibited by cloxacillin or boronic acid (plasmid-mediated AmpC and OXA-ESBLs). $ESBL_{CARBA}$ are enzymes with hydrolytic activity against carbapenems. There are over 150 different ESBLs described.

2.4.1. TEM

TEM type ESBLs are derivatives of TEM-1 and TEM-2. The first TEM beta-lactamase gene, designated as *bla*TEM-1, was isolated from a strain of *E. coli* in 1963 in Greece (Datta and Kontomichalou, 1965). TEM-1, which is not an ESBL, can hydrolyze penicillins and first-generation cephalosporins; however, is unable to hydrolyze the oxyimino cephalosporins or monobactams (Sturenburg and Mack, 2003). TEM-2 was the first derivative of TEM-1 and

had a single amino acid substitution at position 39; however it had the same hydrolytic profile as TEM-1. TEM-3 was the first TEM-type beta-lactamase that showed the ESBL phenotype. Since that time the number and variety of extended-spectrum TEM-types has increased rapidly. More than 200 TEM type beta-lactamases have been described and new genes continue to appear (http://www.lahey.org/Studies/temtable.asp). These TEM variants differ in amino acid sequence, and many of them cause different resistance phenotypes. TEM-type ESBLs are most frequently found in *E. coli* and *K. pneumoniae*, but they are also described in other Gram-negative bacteria (Bradford, 2001). TEM-type ESBLs have been identified in non-Enterobacteriaceae Gram-negative bacteria such as *Pseudomonas aeruginosa* (TEM-42) as well (Mugnier et al., 1996).

2.4.2. SHV

The first emergence of an SHV ESBL was reported in Germany (Knothe *et al*, 1983), which was called SHV-2. This enzyme was found to differ from SHV-1 (parent enzyme) by replacement of glycin with serine at position 238 resulting in enhancement of the affinity of the SHV-1 beta-lactamase to the oxyimino-cephalosporins. The majority of SHV variants showing an ESBL phenotype are identified by the substitution of a serine for glycine at position 238 and some of SHV variants have a substitution of lysine for glutamate at position 240. The serine residue at position 238 is critical for efficient hydrolysis of ceftazidime and lysine residue at position 240 is critical for efficient hydrolysis of cefotaxime (Huletsky et al., 1993). Currently, more than 40 SHV-type ESBLs have been described (http://www.lahey.org/Studies/) where changes in amino acid sequence confer the ability to hydrolyze the new cephalosporins (Sturenburg and Mack, 2003).

SHV-type ESBLs are mostly found in *K. pneumoniae,* but have also been found in *E. coli, Citrobacter diversus* and *P. aeruginosa*. In Hungary, SHV-5 and SHV-2a are the predominant SHV- type ESBLs produced by *Klebsiella spp.* (Toth et al., 2005).

2.4.3. CTX-M

The CTX-M type beta-lactamases was first recognized in 1989 as a new ESBL family member (Bonnet, 2004). The origin of CTX-M-type ESBLs was completely different from that of TEM- or SHV-type ESBL (Bonnet, 2004). The CTX-M family of enzymes are thought to have derived from initial transfer of the chromosomal beta-lactamase gene from *Kluyvera spp*. to conjugative plasmids that readily disseminated among different members of the Enterobacteriaceae and other Gram-negative bacteria (Canton and Coque, 2006). These original mobilized *bla*$_{\text{CTX-Ms}}$ genes affected cefotaxime to a higher degree than ceftazidime, and that is where the name came from, cefotaximase (Canton et al., 2008). From an evolutionary point of view, CTX-Ms, similarly to other ESBLs, later diverged by point mutations as a consequence of antibiotic selective pressure once *bla*$_{\text{CTX-M}}$ genes were mobilized from *Kluyvera* spp. and were incorporated into mobile genetic elements, which also gave them the opportunity to enhance the hydrolytic activity against ceftazidime like CTX-M-15, through acquisition of insertion sequences acting as strong promoters (Canton et al., 2012).

To date, there are over 160 CTX-M variants (http://www.lahey.org/Studies/other.asp) recorded and they are divided into five different groups according to their amino-acid sequences: i) CTX-M-1 (emerged in Germany in 1989), ii) CTX-M-2 (emerged in Japan in 1986 and then in Argentina in 1989) , iii) CTX-M-8 (emerged in Brazil in 1996-1997), iv) CTX-M-9 (emerged in Spain in 1994) and v) CTX-M-25 (emerged in Canada in 2000) (Canton et al., 2008).

There has been a rapid spread of the CTX-M beta-lactamases, and they have been detected all over the world. The reason for this rise may be a) extraordinary dissemination of the corresponding *bla*$_{\text{CTX-M}}$ genes in highly mobilizable genetic platforms (plasmids and transposons), b) the presence of these platforms within successful clones (Canton and Coque,

2006; Rogers et al., 2011), c) the co-resistance phenomenon in CTX-M producing isolates, especially to fluoroquinolones and aminoglycosides, which may facilitate a co-selection process (Canton et al., 2012). The CTX-M-15 enzyme, which belongs to group 1, is considered to be the predominant ESBL type in most of the world, which has been referred as the ‘’CTX-M pandemic’’ (Canton and Coque, 2006).

2.4.4. Other types of ESBL

Whereas the majority of ESBLs are coming from TEM or SHV beta-lactamases or are members of the CTX-M family, a few other ESBLs have been described that are not closely related to any of the above types of beta-lactamases. They are also plasmid-mediated, but are not derivatives of any other known beta-lactamase.

One of them is PER, which shares about 25% homology with the TEM and SHV-type ESBLs (Nordmann and Naas, 1994). PER-1 beta-lactamase was first detected in strains of *P. aeruginosa* isolated in Turkey (Nordmann et al., 1993). PER-2 which shares 86% homology to PER-1 has been detected more frequently in South America (Bradford, 2001).

Another type that is closely related to PER is VEB-1, which was first found in isolate of *E. coli* from Vietnam (Poirel et al., 1999). Other VEB enzymes have also been detected in Kuwait and China (Jiang et al., 2005; Poirel et al., 2001a).

GES is a type of ESBLs that has recently been found in South America, Europe, South Africa and Japan (Castanheira et al., 2004; Correia et al., 2003; Poirel et al., 2001b; Vourli et al., 2004). GES-1 beta-lactamase was first detected in a *K. pneumoniae* isolate obtained in France in 1998 (Poirel et al., 2000). The $bla_{\text{GES-1}}$ gene was subsequently detected in *P. aeruginosa* from France (Dubois et al., 2002), and in *K. pneumoniae* from Portugal (Duarte et al., 2003). Another variant, GES-2, was found in *P. aeruginosa* isolated in 2000 in South Africa (Poirel et al., 2001b), notably with the ability to confer intermediate resistance to imipenem.

2.5. Integrons

As it was discussed earlier, ESBLs are enzymes that are mediated by genes located on plasmids. However ESBL encoding genes can be also located on or associated with integrons, which may facilitate the spread of such genetic elements (Machado et al., 2005). Integrons are mobile genetic elements which allow the integration of antimicrobial drug resistance genes via site-specific recombination events. Integrons are capable of recognizing, capturing and expressing multiple resistance genes in cassette structures, therefore play an important role in the dissemination of antimicrobial resistance (White et al., 2001). Five integron classes related to antibiotic resistance have been described based on the homology of their integrase genes (Machado et al., 2005). All integrons are composed of three key elements: 1) *intI* gene which encodes an integrase, 2) a primary recombination site (*attI*), 3) a promoter (Pc) that directs transcription of captured genes. Class 1 integrons (Figure 1) show the greatest diversity of antibiotic resistance gene cassettes, and have been mainly associated with functional and non-functional transposons derived from *Tn*402 (Brown et al., 1996; Radstrom et al., 1994). The non-functional type is the main common structural organization which has been described in clinical isolates (Gillings et al., 2009). In addition, these structures are frequently associated either with plasmids or larger transposons, such as those of the *Tn*3 family (*Tn*21, *Tn*1696) aiding their dispersion (Labbate et al., 2009). Class 2 integrons show lower diversity than class 1 integrons, primarily because of the presence of a stop codon in the class 2 integrase (*intI2*), resulting in a truncated and non-functional protein. Because of this feature in class 2 integrons, they have quite a stable gene cassette array, mainly composed of gene cassette *dfrA1*, *sat2*, *ant(3'')-Ia* and *orfX* (Hansson et al., 2002). Class 2 integrons are almost always embedded in *Tn*7 transposons and their derivatives. These two classes, are the most frequently found integrons in nosocomial and community settings, and are frequently found in ESBL-producing Enterobacteriaceae (Machado et al.,

2005; Su et al., 2006). Class 3 integron carrying the *IMP-1* metallo-beta-lactamase gene, was first detected in *Serratia marcescens* isolate in Japan in 1993 (Arakawa et al., 1995). The class 3 integron platform also has been captured by a *Tn402* transposon, though in the reverse orientation as compared to the class 1 capture event (Collis et al., 2002). Such class 3 integrons are relatively common in Japan (but not very common elsewhere), where they have spread into some of human pathogens and commensals (Gillings, 2014). The worldwide prevalence of the other classes of integrons remains low (Deng et al., 2015).

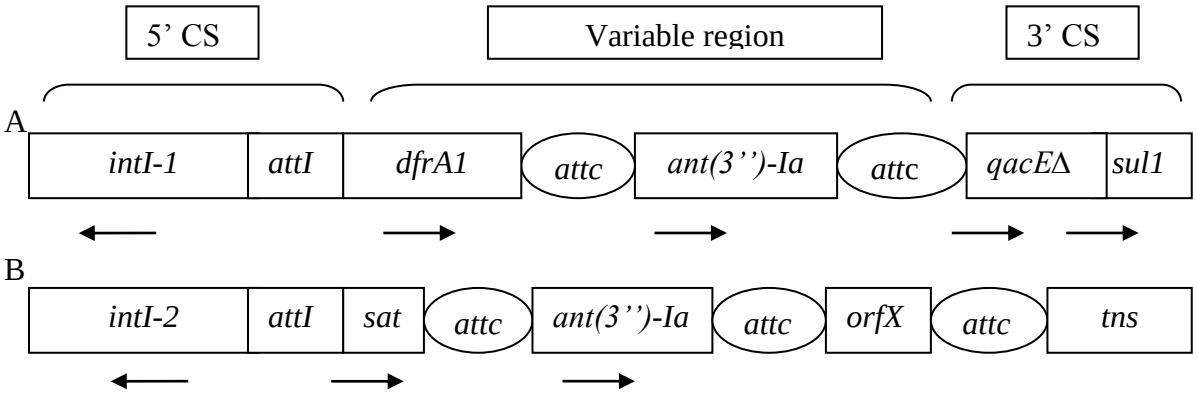


Figure 1. Schematic representation of class 1 and 2 integrons. (A) Class 1 integron: In the 5′ conserved segment (5′ CS), there is a promoter located within *intI1*, which drives transcription of the genes within gene cassettes. The gene product of *intI1* catalyzes recombination between a primary recombination site (*attI*) and a corresponding 59-base element site (59-be or *attC*) which is carried on mobile gene cassettes. In the 3' conserved segment (3' CS), there is a quaternary ammonium resistance gene (*qacE*Δ1) and a sulfonamide resistance gene (*sul1*). The arrows indicate the direction of transcription for each gene. (B) Class 2 integron: its 5' CS is similar to the class 1integron and its 3′ CS contains five transposon genes (*tnsA*, *tnsB*, *tnsC*, *tnsD* and *tnsE*).

2.6. Epidemiology of ESBLs

There are marked geographical differences in the proportion of ESBL producing enterobacteria. In Europe the prevalence of bacteria which are resistant to extended-spectrum cephalosporins varies significantly between countries; they are less frequent in the northern

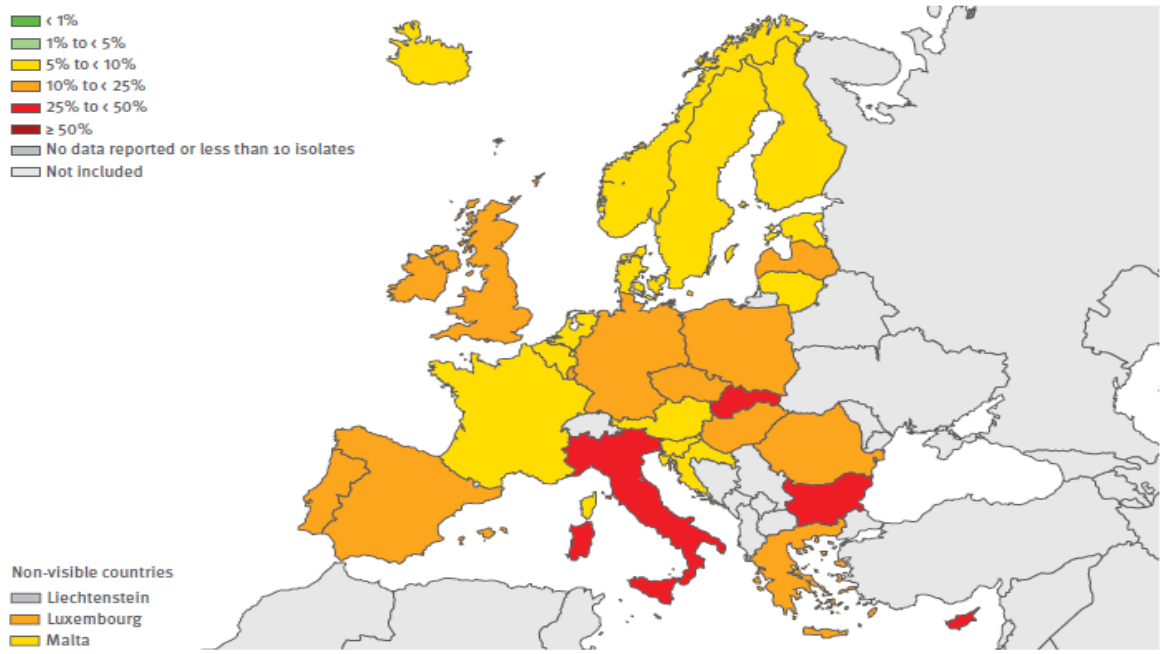
European countries and more common in the southern and eastern parts of Europe (Canton et al., 2008; Coque et al., 2008a).

According to an Annual Epidemiological Report from the European Antimicrobial Resistance Surveillance Network (EARS-Net), the percentages of isolates resistance to third-generation cephalosporins in 2013 ranged from 5.0% (Iceland) to 39.6% (Bulgaria) for *E. coli* (Figure 2a) and from 0 % (Iceland) to 70.1 % (Greece) for *K. pneumoniae* (Figure 2b), in invasive bacterial isolates, with a north-to-south gradient (According to EARS-Net database 85 % to 100 % of the reported *K. pneumoniae* isolates are ESBL-positive). Since 2000, the CTX-M enzymes have emerged globally and became pandemic, but the dominant enzyme differs between countries (Canton and Coque, 2006). Isolates with the CTX-M-9 group are frequent in Spain and isolates with the CTX-M-3 enzymes have been described mainly in Eastern Europe, although clones producing CTX-M group 1 (including the CTX-M-15 type) are the most widespread throughout Europe (Bonnet, 2004; Canton et al., 2008; Coque et al., 2008a; Coque et al., 2008b). SHV-5 beta-lactamase is very common worldwide and has been found in France, Greece, Poland, Hungary, South Africa, the UK and US (Bradford 2001). CTX-M-3, SHV-2 and SHV-5 are usually widely spread in eastern European countries (Canton et al., 2008; Damjanova et al., 2007). SHV-12 is the most common ESBL found in Korea (Bradford, 2001) and it is also one of the most prevalent enzymes associated with nosocomial *K. pneumoniae* in Spanish, Italian and Polish hospitals and is also increasingly reported in *E. coli* isolates from community.acquired infections (Coque et al., 2008a).

The prevalence of ESBL-producers outside Europe also varies between countries. In the African continent, CTX-M is dominant. In Tanzania the first study of ESBLs in neonates was performed in 2001-2002, and it was found that 25.0% of the *E. coli* and 17.0% of the *K. pneumoniae* were ESBL-producers, mainly CTX-M-15 and TEM-63 type ESBL were found (Blomberg et al., 2005). A study conducted at a tertiary hospital in Nigeria, found that among

the overall ESBL producing isolates, 35% were of community origin and 65% from hospitals (Afunwa et al., 2011). In another study conducted at a tertiary hospital in Tanzania, the ESBL prevalence was 64.0% in *K. pneumoniae* and 24.0% in *E. coli* (Mshana et al., 2009). Herindrainy et al. observed that 10.0% of non-hospitalized patients in Madagascar carried ESBLs (mainly CTX-M-15) (Herindrainy et al., 2011). In a study from USA, which included 26 hospitals from 20 states participating in the SENTRY Program showed that the resistance levels to cephalosporins and/or aztreonam in invasive strains of the family Enterobacteriaceae was 6.4%, and rates of CTX-M-producing strains increased from 26.6% in 2007 to 43.8% in 2010 (Castanheira et al., 2013). In a study from hospitals in Canada, the rate of ESBL-producing *E. coli* was below 5.0%; however, they were often multi resistant and associated with the pandemic ST131 clone (Simner et al., 2011). In India, the prevalence of ESBL production was 69.0% in clinical *E. coli* and in 41.0% of *K. pneumoniae* isolates (Pathak et al., 2012). In Thailand high carriage of ESBL-producers (52.0%) among healthy volunteers has been reported (Oteo et al., 2010).

a)



b)

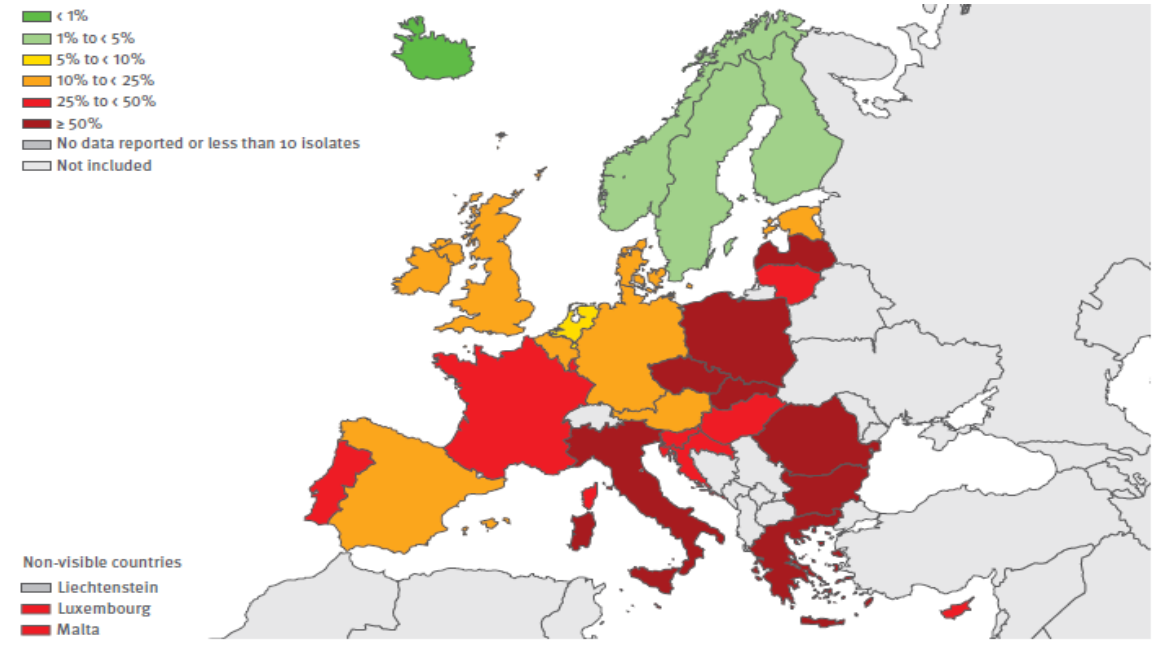


Figure 2. Percentage of invasive *E. coli* (a) and *K. pneumoniae* (b) isolates resistant to third-generation cephalosporins, EU/EEA, 2013 (from EARS-net, last visited October 2015, http://ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx)

2.6.1. Hungary

The first detection of ESBL-producing bacteria in Hungary took place in 1996 in clinical isolates of *K. pneumoniae* (Pragai et al., 1998). Between 2002 and 2003 nosocomial outbreaks caused by *K. pneumoniae* and *K. oxytoca* producing SHV-2a and SHV-5 have been reported, as a result of dissemination of identical allodemic R-plasmids (Tóth et al., 2005; Damjanova et al., 2006). Between 1998 and 2004, outbreaks caused by SHV-type ESBL-producing *K. pneumoniae,* were reported exclusively from neonates (Damjanova et al., 2007). In 2005, eruptive and extensive dissemination of CTX-M-15 producing *K. pneumoniae* clones (mainly ST11, ST15, ST 147) was observed causing both outbreaks and sporadic infection in adult patients across the country (Damjanova et al., 2006; Damjanova et al., 2008). One year later another epidemic clone (ST274) emerged in various regions of the country (Damjanova et al., 2011). In a study from Pecs, Melegh and colleagues found a new CTX-M-15 producing *K. pneumoniae* clone (ST101) which showed high level resistance to ciprofloxacin (Melegh et al., 2015). The result from an investigation of fourteen outbreaks caused by ESBL-producing *K. pneumoniae* in Hungary between 2005 and 2008, showed that the outbreaks in neonatal intensive care units (where fluoroquinolone-type antibiotics were not in use), remained polyclonal and strains retained SHV-type ESBLs, while in the adult outbreaks, CTX-M-type ESBL clones (ST15, ST147) were involved (Szilagyi et al., 2010). It was also shown that the use of fluoroquinolone type antibiotics was responsible for the expansion of the major clone of ESBL-producing *K. pneumoniae* (Toth et al., 2014). There are very little data about the epidemiology of ESBL-producing *E. coli* in Hungary. The most common types of ESBL-producing *E. coli* reported were CTX-M-15 in human and CTX-M-1 in animal isolates (Toth et al., 2013).

2.7. Faecal carriage of ESBL-producers and risk factors for colonization or infections associated with these bacteria

The first reports on human faecal carriage with ESBL were from Spain in outpatients, in 2001 (Mirelis et al., 2003). During the period 1991-2003, there was a gradual increase in the faecal carriage rate in outpatient settings in Spain from 0.7% to 5.5% (Valverde et al., 2004). Since then, asymptomatic faecal carriage of ESBL-producing bacteria in the hospital and the community has been reported in different studies with wide differences in carriage rates between geographic areas. The highest prevalence rates have been reported from Thailand 69.3%, Egypt 63.3% and China 50.5% (Woerther et al., 2013). In Europe the highest carriage rate (11.6%) was observed among patients upon admission to a geriatric unit in Belgium in 2011 (Schoevaerdts et al., 2012). The duration of carriage can be very long, up to several years in some cases (Tham et al., 2012), which constitutes a critical factor in the epidemiology of ESBLs both in hospital and community settings.

Patient-to-patient transmission of ESBL producers occurs frequently, but *K. pneumoniae* appears to have a higher propensity to be transmitted than *E. coli* (Harris et al., 2007a; Harris et al., 2007b). Transmission of such bacteria usually occurs via the faecal-oral route, either directly or indirectly through hand contact with healthcare workers. Environmental reservoirs (sinks, food) are also considered emerging vehicles of transmission of ESBL producers in hospitals (Calbo et al., 2011; Lowe et al., 2012). Many studies have identified risk factors for acquiring and being infected with ESBL-producers. Risk factors for acquiring ESBL while admitted to hospital include a history of extended antibiotic courses (especially cephalosporins and fluoroquinolones) (Aldeyab et al., 2012; Ofner-Agostini et al., 2009; Pena et al., 1997; Shaikh et al., 2015), prolonged hospital stay (Lautenbach et al., 2001; Ofner-Agostini et al., 2009; Shaikh et al., 2015), admission to intensive care unit (Cornejo-Juarez et al., 2015; Flaherty and Weinstein, 1996; Rettedal et al., 2013), underlying host factors (such

as being neutropenic, transplant recipient or neonate (Linares et al., 2008; Logan et al., 2014; Rettedal et al., 2013; Rodriguez-Bano et al., 2010) and the presence of foreign devices (urinary catheter, central venous catheter, mechanical ventilators) (Pena et al., 1997). In non-hospitalized patients, risk-factors associated with ESBL producers included recent antibiotic use (Kang et al., 2012; Reuland et al., 2016) and hospitalization in the past three months (Ben-Ami et al., 2009), residence in a long-term care facility (Ben-Ami et al., 2009), age over 65 years (Ben-Ami et al., 2009), household contacts (Valverde et al., 2008), and male sex (Ben-Ami et al., 2009). Apart from these, a history of travel is also recognized as a risk factor for becoming colonized with ESBL producers (Tangden et al., 2010). Carriage rates, and therefore the risk of colonization in travellers returning home, increased significantly after travel to Egypt, India and Thailand (Tham et al., 2010). In a study conducted by Van der Bij and Pitout, North Africa and the eastern Mediterranean are also associated with a higher carriage risk in returned travellers (van der Bij and Pitout, 2012).

2.8. Clinical impact of ESBLs

ESBL producers have a wide clinical significance and high impact in healthcare systems especially in low income countries (as poor access to drinking water, poverty, and a high population density are efficient driving forces for ESBL dissemination) (Woerther et al., 2013). ESBL producers are associated with different kind of infections, such as pneumonia, urinary tract infections, septicaemia, intra-abdominal infections and meningitis (Badal et al., 2013; Dayan et al., 2013). ESBL producers often exhibit co-resistance to several commonly used antibiotic classes, such as fluoroquinolones, aminoglycosides and trimethoprim-sulfamethoxazole. This high resistance rate against drugs commonly used in the empirical treatment of critically ill patients may result in initial treatment failure, delay of adequate therapy and consequent increased morbidity and mortality rate, higher hospital costs and longer hospital stay (Cordery et al., 2008; Cosgrove and Carmeli, 2003; Schwaber et al.,

2006). Thus, the treatment options are often very limited in infections caused by these bacteria. Carbapenems have been regarded as the drug of choice for treatment of serious infections caused by ESBL-producing bacteria; however the disadvantage is the potential selection for carbapenem-resistance in ESBL producers and in other Gram-negative bacteria (Rupp and Fey, 2003).

3. Objectives

The overall aim was to study the epidemiology of bacteria producing ESBLs in asymptomatic individuals, with no sign of ongoing infection that might be attributed to ESBL-producing bacteria.

Specific aims were as follows:

1) To evaluate the prevalence of ESBL-producing Enterobacteriaceae among applicants for LTC facilities and individuals screened for employment eligibility purposes and to compare the prevalence of faecal carriage and characteristics of these enzymes in these two groups (paper I).

2) To survey the faecal carriage rate of ESBL producers among healthy individuals in the same geographic area a few years later and to compare the prevalence and characteristics of ESBL producers to our previous results (paper III).

3) To assess dynamics of ESBL carriage in patients by investigating the prevalence of faecal colonization with ESBL producers among pediatric and adult inpatients of different wards or ICUs, as well as among outpatients and screened medical students and comparing the characteristics of ESBL producers in these populations (paper II).

4. Materials and Methods

4.1. Samples and isolates

We investigated three batches of samples (first batch; screened asymptomatic carriers 2009-10, second batch; screened inpatients, outpatients and medical students 2010-13, third batch; screened asymptomatic carriers 2013-14, Table 3). The first batch (paper I), contained 1,640 faecal samples sent for screening for enteric pathogens from two groups of asymptomatic individuals investigated between March 2009 and April 2010. Samples originated from 1,109 individuals (300 males and 809 females) being screened for employment eligibility purposes (e.g. jobs at hospitals, kindergartens and food-processing plants) and 531 individuals (218 males and 313 females) who needed to be screened for enteric pathogens prior to admittance to LTC facilities.

The second batch (paper II) of samples included 5581 non-duplicate faecal specimens which were sent for routine diagnostics from October 2010 to February 2013, originating from 4343 inpatients (adult ICU; adult non-ICU; pediatric ICU; pediatric non-ICU and rehabilitation), 814 outpatients and 424 screened medical students (223 foreign and 201 Hungarian students).

The third batch (paper III), contained stool samples from 1004 healthy individuals from the same population as the first batch; 779 from individuals screened for employment purposes (599 females, 180 males) and 225 from applicants for the LTC facilities (140 females, 85 males) between November 2013 and May 2014.

With the exception of international medical students included in our second batch, the vast majority of individuals in our studies were located in North-Eastern Hungary from the EU regions of Northern Hungary and Northern Great Plain.

	Paper I		Paper II							Paper II	
	2009-10 (1st batch)		2010-13 (2nd batch)							2013-14 (3rd batch)	
	LTC applicants	Employment screening group	Adult ICU	Adult non-ICU	Pediatric ICU	Pediatric non-ICU	Rehabilitation	Out-patient	Screened medical student	LTC applicants	Employment screening group
Number of samples	531	1109	330	1397	619	1864	133	814	424	225	779
Median age (range)	75 (0-100)	34 (15-68)	68 (20-96)	69 (0-101)	<1 (0-16)	2 (0-22)	68 (0-93)	28 (0-91)	25 (20-40)	81 (0-103)	34 (14-61)
25 percentile (year)	63	25	56.75	55	<1	1	54	13.75	23	73	24
75 percentile (year)	83	44	76	80	1	6	76	52	26	86	44

Table 3. Demographic data of studied populations. ICU: intensive care unit; LTC: long-term care.

Faecal samples were inoculated directly onto eosin methylene blue agar plates supplemented with 2 mg/l cefotaxime. All colonies with different morphology were further identified by means of biochemical tests; enterobacterial isolates were then processed further. Antimicrobial susceptibility was determined by the EUCAST disk diffusion method using ertapenem, meropenem, imipenem, cefotaxime, ceftazidime, cefepime, ciprofloxacin, trimethoprim–sulfamethoxazole (co-trimoxazole), doxycycline, colistin, amikacin, gentamicin, tobramycin and tigecyclin. All isolates showing a decreased susceptibility to at least one third generation cephalosporin or to cefepime were tested for ESBL phenotype using double disk synergy test (Oxoid, Basingstoke, UK) and then re-identified using a MALDI Biotyper (Bruker, Bremen, Germany).

Carbapenem-resistant isolates were also tested using the Hodge test as specified in the Clinical and Laboratory Standards Institute guidelines.

In the case of isolates displaying an ESBL phenotype, DNA was extracted by heating a loopful of bacterial cells in 200 µl TE buffer; (100 Mm Tris and 10 Mm EDTA) at 98°C for 15 minutes. PCR amplifications were carried out in a MyCycler PCR machine (BioRad, Hercules, CA, USA). Primer sequences are listed in Tables 4 and 5. Amplified DNA-products were analyzed on a 1% agarose gel (Sigma, St. Louis, MO, USA) stained with ethidium bromide. Samples were electrophoresed in TBE buffer at 100 V for 70 minutes. The gel was examined using a UV transilluminator and photographed. For sequencing, PCR products were then purified with a PCR purification kit (Isolate PCR and Gel Kit, Bioline, Taunton, MA, USA) following the manufacturer's instructions. The resulting sequences were compared with known sequences using CLC DNA Workbench software (CLC Bio, Aarhus, Denmark) and the Basic Local Alignment Search Tool (BLAST, NCBI).

(Strain collection has been done by co-authors).

4.2. Identification of ESBL genes

The *bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CTX-M}$ genes were detected by PCR, as described previously (Bedenic et al., 2001; Edelstein et al., 2003; Jouini et al., 2007); *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were identified by sequencing (Macrogen, Amsterdam, The Netherlands) using the same primers, while *bla*$_{CTX-M}$ genes were identified by the sequencing of products of group-specific PCRs (Pitout et al., 2004b). Primer sequences and annealing temperatures are listed in Table 4. Sequence alignment and analyses were performed using CLC DNA Workbench.

4.3. Aminoglycoside resistance genes and characterization of integrons

The aminoglycoside resistance genes *aac(3')-IIa (aacC2)*, *aac(6')-Ib (aacA4), aph(3')-Ia (aphA1), ant(2')-Ia (aadB), ant(3')-Ia (aadA1), armA* , *rmtA* and *rmtB* were detected by PCR, as previously described (Bogaerts et al., 2007; Frana et al., 2001; Hannecart-Pokorni et al., 1997; Noppe-Leclercq et al., 1999). The detection of class 1 and class 2 integrons was performed by PCR assays according to Mazel et al (Mazel et al., 2000) and the amplification and sequencing of the variable regions were performed as described by White et al (White et al., 2001), using newly designed internal primers when necessary. Primer sequences are listed in Table 4. Sequences were assembled in the CLC DNA Workbench, and gene cassettes were identified using the BLAST (http://blast.ncbi.nlm.nih.gov/Blast). Identification of class 1 and class 2 integrons with variable regions of the same size was performed by restriction analysis using at least two enzymes for each cassette array, including *Eco*RI, *Hind* III, *Mse*I and RsaI (Thermo Scientific, Waltham, MA, USA).

Target gene	Primer name	Sequence (5'→3')	Amplification size (bp)	Annealing Temp (°C)	$MgCl_2$ (25mM)	Primer cc[c] (µM)	Reference
bla$_{TEM}$	bla$_{TEM}$-F bla$_{TEM}$-R	ATTCTTGAAGACGAAAGGGC ACGCTCAGTGGAACGAAAAC	1150	56	6	10	(Jouini et al., 2007)
bla$_{SHV}$	bla$_{SHV-F}$ bla$_{SHV-R}$	CGCCGGGTTATTCTTAATTTGTCGC TCTTTCCGATGCCGCCGCCAGTCA	1016	60	3	10	(Bedenic et al., 2001)
bla$_{CTX-M}$	pan$_{CTX-M-F}$ pan$_{CTX-M-R}$	TTTGCGATGTGCAGTACCAGTAA CGATATCGTTGGTGGTGCCATA	544	65	6	10	(Edelstein et al., 2003)
bla$_{CTX-M-group\ I}$[a]	CTXM1-F3 CTXM1-R2	GACGATGTCACTGGCTGAGC AGCCGCCGACGCTAATACA	499	55	8	10	(Pitout et al., 2004b)
bla$_{CTX-M-group\ II}$[a]	TOHO1-2F TOHO1-1R	GCGACCTGGTTAACTACAATCC CGGTAGTATTGCCCTTAAGCC	351	55	8	10	(Pitout et al., 2004b)
bla$_{CTX-M-group\ III}$[a]	CTXM825F CTXM825R	CGCTTTGCCATGTGCAGCACC GCTCAGTACGATCGAGCC	307	55	4	10	(Pitout et al., 2004b)
bla$_{CTX-M-group\ IV}$[a]	CTXM914F CTXM91R	GCTGGAGAAAAGCAGCGGAG GTAAGCTGACGCAACGTCTG	474	62	4	10	(Pitout et al., 2004b)
aac(6')-Ib	aac(6')-Ib-F aac(6')-Ib-R	GTTACTGGCGAATGCATCACA TGTTTGAACCATGTACACGGC	216	58	8	25	(Frana et al., 2001)
aac(3')-IIa	aac(3')-IIa-F aac(3')-IIa-R	ATGCATACGCGGAAGGC TGCTGGCACGATCGGAG	822	58	4	25	(Noppe-Leclercq et al., 1999)
ant(2")-Ia	ant(2")-Ia –F ant(2")-Ia-R	GAGCGAAATCTGCCGCTCTGG CTGTTACAACGGACTGGCCGC	320	67	5	25	(Frana et al., 2001)
ant(3")-Ia	ant(3")-Ia-F ant(3")-Ia-R	TCGACTCAACTATCAGAGG ACAATGGTGACTTCTACAGCG	244	58	8	25	(Hannecart-Pokorni et al., 1997)
aph(3')-Ia	aph(3')-Ia-F aph(3')-Ia-R	CGAGCATCAAATGAAACTGC GCGTTGCCAATGATGTTACAG	623	58	5	25	(Noppe-Leclercq et al., 1999)
armA	met-F met-F	CAAATGGATAAGAATGATGTT TTATTTCTGAAATCCACT	776	55	4	25	(Bogaerts et al., 2007)
rmtA	rmtA-F rmtA-R	CTAGCGTCCATCCTTTCCTC TTTGCTTCCATGCCCTTGCC	635	55	4	25	(Bogaerts et al., 2007)
rmtB	rmtB-F rmtB-R	ATGAACATCAACGATGCCCT CCTTCTGATTGGCTTATCCA	769	55	4	25	(Bogaerts et al., 2007)
Int-1	Int-1-F Int-1-R	GGTCAAGGATCTGGATTTCG ACATGCGTGTAAATCATCGTC	500	62	10	10	(Mazel et al., 2000)
Int-2	Int-2-F Int-2-R	CACGGATATGCGACAAAAAGGT GTAGCAAACGAGTGACGAAATG	800	62	6	10	(Mazel et al., 2000)

Class 1 integron variable region	CS-F CS-R	GGCATCCAAGCAGCAAG AAGCAGACTTGACCTGA	Variable	55	8	10	(White et al., 2001)
Class 2 integron variable region	HEP-F HEP-R	CGGGATCCCGGACGGCATGCACGATTT GATGCCATCGCAAGTACGAG	Variable	60	6	10	(White et al., 2001)
UidA	Ecspec-F Ecspec-R	ATCACCGTGGTGACGCATGTCGC CACCACGATGCCATGTTCATCTGC	486	50	16	10	(Heininger et al., 1999)
K. pneumoniae 16S–23S ITS[b]	Pf Pr2	ATTTGAAGAGGTTGCAAACGAT CCGAAGATGTTTCACTTCTGATT	260	57	12	10	(Liu et al., 2008)

Table 4. List of primes and annealing temperatures utilized in the PCR analysis.[a] Group I includes CTX-M-1, 3, 10 -12, 15 (UOE-1), 22, 23, 28, 29, and 30. Group II includes CTX-M-2, 4, 7, and 20 and Toho-1. Group III includes CTX-M-8. Group IV includes CTX-M-9, 13, 14, 16-19 and 21, and 27 and Toho-2. Group V includes CTX-M-25 and 26. [b] Internal transcribed spacer.[c] Concentration.

4.4. Phylogenetic analysis of *E. coli* and virulence genes

Phylogenetic groups of *E. coli* were determined using the multiplex PCR method developed by Clermont et al. (Clermont et al., 2000). Three markers were used: *chuA*, *yjaA* and TSPE4C2, primers sequences are listed in Table 5; using these markers *E. coli* were grouped into A, B1, B2 and D phylogenetic groups (Figure 3). The pandemic O25b-ST131 clone was screened in the isolates of the phylogenetic group B2 with a PCR-based assay (Clermont et al., 2009). A multiplex PCR assay was used to determine the presence of virulence factor genes characteristic for enterovirulent *E. coli* pathotypes (Persson et al., 2007). We used PCR with previously described primers (Table 5) for the first batch of samples to search for genes coding for the putative extra-intestinal virulence factors of *E. coli* , including adhesins (*papC*, *fimH*, *sfaS* and *sfa/focDE*), toxins (*cnf1* and *cvaC*), factors related to iron acquisition (*iutA* and *fyuA*), the capsule system (*kpsMT II*) and miscellaneous factors (*ibeA*, PAI, *traT* and *csgA*) (Braun and Vidotto, 2004).

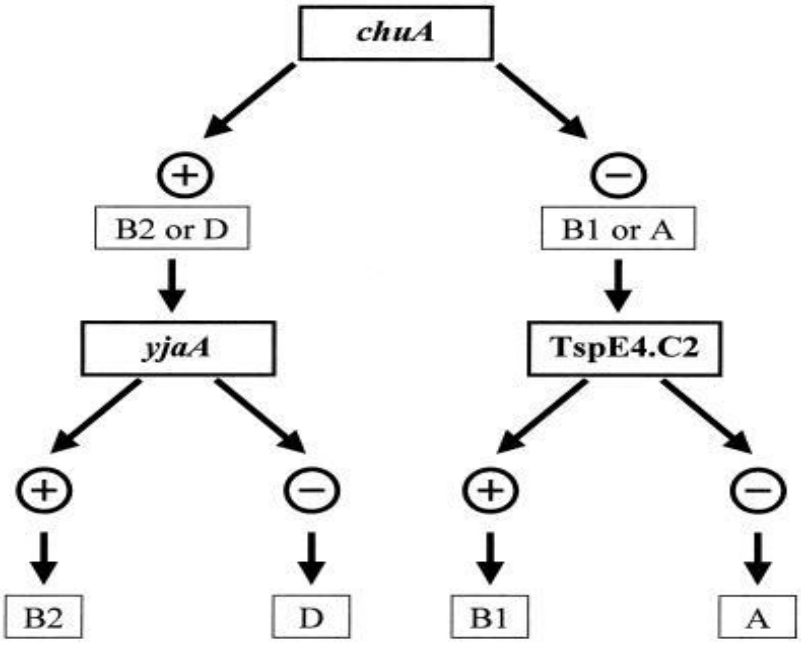


Figure 3. Dichotomous decision tree to determine the hylogenetic group of an *E. coli* strain, by using the results of PCR amplification of the *chuA* and *yjaA* genes and DNA fragment TSPE4.C2 (Clermont et al., 2000).

Target gene	Primer name	Sequence (5'→3')	Amplification size (bp)	Annealing Temp (°C)	$Mgcl_2$ (25Mm)	Primer cc[a] (µM)	Reference
papC	pap1 pap2	GACGGCTGTACTGCAGGGTGTGGCG ATATCCTTTCTGCAGGGATGCAATA	328	63	5	20	(Braun and Vidotto, 2004)
fimH	FimH-F FimH-R	TGCAGAACGGATAAGCCGTGG GCAGTCACCTGCCCTCCGGTA	508	63	5	20	(Braun and Vidotto, 2004)
sfaS	SfaS-F SfaS-R	GTGGATACGACGATTACTGTG CCGCCAGCATTCCCTGTATTC	240	63	5	20	(Braun and Vidotto, 2004)
sfa/focDE	sfa1 sfa2	CTCCGGAGAACTGGGTGCATCTTAC CGGAGGAGTAATTACAAACCTGGCA	410	63	5	20	(Braun and Vidotto, 2004)
cnf1	cnf1 cnf2	AAGATGGAGTTTCCTATGCAGGAG CATTCAGAGTCCTGCCCTCATTATT	498	63	5	20	(Braun and Vidotto, 2004)
cvaC	ColV-C-F ColV-C-R	CACACACAAACGGGAGCTGTT CTTCCCGCAGCATAGTTCCAT	680	63	5	20	(Braun and Vidotto, 2004)
iutA	AerJ –F AerJ-R	GGCTGGACATCATGGGAACTGG CGTCGGGAACGGGTAGAATCG	300	63	5	20	(Braun and Vidotto, 2004)
fyuA	FyuA-F FyuA-R	TGATTAACCCCGCGACGGGAA CGCAGTAGGCACGATGTTGTA	880	63	5	20	(Braun and Vidotto, 2004)
kpsMT II	KpsII-F kpsII-R	GCGCATTTGCTGATACTGTTG CATCCAGACGATAAGCATGAGCA	272	63	5	20	(Braun and Vidotto, 2004)
ibeA	ibe10-F ibe10-R	AGGCAGGTGTGCGCCGCGTAC TGGTGCTCCGGCAAACCATGC	170	63	5	20	(Braun and Vidotto, 2004)
PAI	RPAi-F RPAi-R	GGACATCCTGTTACAGCGCGCA TCGCCACCAATCACAGCCGAAC	930	63	5	20	(Braun and Vidotto, 2004)
traT	TraT-F TraT-R	GGTGTGGTGCGATGAGCACAG CACGGTTCAGCCATCCCTGAG	290	63	5	20	(Braun and Vidotto, 2004)
csgA	M464-F M465-R	ACTCTGACTTGACTATTACC AGATGCAGTCTGGTCAAC	200	50	5	20	(Braun and Vidotto, 2004)
ChuA	ChuA 1 ChuA 2	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279	55	12	10	(Clermont et al., 2000)
yjaA	YjaA 1 YjaA 2	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211	55	12	10	(Clermont et al., 2000)
TSPE4.C2	TspE4.C2 1 TspE4.C2 2	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152	55	12	10	(Clermont et al., 2000)
pabB	O25pabBspe-F O25pabBspe-R	TCCAGCAGGTGCTGGATCGT GCGAAATTTTTCGCCGTACTGT	347	65	6	10	(Clermont et al., 2009)
trpA	trpA-F trpA-R	GCTACGAATCTCTGTTTGCC GCAACGCGGCCTGGCGGAAG	427	65	6	10	(Clermont et al., 2009)

Human *estA*	StFh StRh	TTTCGCTCAGGATGCTAAACCAG CAGGATTACAACACAATTCACAGCAGTA	151	57	2.6	0.4	(Persson et al., 2007)
Porcine *estA*	StFp StRp	CTTTCCCCTCTTTTAGTCAGTCAACTG CAGGATTACAACAAAGTTCACAGCAG	160	57	2.6	0.4	(Persson et al., 2007)
vtx1	PS3 PS4	GTTTGCAGTTGATGTCAGAGGGA CAACGAATGGCGATTTATCTGC	260	57	2.6	0.25	(Persson et al., 2007)
eae	PS5 PS6	GGYCAGCGTTTTTTCCTTCCTG TCGTCACCARAGGAATCGGAG	377	57	2.6	0.15	(Persson et al., 2007)
vtx2	PS7 PS8	GCCTGTCGCCAGTTATCTGACA GGAATGCAAATCAGTCGTCACTC	420	57	2.6	0.5	(Persson et al., 2007)
eltA	PS9 PS10	AAACCGGCTTTGTCAGATATGATGA TGTGCTCAGATTCTGGGTCTCCT	479	57	2.6	0.45	(Persson et al., 2007)
ipaH	PS11 PS12	TTGACCGCCTTTCCGATACC ATCCGCATCACCGCTCAGAC	647	57	2.6	0.1	(Persson et al., 2007)
16S rDNA	PS13 PS14	GGAGGCAGCAGTGGGGAATA TGACGGGCGGTGTGTACAAG	1062	57	2.6	0.25	(Persson et al., 2007)

Table 5. Primers and annealing temperatures used in the PCR reactions. R = A or G; Y = C or T. [a] Concentration.

4.5. Genetic relatedness

For the first batch of samples (paper I), the epidemiological relationship was analyzed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) with ERIC2 and ERIC1R primers (Versalovic et al., 1991) and pulsed-field gel electrophoresis (PFGE). Plugs were prepared as described earlier (Mozes et al., 2014), macrorestriction was performed using XbaI (Fermentas, Vilnius, Lithuania) in a CHEF DRIII machine (Bio-Rad, Hercules, CA, USA) in 1% SeaKem Gold agarose (Lonza, Basel, Switzerland) at 14° C. Electrophoresis was performed at 6 V/cm, with a reorientation angle of 120°, and switch times were ramped between 2 and 64 s for 20 h for both species. Gels were stained with ethidium bromide and visualized under UV light. Banding patterns were analyzed with Fingerprinting II software (Bio-Rad, Hercules, CA, USA). The threshold for probable relatedness was set at >85% similarity. The Simpson index of diversity (D) was calculated to assess diversity, as described earlier (Hunter and Gaston, 1988).

(PFGE analysis has been performed with the help of my co-authors).

4.6. Prevalence of ESBL-infected patients and antibiotic consumption

For comparison to the data of the second batch of samples (paper II), monthly numbers of ESBL-infected patients were collected from data of the Bacteriology Laboratory and expressed as number of ESBL-infected patients per positive samples, incidence density of ESBL producers per 100 bed-days and proportion of ESBL producers among *Klebsiella spp*. and *E. coli*. Prevalence data were also collected on *K. pneumoniae* and *E. coli* as well as on adult and pediatric patients separately. Monthly antibiotic consumption was calculated as defined daily doses/100 bed-days for the major antibiotic groups (Monnet, 2006).

(Antibiotic consumption data collection has been done by co-authors).

4.7. Statistical analysis

Prevalences were compared by means of the χ 2 test or the Fisher exact test as appropriate. Age distribution in the different groups was compared by means of the Kolmogorov-Smirnov test. The distribution of genes coding for ESBLs, aminoglycoside modifying enzyme genes and co-resistance patterns were all analyzed by χ 2 test, and the pairwise comparisons derived were adjusted with the Bonferroni correction. The association of genes with each other or with different characteristics was analysed using the Pearson correlation. PaSt v3.0 was used to perform the statistical tests (Hammer et al., 2005).

Time series data of monthly antibiotic consumptions, prevalence of ESBL carriers and prevalence of ESBL-infected patients were used in different combinations to build dynamic regression models (paper II) using the Pankratz methodology (Pankratz, 1991). First, pairwise dynamic regression models were built to assess the relationship between i) variables characterizing ESBL-infected patients as explanatory and ESBL carriage rates as dependent variables, ii) these two sets of variables tested in reverse order, iii) using consumption of different antibiotics as explanatory variables against ESBL carriage rates or iv) against variables characterizing ESBL-infected patients. Later models with multiple explanatory variables were constructed using v) rates of infection with ESBL-producing *E. coli* and *K. pneumoniae* as separate explanatory variables and vi) rates of infection in pediatric and adult patients as separate explanatory variables. Causal relationship between two time-series was assessed using Granger causality tests. Time-series analysis was performed in the software environment Eviews 3.1.

(Time-series analysis has been done with the help of my supervisor).

5. Results

5.1. Prevalence of ESBL-producer carriage

The overall prevalence of ESBL carriers among individuals screened for enteric pathogens in 2009-2010 and in 2013-2014 (first and third batches) was comparable (3.7% (60/1640) and 3.0% (30/1004), respectively). The LTC groups carried ESBL-producers significantly more frequently than the employment screening groups (7.2%, 38/531 vs. 2.0%, 22/1109, $p<0.001$ and 5.3%, 12/225 vs. 2.3%, 18/779, $p=0.019$ respectively, Figure 4).

In the first batch, among the 63 ESBL-producing isolates from the 60 patients, the species found were *E. coli* (43 isolates), *K. pneumoniae* (18 isolates), *Klebsiella oxytoca* (1 isolate) and *Proteus mirabilis* (1 isolate). Three individuals, all from the LTC group, harboured multiple ESBL-producing isolates simultaneously (*K. pneumoniae* and *E. coli* in 2 individuals and *K. oxytoca* and *E. coli* in 1 individual). Among the LTC group, the same proportion of *E. coli* and *K. pneumoniae* isolates was found (21/41 vs. 18/41), while among individuals on employment screening only *E. coli* isolates occurred.

In the same population of individuals sampled in 2013-14 (third batch), altogether 32 isolates producing ESBLs were found. Similarly to the first batch, the same proportion of *E. coli* and *K. pneumoniae* occurred in the LTC group (5/14 vs. 5/14, and two applicants carried both species simultaneously), and only *E. coli* isolates were found in the employment screening group. Other species did not occur.

Prevalence of faecal carriage of ESBL-producers in the second batch was 7.4% (323/4343) among inpatients which was significantly higher than outpatients and screened medical students (3.1%, 25/814 and 2.6%, 11/424 respectively; $p<0.001$). Among inpatient subgroups (Figure 4), adults showed significantly higher carriage rates of ESBL producers (12.0%, 222/1853) than pediatric patients (4.1%, 101/2490; $p<0.001$). The highest prevalence was found in rehabilitation wards (27.1%, 36/133), followed by ICU patients (9.3%, 88/949);

lowest prevalences found in non-ICU patients (6.1%, 199/3261). These differences were statistically significant in any pairwise comparison (p<=0.001). The prevalence rates were comparable in medical screening students and outpatient study groups (1.0%-4.0%) as well as in pediatric non-ICU (3.0%, 56/1864), however significantly higher, in pediatric ICU (6.8%, 42/619) and more significantly higher in adult ICU and non-ICU (13.9%, 46/330 and 10.2%, 143/1397; respectively). Among the screened medical students non-Hungarian medical students showed higher prevalence rate than Hungarian students (4.0% and 1.0%, respectively; p=0.049). The rate of carriage of ESBL producers was not affected by the region where patients lived (except non-Hungarian medical students), by their gender or by their age in any of the comparisons.

Among the 369 ESBL-producers from the 359 patients, the most common species were *K. pneumoniae* and *E. coli* (185 and 179 isolates, respectively), followed by two *Proteus mirabilis*, two *Citrobacter brakii* and one *K. oxytoca* isolate. Ten individuals among inpatients harboured *K. pneumoniae* and *E. coli* simultaneously. In inpatients, proportion of *E. coli* and *K. pneumoniae* was similar (46.0%, 153/333 vs. 52.9%, 176/333), while among outpatients and medical students *E. coli* was more frequent (64.0%, 16/25 vs. 32.0%, 8/25; p=0.024 and 90.9%, 10/11 vs. 9.1%, 1/11, p<0.001, respectively). Among adult non-ICU and pediatric ICU patients, the proportion of *K. pneumoniae* and *E. coli* was comparable (43.9%, 65/148 vs. 54.7%, 81/148 and 42.1%, 24/43 vs. 31.6%. 18/43, respectively); however *K. pneumonia*e was more prevalent among adult ICU (75.5%, 37/49 vs. 24.5%, 12/49; p<0.001) and rehabilitation patients (75.0%, 27/36 vs. 22.2%, 8/36; p<0.001). In contrast, *E. coli* prevalence was significantly higher among pediatric non-ICU (P=0.030, Figure 8).

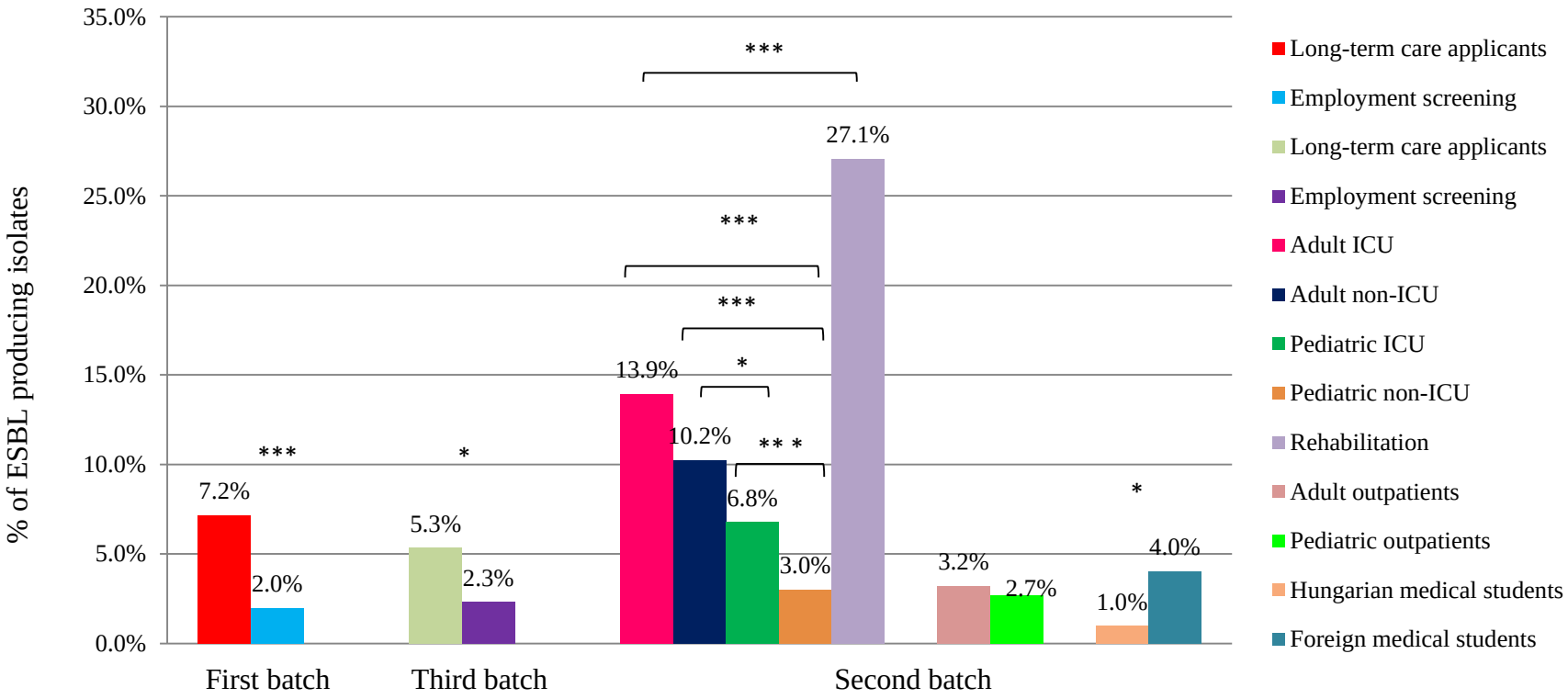


Figure 4. Prevalence of ESBL producing isolates among different groups in the three batches (paper I, II and III). ICU: intensive care unit. * $p < 0.05$; * * * $p < 0.001$.

5.2. Characterization of ESBL producing isolates

Carriage of ESBL genes of the *bla*$_{\text{CTX-M}}$ family was dominant in all study groups of all sample batches (Figure 5). In the first batch, out of the 63 ESBL-positive isolates 50 harboured a *bla*$_{\text{CTX-M}}$ gene; the majority of which were *bla*$_{\text{CTX-M-15}}$ (68.0%, 34/50). Other *bla*$_{\text{CTX-M}}$ types were found only in *E. coli* isolates, of which one isolate harboured *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{CTX-M-2}}$ simultaneously. All *K. pneumoniae* and five *E. coli* isolates (11.6%, 5/43) carried *bla*$_{\text{SHV-12}}$; in the case of *K. pneumoniae*, eleven isolates harboured both *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{SHV-12}}$ (61.1%; 11/18). The distribution of the ESBL genes was significantly different between the two study groups (p=0.001, Figure 5). In the LTC group *bla*$_{\text{CTX-M-15}}$ was found more frequently than in the employment screening group (68.3% vs. 22.7%, respectively; p<0.001), while the frequencies of other genes were comparable. When comparing ESBL gene distribution among *K. pneumoniae* (all from the LTC group), *E. coli* from the LTC group and *E. coli* from the employment screening, a significant difference was found in pairwise comparisons (p<0.001 to p=0.026). In *E. coli* from the LTC group, *bla*$_{\text{CTX-M-15}}$ gene was found in 76.2% (16/21) of the isolates, while in the employment screening group only in 22.7% (5/22; p<0.001). In addition, *K. pneumoniae* carried *bla*$_{\text{SHV-12}}$ more frequently than *E. coli* either among the LTC group or in the employment screening group (p<0.001).

In the same population of individuals sampled in 2013-14 (the third batch), all isolates carried CTX-M type ESBLs, with the dominance of *bla*$_{\text{CTX-M-15}}$ (84.4%, 27/32). Diversity of ESBL genes was lower than the first batch (Figure 5). All *K. pneumoniae* isolates carried *bla*$_{\text{CTX-M-15}}$ (two of them harboured *bla*$_{\text{SHV-5}}$ and other two harboured *bla*$_{\text{SHV-110}}$ simultaneously), while six of seven *E. coli* isolates (85.7%) from the LTC group and 14 of 18 isolates (77.8%) of the employment screening group carried *bla*$_{\text{CTX-M-15}}$. Comparing with the first batch, *bla*$_{\text{CTX-M-15}}$ in *E. coli* from the employment screening group in 2013-14, became significantly more frequent (p=0.001), however no significant differences found between *E. coli* in the LTC

group for both batches. The remaining one and four *E. coli* carried $bla_{\text{CTX-M-1}}$. All of these rates were statistically comparable.

In the second batch, the most common gene was also $bla_{\text{CTX-M-15}}$ in both major species and in all groups (Figure 5). In *E. coli*, other frequent genes were $bla_{\text{CTX-M-1}}$ and $bla_{\text{SHV-12}}$, while in *K. pneumoniae* $bla_{\text{CTX-M-15}}$ was followed by $bla_{\text{SHV-12}}$ in all inpatient groups. One *K. pneumoniae* isolate harboured $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-12}}$ simultaneously. Adult inpatients carried $bla_{\text{CTX-M-15}}$ more frequently than children, regardless of ward type ($p<0.001$). Diversity of ESBL genes in *E. coli* was higher in children than in adults (Figure 5).

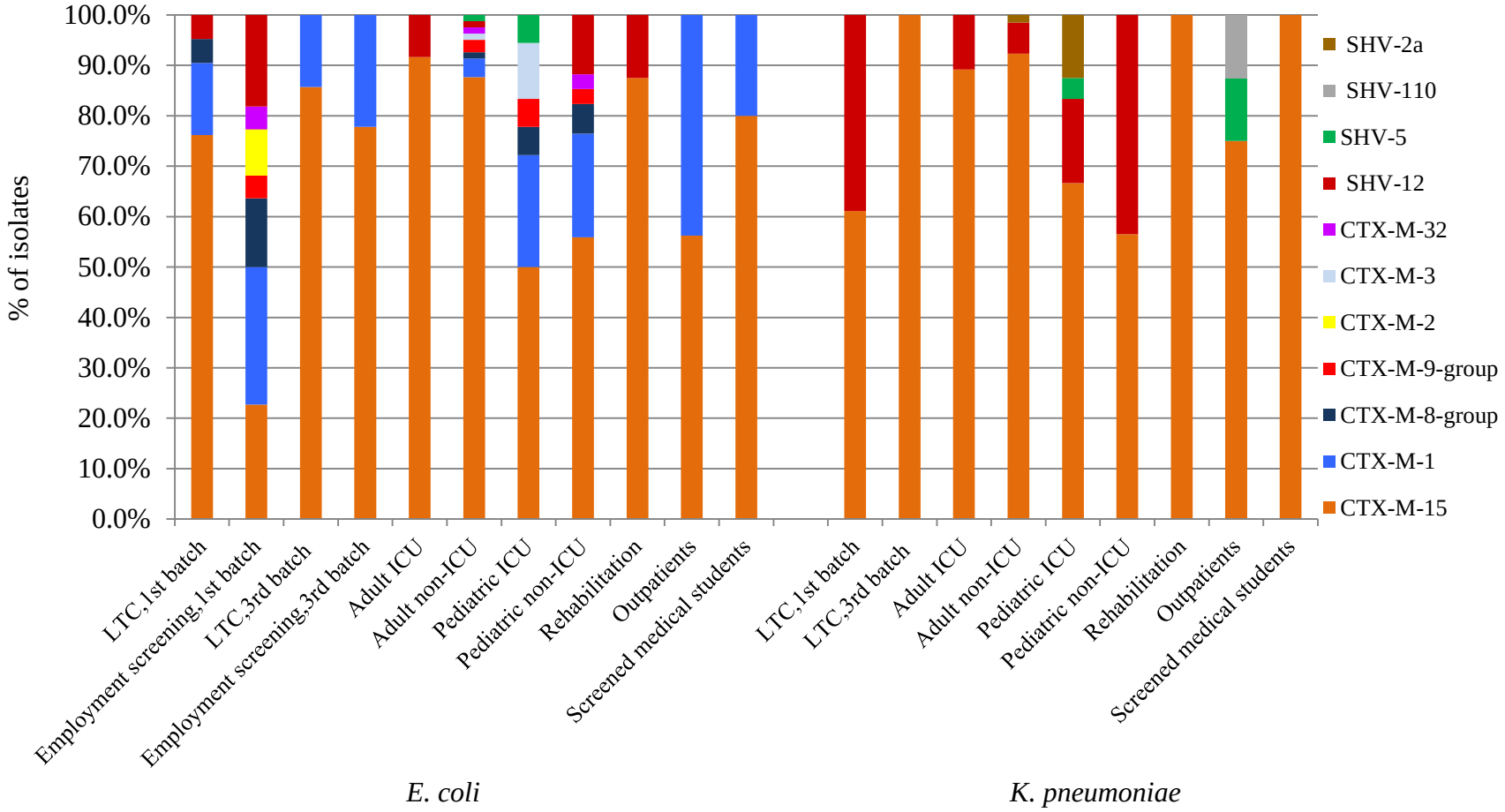


Figure 5. Distribution of ESBL genes in different groups in *E. coli* and *K. pneumoniae* (paper I, II and III). ICU: intensive care unit; LTC: long--term care.

5.3. Resistance patterns and aminoglycoside resistance genes

Resistance to the tested antibiotic classes was common in all three batches. All ESBL-producing isolates were susceptible to colistin; carbapenem nonsusceptibility (Hodge test negative) was detected in *K. pneumoniae* isolates (one harbouring $bla_{\text{SHV-12}}$ in the first batch and eleven harbouring $bla_{\text{ctx-m-15}}$ and two harbouring $bla_{\text{SHV-12}}$ in the second batch; none in the third batch).

In 2009-2010 (in the first batch) *K. pneumoniae* was resistant to all the tested antibiotics, except for carbapenems and colistin, and it carried the *aac(6')-Ib* gene but no other tested aminoglycoside resistance genes. In case of *E. coli* there was marked variability in the patterns of resistance to ciprofloxacin, amikacin, tobramycin and trimethoprim-sulfamethoxazole ($p=0.048$ to $p<0.002$) between isolates from individuals on employment screening and applying for LTC admission (Figure 6). Considering commensal vs. pathogenic *E. coli* isolates as classified by phylogroups, the resistance pattern was similar (but no significant differences in resistance to ciprofloxacin and trimethoprim-sulfamethoxazole). In contrast to *K. pneumoniae*, isolates resistant to all non-beta-lactam antibiotics (except colistin) were not found in *E. coli* from either group. In the *E. coli* isolates, the presence of the $bla_{\text{CTX-M-15}}$ gene correlated positively with ciprofloxacin, amikacin and tobramycin resistance, while the gene $bla_{\text{CTX-M-1}}$ correlated negatively with amikacin and tobramycin resistance (Table 6).

Figure 6. Resistance rates in ESBL-producing isolates obtained from long-term care facility applicants and individuals on employment screening (paper I and III). amik: amikacin; cip: ciprofloxacin; dox: doxycycline; gen: gentamicin; tig: tigecycline; tmp + smx: trimethoprim-sulfamethoxazole (co-trimoxazole); tob: tobramycin.* $p < 0.05$; * * $p < 0.01$; * * * $p < 0.001$.

	bla$_{CTX-M-15}$	*bla*$_{CTX-M-1}$
Ciprofloxacin resistance	r=0.39 **	S
Gentamicin resistance	NS	S
Amikacin resistance	r=0.52 ***	r=-0.40 **
Tobramycin resistance	r=0.52 ***	r=-0.42 **
Tigecycline resistance	NS	NS
Carriage of aac(3')-IIa	r=0.34 *	NS
Carriage of aac(6')-Ib	r=0.70 ***	r=-0.37 *
Carriage of *aph(3')-Ia*	r=-0.36 *	NS
Carriage of *ant(3'')-Ia*	r=-0.35 *	NS
Carriage of type I integron	r=-0.51 ***	NS

Table 6. Correlation of carriage of *bla*$_{CTX-M-15}$ and *bla*$_{CTX-M-1}$ with resistance to antibiotics and aminoglycoside resistance genes in *E. coli* isolates (paper I). NS: not significant; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; shading indicates negative correlation.

All isolates were negative for *ant(2')-Ia, armA, rmtA* and *rmtB* genes. In *E. coli* from the LTC group, aminoglycoside resistance genes were distributed unevenly (p=0.017, Figure 7), with more common carriage of *aac(6')-Ib* than *aph(3')-Ia* and *ant(3')-Ia* (p =0.048 for both comparisons), but not more common than the carriage of *aac(3')-IIa*. The distribution of aminoglycoside resistance genes was also uneven ($p<0.001$) in the case of *E. coli* from the employment screening group, but in these isolates *ant(3')-Ia* was more frequent than *aac(6')-Ib* and *aph(3')-Ia* (p=0.005 and p=0.02, respectively). Regarding commensal and pathogenic *E. coli* isolates, pathogenic isolates showed a pattern very similar to that of the isolates from the LTC group, but the dominance of *aac(6')-Ib* over *aph(3')-Ia* and *ant(3')-Ia* was more marked (p=0.004 for both comparisons). The commensal isolates were similar to the isolates from the employment screening group.

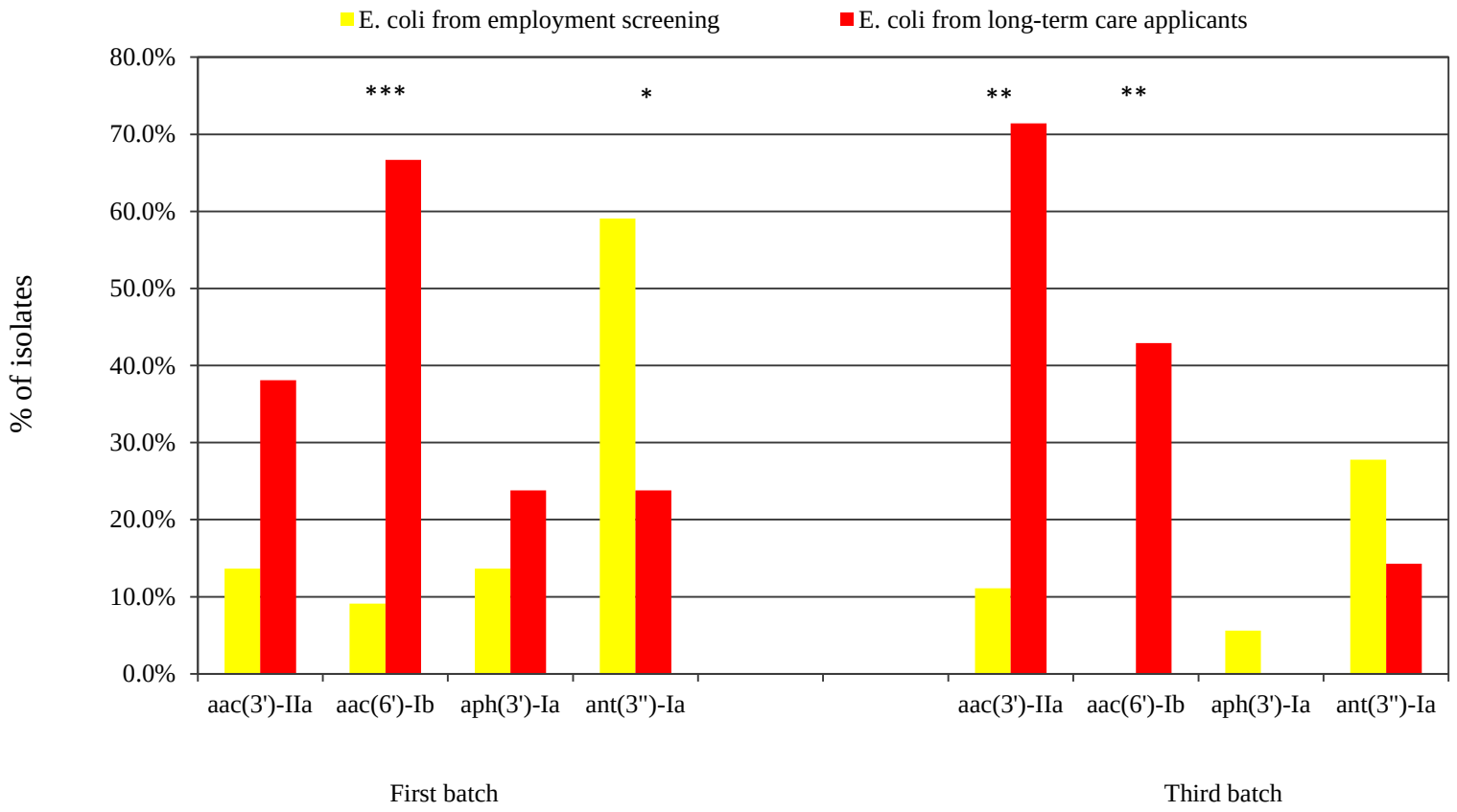


Figure 7. The prevalence of aminoglycoside resistance genes in the two groups (paper I and III).

* $p<0.05$; ** $p<0.01$, *** $p<0.001$.

In the consecutive study of the same population in 2013-14 (third batch), resistance to amikacin, co-trimoxazole, ciprofloxacin and gentamicin was found in 13, 12, 11 and 8 of the 14 isolates in the LTC group respectively, while these numbers were 6, 11, 11 and 6 of the 18 isolates in the employment screening group. The difference in susceptibility also showed when comparing only *E. coli* isolates; six and all of the seven isolates in the LTC group were ciprofloxacin and amikacin resistant, respectively, while eleven were ciprofloxacin resistant and six were amikacin resistant of the 18 *E. coli* isolates from the employment screening group (Figure 6). Similarly to the first batch, all isolates were negative for *ant(2')-Ia*, *armA*, *rmtA* and *rmtB* genes. The genes *aac(3')-IIa* and *aac(6')-Ib* were more dominant in the LTC group than in the employment screening group (10/14 vs. 2/18, $p<0.001$ and 5/14 vs. 0/18, $p=0.001$, respectively, Figure 7). This was also evident when comparing only *E. coli* isolates from the two groups (5/7 vs. 2/18, $p=0.007$ and 3/7 vs. 0/18, $p=0.015$, respectively).

In the second batch, resistance rates to ciprofloxacin and aminoglycosides were significantly higher among adults both in ICUs and non-ICUs than among children, outpatients and screened students ($p<0.001$; Figure 8). The genes *aac(3')-IIa* and *aac(6')-Ib* were more frequent in adults ($p<0.001$), while in children *aph(3')-Ia* was found more frequently ($p<0.001$, Figure 9). Resistance to ciprofloxacin and to aminoglycoside antibiotics correlated with the presence of $bla_{\text{CTX-M-15}}$ gene ($r=0.42$-0.54; $p<0.001$). *K. pneumoniae* showed significantly higher resistance to all antibiotics tested than *E. coli* ($p<0.001$), ertapenem resistance (altogether thirteen isolates: six, four, two and one isolate from adult-ICU, pediatric-ICU, adult non-ICU and rehabilitation ward, respectively) and *rmtA* (one isolate from adult non-ICU) was detected only in *K. pneumoniae* from inpatients. The genes *aac(3')-IIa, aac(6')-Ib* and *ant(3'')-Ia* were also significantly more frequent in *K. pneumoniae* ($p<0.001$). In case of *E. coli* isolates, resistance rates to ciprofloxacin and aminoglycosides were significantly higher in extraintestinal pathogenic than in commensal strains ($p<0.004$).

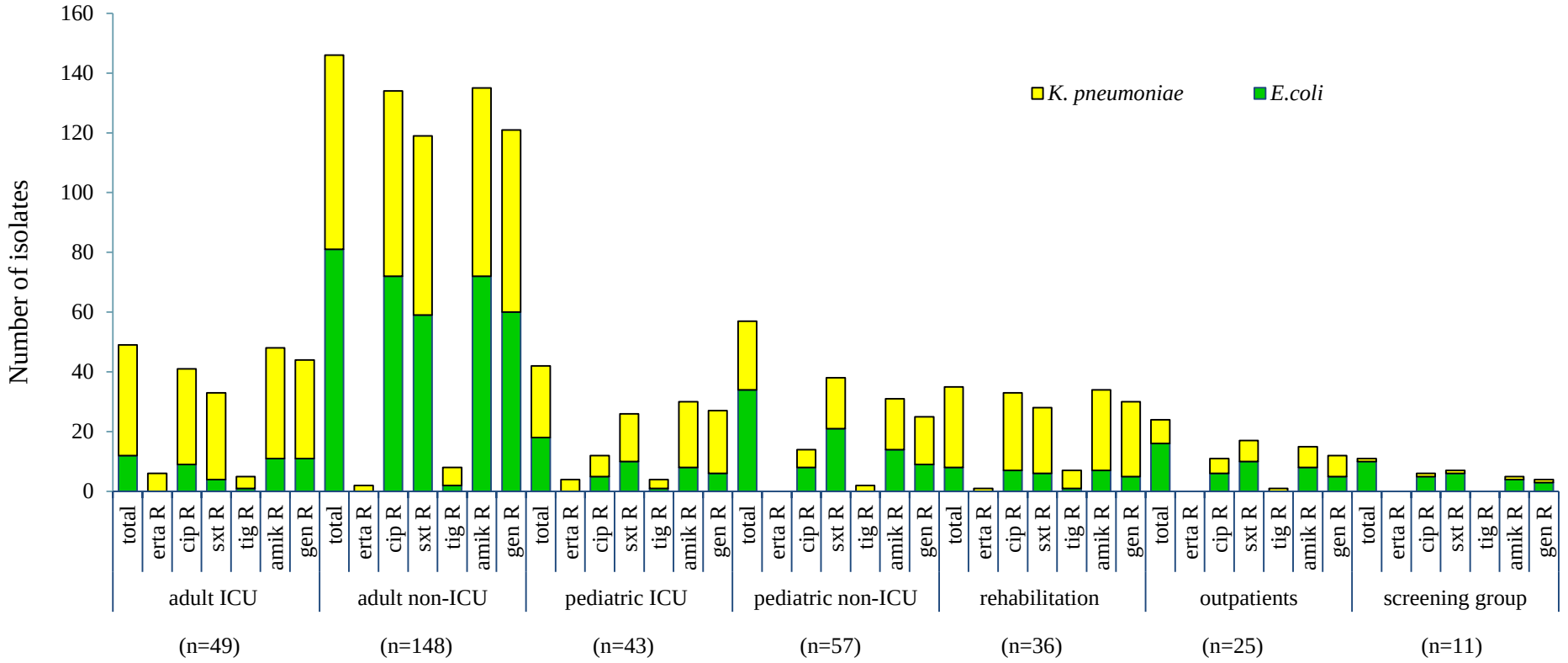


Figure 8. Proportions of ESBL-producing *E. coli* and *K. pneumoniae* in the different study groups and number of isolates resistant to different antibiotics (paper II). ICU: intensive care unit, erta R: ertapenem resistant, cip R: ciprofloxacin resistant, sxt R: co-trimoxazole resistant, tig R: tigecycline resistant, amik R: amikacin resistant, gen R: gentamicin resistant.

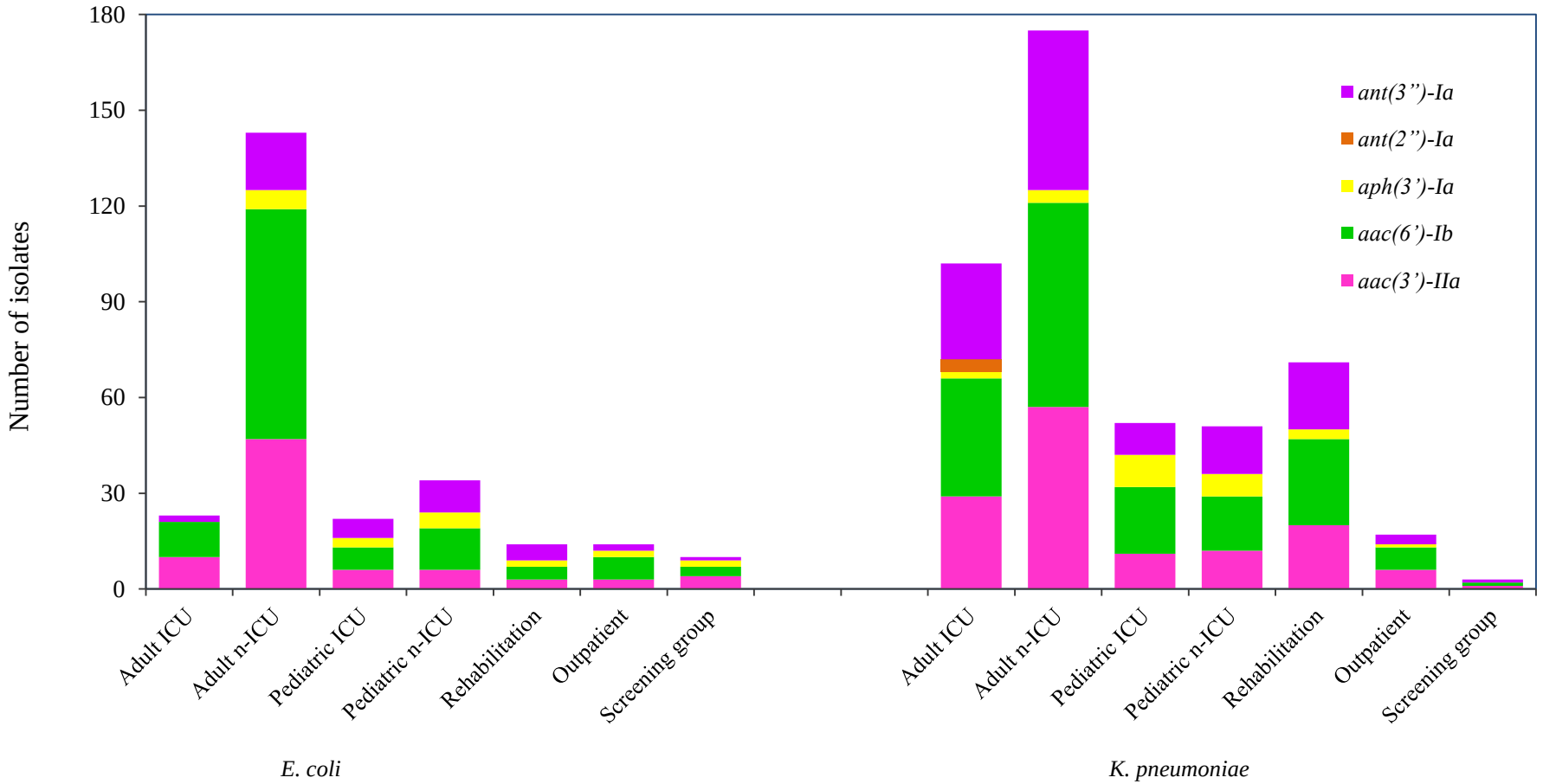


Figure 9. The prevalence of aminoglycoside resistance genes among patients and screened medical students (paper II). ICU: intensive care unit.

5.4. Characterization of integrons

In the first batch, class 1 integrons were found more frequently than class 2 integrons (55.6% and 6.3%, respectively, p<0.001).The carriage rate of class 1 integrons was significantly higher in the *E. coli* from the employment screening group than from the LTC group (68.2%, 15/22 vs. 28.6%, 6/21; p=0.010). The majority of *K. pneumoniae* carried a class 1 integron with a single *ant(3')-1b* gene. Class 2 integrons were found in four of the isolates (three among the LTC group and one in an isolate for employment screening); of these, two *E. coli* isolates harboured both integron types (one isolate was from the LTC group and the other from the employment screening group). Carriage of class 1 integrons in *E. coli* was correlated negatively with carriage of $bla_{\text{CTX-M-15}}$. Seven (Figure 10 and 11) and two different gene cassette arrays (*dfrA1-sat2-ant(3")-Ia* and *sat2-ant(3")-Ia*) were identified amongst the class 1 and 2 integrons, respectively. These arrays did not show any association with the ESBL gene carried or the study group of origin.

Overall carriage rate of class 1 and 2 integrons in the same population group in 2013-14 (third batch), was 40.6% (13/32) and 9.4% (3/32) respectively. Similar to the first batch class 1 integrons were found more frequently than class 2 integrons (p=0.008).Class 1 integrons were detected in four *K. pneumoniae* and three *E. coli* from the LTC group, while in six of *E. coli* isolates from the employment screening group; gene cassette arrays are shown in Figure 10 and 11. Class two integrons were found in two *E. coli* with a uniform gene cassette array of *dfrA1-sat2-ant(3")-Ia* (one from the LTC group and two from the employment screening group).

In the second batch, class 1 and 2 integrons were found in 74.5% (275/369) and 4.1% (15/369) of isolates, respectively. Carriage of class 1 integrons was significantly more frequent in *K. pneumoniae* than in *E. coli* (81.1%, 150/185 vs. 68.7%, 123/179; p=0.006); and in isolates from adults than in isolates from children both in ICU and non-ICU (79.6%

and 85.8% vs. 53.5% and 61.4%, respectively; p=0.018 to p<0.001). Rates of integron carriage were comparable between inpatients and outpatients. A single class 2 integron (*sat2-ant(3")-Ia*) as well as nine different class 1 integrons (Figure 10, 11) were identified, their distribution among the different study groups is shown in Figure 11.

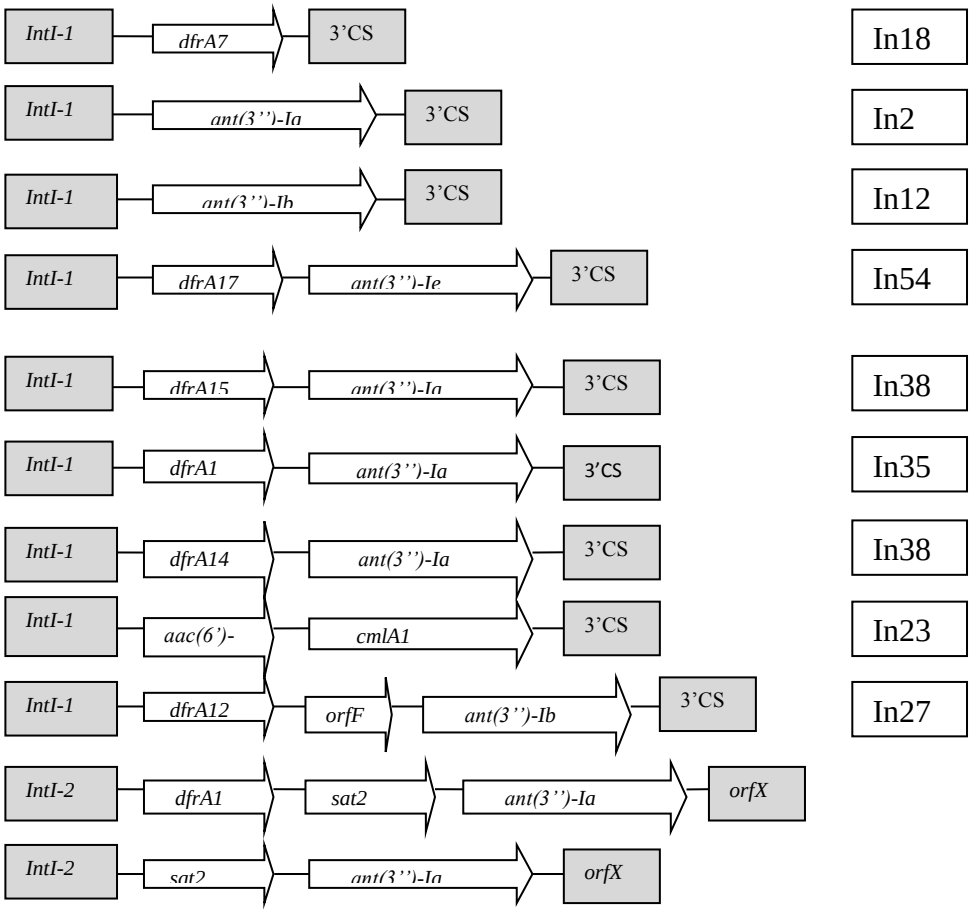


Figure 10. Schematic representation of the various gene cassette arrangements found in class 1 and class 2 integrons. The arrows indicate the different gene cassettes. The grey boxes represent the 3 ′ and 5 ′ conservative segments. Integron numbers for class 1 integrons according to the Integrall database (http://integrall.bio.ua.pt/?nomenclature) are also shown.

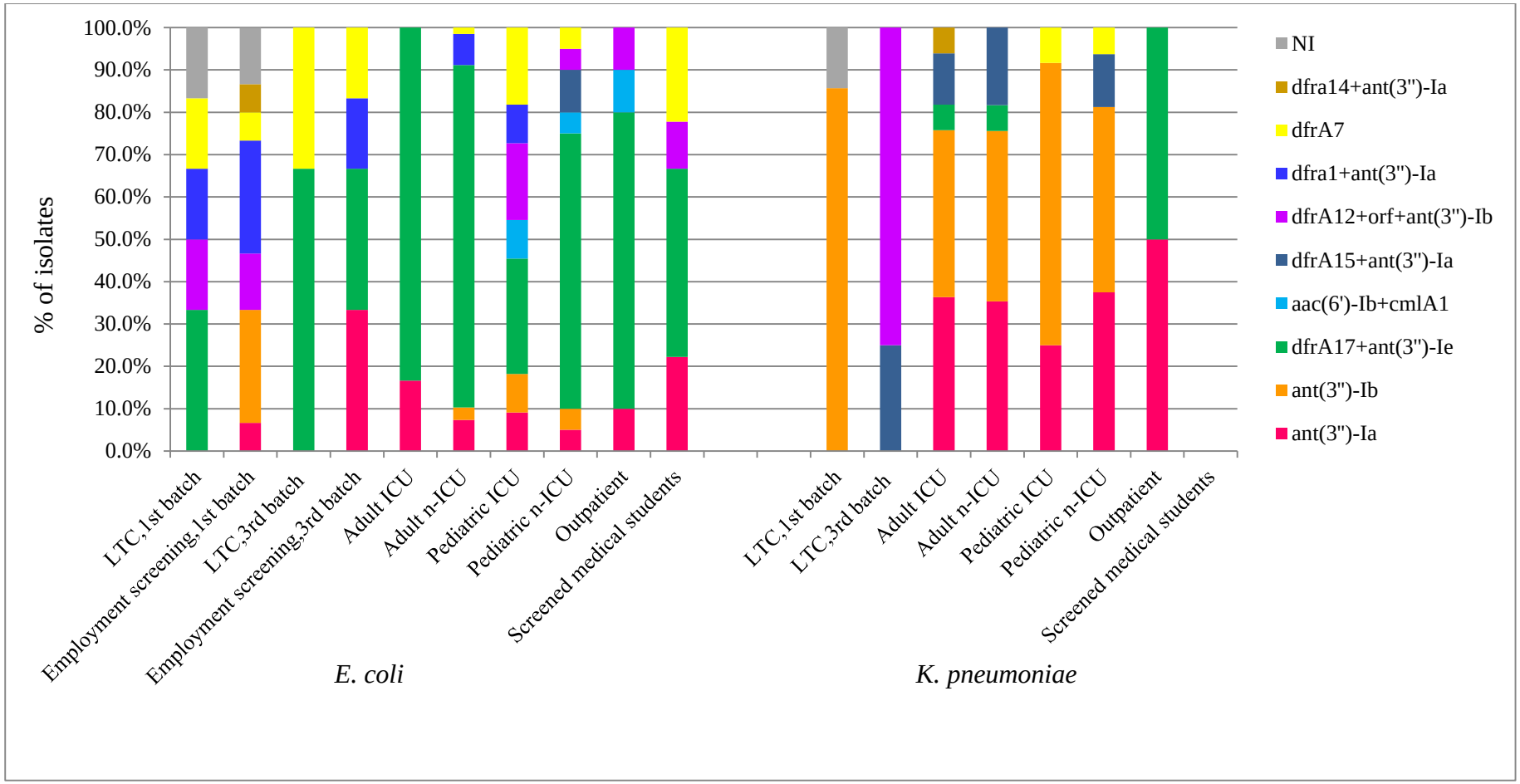


Figure 11. Gene cassette arrays of class I integrons in *E. coli* and *K. pneumoniae* isolated from different study groups (paper I, II and III). LTC: long-term care; ICU: intensive care unit; NI: not identified.

5.5. Phylogrouping of *E. coli* and analysis of virulence genes

Phylogenetic analysis in the first batch showed that the majority of the isolates belonged to group A (46.5%). Group B2, B1 and D were found for 25.6%, 23.3% and 4.6% of the strains, respectively (Figure 12). The commensal *E. coli* phylogroup A was more frequent in the employment screening group (14/22 vs. 6/21; p=0.021), while the pathogenic phylogroup B2 was more frequent in the LTC applicants (9/21 vs. 2/22; p=0.016). All phylogroup B2 isolates belonged to the $bla_{\text{CTX-M-15}}$ producer O25b:ST131 pandemic clone. None of the isolates belonged to the enterovirulent pathogroups. Distribution of the tested extraintestinal virulence genes according to phylogroup is shown in Table 7. Isolates associated with pathogenic phylogroups (group B2 and D) showed a higher prevalence of the genes *kpsMT II* (p=0.002) and *fyuA* (p<0.001) than those from commensal phylogroups (group A and B1). The genes *sfaS*, *cnf1*, *ibeA*, *sfa/focDE* and PAI were completely absent from the commensal isolates. The comparison *E. coli* isolates in two groups yielded similar results, but only the prevalence of PAI was significantly higher among the LTC group (p=0.001). Virulence factor patterns were not linked to the type of ESBL gene carried.

Gene	Number of positive isolates per phylogroup				
	A (n=20)	B1(n=10)	B2 (n=11)	D (n=2)	Total (n=43)
iutA	6	6	8	1	21 (48.8%)
papC	1	2	3	1	7 (16.3%)
kpsMT II	5	1	8	1	15 (34.9%)
fyuA	3	4	10	1	18 (41.9%)
sfaS	0	0	3	0	3 (7.0%)
cvaC	5	6	4	0	15 (34.9%)
traT	9	10	9	1	29 (67.4%)
cnf1	0	0	2	0	2 (4.6%)
ibeA	0	0	3	0	3 (7.0%)
sfa/focDE	0	0	3	0	3 (7.0%)
PAI	0	0	10	1	11 (25.6%)
fimH	15	10	11	2	38 (88.4%)
csgA	19	10	2	1	32 (74.4%)

Table 7. Distribution of virulence genes according to *E. coli* phylogenetic groups (paper I).

Phylogroup distribution for the same population studied in 2013-14 (third batch) is shown in Figure 12. In the employment screening group from the third batch, phylogroup B2 became significantly more dominant than in the first batch (44.4% vs. 9.1%; p=0.025), while in the LTC group, phylogroup D was found more frequently (42.9% vs. 4.8%; p=0.038). All phylogroup B2 isolates in both groups belonged to the $bla_{\text{CTX-M-15}}$ producer O25b:ST131 pandemic clone.

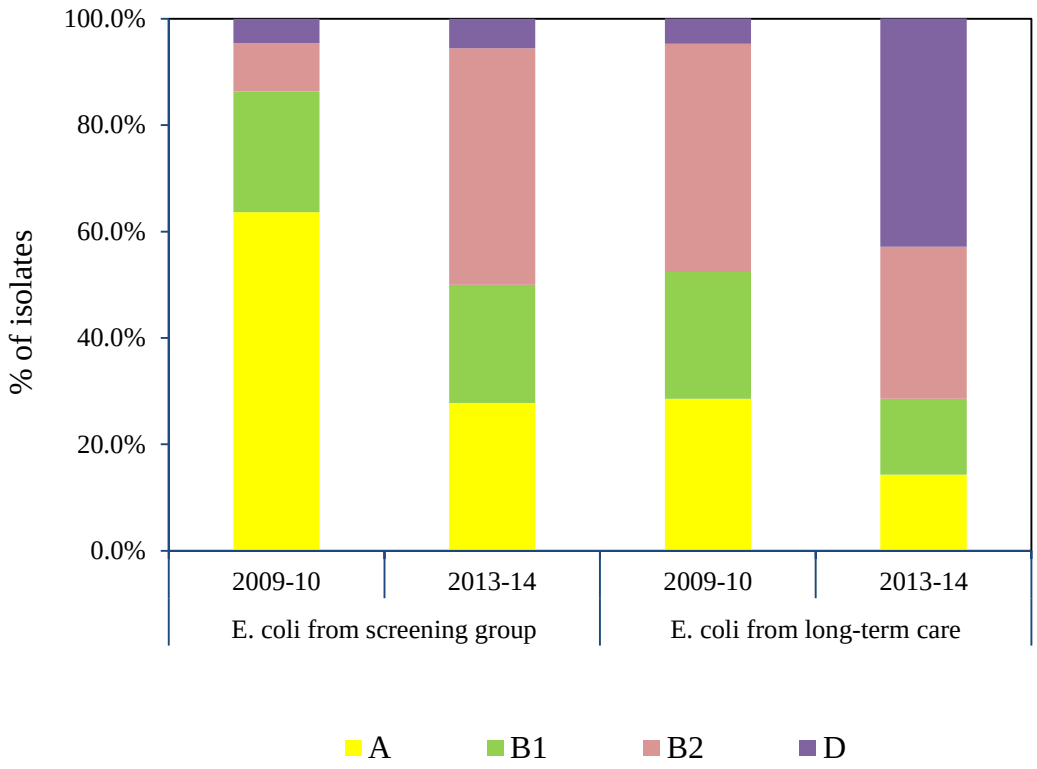


Figure 12. Comparison of the distribution of the phylogroups of *E. coli* isolates in the two groups of individuals in 2009-10 and 2013-14 (paper I and III).

Distribution of *E. coli* isolates among the four phylogroups in the second batch is shown in Table 8. Among inpatients, pathogenic phylogroups B2 and D were more common in adults, ($p<0.001$), while phylogroup B1 and A were more frequently found in children ($p<0.001$). We observed lower proportion of pathogenic phylogroups among pediatric non-ICU as compared to adult non-ICU. Clone ST131 was detected in 95.6.0% (43/45) of the phylogroup B2 isolates harbouring *bla*$_{\text{CTX-M-15}}$. A single phylogroup B1 isolate from pediatric non-ICU, carried an *eae* gene, thus proved to be an enteropathogenic *E. coli*.

	A (n=27)	B1 (n=31)	B2 (n=45)	D (n=76)
Adult ICU	1	3	4	4
Adult non-ICU	3	13	20	45
Pediatric ICU	6	4	3	5
Pediatric non-ICU	7	8	9	10
Rehabilitation	3	0	2	3
Outpatients	6	1	4	5
Screened medical students	1	2	3	4

Table 8. Distribution of *E. coli* isolates among the four phylogenetic groups (paper II). ICU:intensive care unit.

5.6. Genetic diversity (paper I)

The result of ERIC-PCR (Figure 13) and PFGE (Figure 14; D=0.99 and 0.96, respectively) showed that the genetic diversity among *E. coli* isolates was high. One isolate was not typeable with ERIC-PCR but its PFGE analysis was successful. The diversity of *E. coli* between the two groups was comparable both with ERIC-PCR and PFGE analysis (D=0.99 vs. 0.99 and D=0.94 vs. 0.95, respectively). Isolates in the same cluster were not uniform considering ESBL genes and carriage of integrons. In contrast, *K. pneumoniae* isolates were markedly less diverse, both with ERIC-PCR (Figure 15) and PFGE (Figure 16; D=0.58 and 0.31, respectively); a PFGE clone containing 15 of the 18 isolates was detected. Notably, one of the distinct isolates was the carbapenem nonsusceptible isolate.

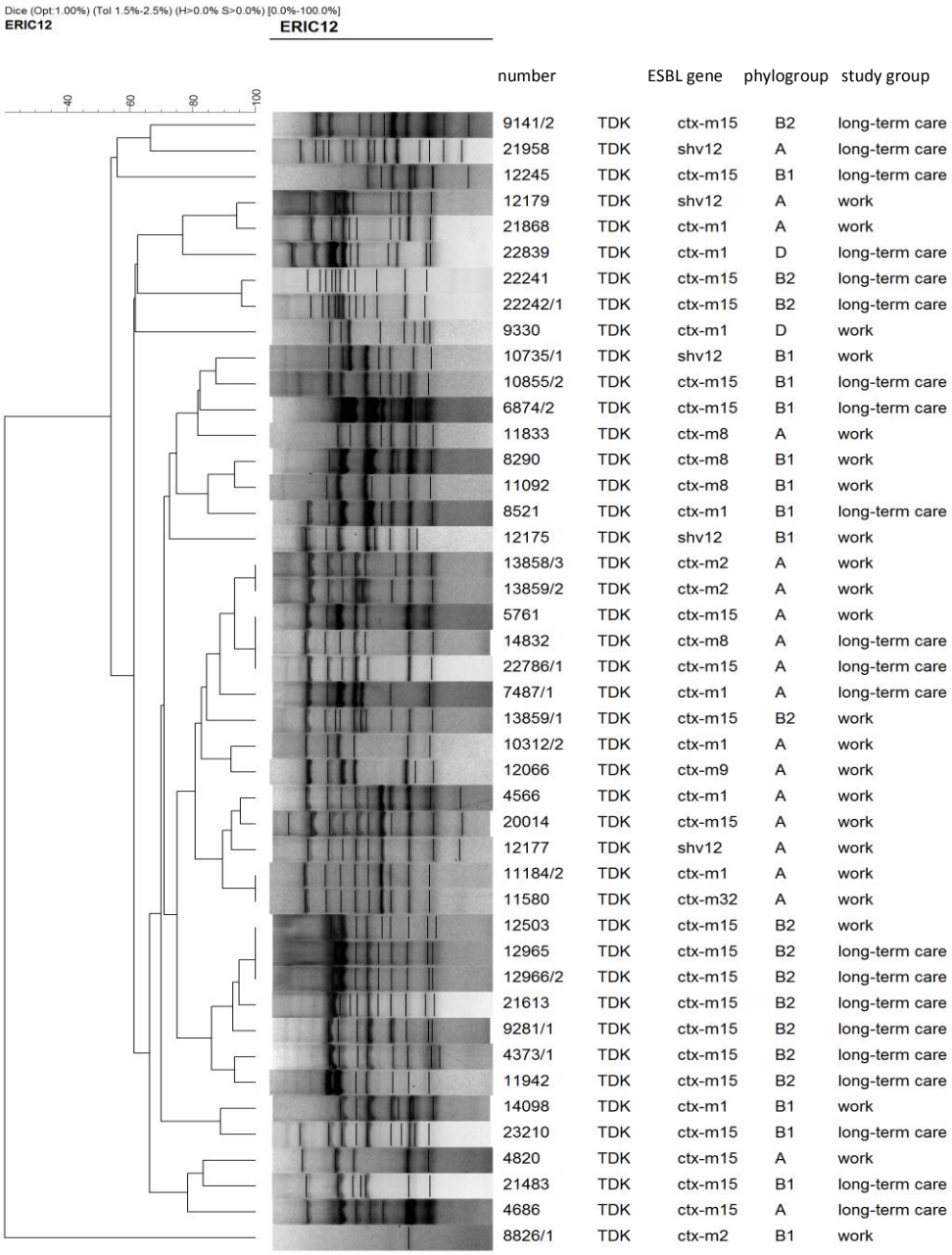


Figure 13. Dendrogram generated from macrorestriction patterns of the *E. coli* isolates (ERIC-PCR).

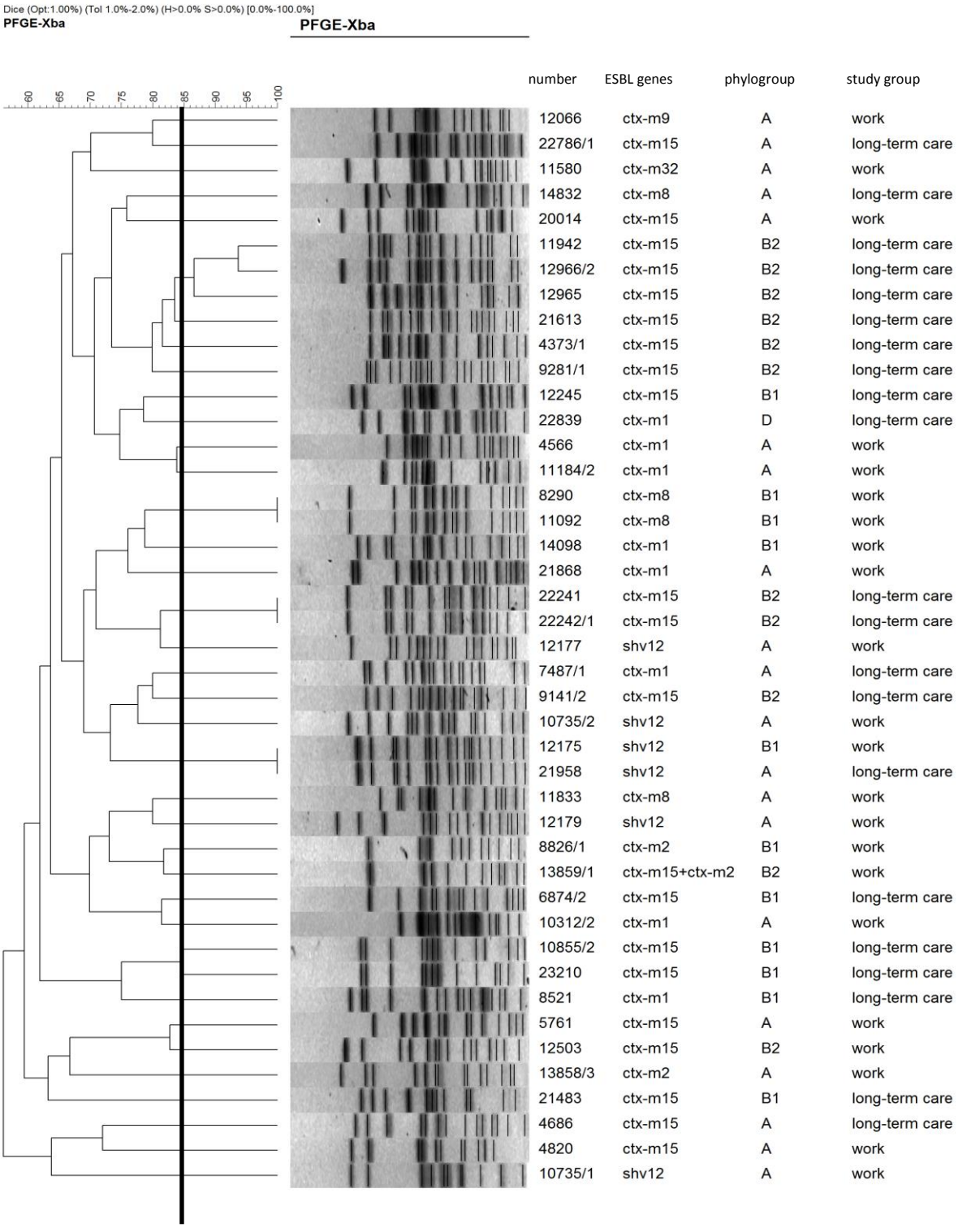


Figure 14. Dendrogram generated from macrorestriction patterns of the *E. coli* isolates (PFGE, paper I).

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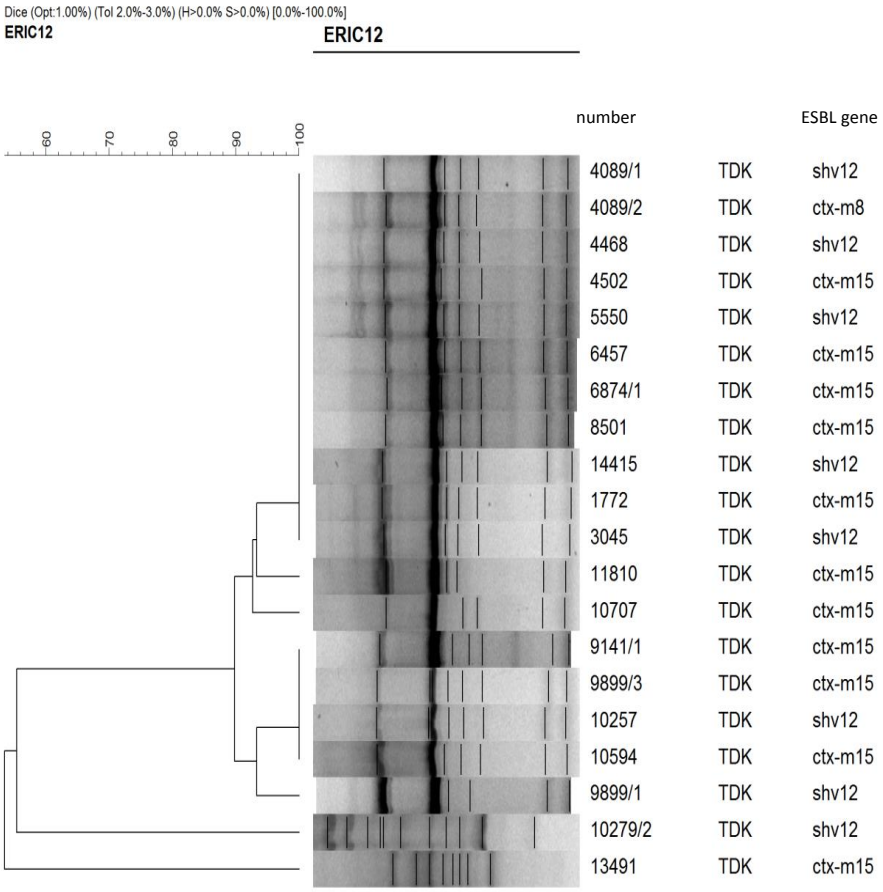

Figure 15. Dendrogram generated from macrorestriction patterns of *K. pneumoniae* isolate (ERIC-PCR).

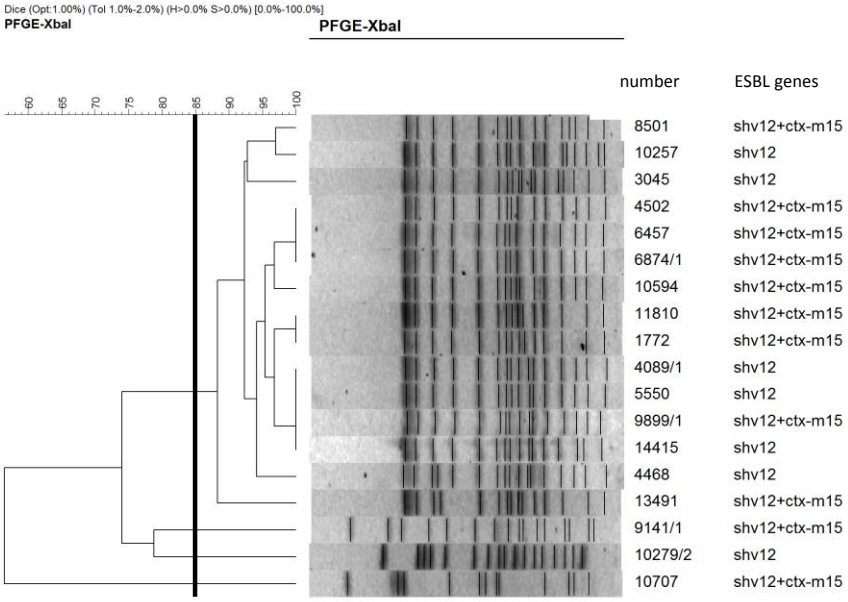

Figure 16. Dendrogram generated from macrorestriction patterns of *K. pneumoniae* isolate (PFGE, paper I).

5.7. Prevalence of ESBL-infected patients and antibiotic consumption (temporal patterns of carriage dynamics, paper II)

Among patients (in the second batch), trend analysis of monthly prevalences revealed two stages within the study period. Between October 2010 and October 2011 carriage rates showed increasing tendency, while during the second part (from November 2011 to February 2013) it oscillated around a level; this applies both to *E. coli* and *K. pneumoniae*. Prevalence in adults showed an increasing trend in both stages, but in pediatric patients the initial rise was followed by a decreasing tendency. An increasing trend was observed initially both in ICUs and non-ICUs, followed by a decrease in the ICUs, while by oscillation around a level in non-ICUs. The proportion of CTX-M producers among *K. pneumoniae* steadily increased throughout the study, but among *E. coli* the initial decrease was followed by an increasing trend both in colonized and infected individuals.

According to time-series models and Granger causality, infected patients are more likely to be sources for carriage than carriers for infections; carriage of *K. pneumoniae* Granger-caused infection only in adults, while carriage of *E. coli* only in children (Table 9).

For *K. pneumoniae*, the effect of infections manifested with longer lags than for *E. coli*; in composite models only the effect of adults and *K. pneumoniae* infections was significant on ESBL carriage. *K. pneumoniae* infections predicted carriage in adults as well as in children, the effect of *E. coli* infections was only significant in adult carriage (Table 10).

Dependent variable(s)	Regressor(s)	lag	ARIMA terms	coefficient	T-statistic	probability	probability of dependent not Granger causing regressor	probability of regressor not Granger causing dependent
Prevalence of infections by ESBL producers	ESBL carriage rate	-2	1,0,3	-0.02±0.02	-1.37	0.184	0.034	0.477
Prevalence of infections by ESBL producing K. pneumoniae	ESBL carriage rate	-3	1,0,3	-0.01±0.02	-0.24	0.811	0.052	0.114
Prevalence of infections by ESBL producing E. coli	ESBL carriage rate	-3	[1,2],0,0	-0.06±0.04	-1.65	0.114	0.420	0.939
ESBL carriage rate	Prevalence of infections by ESBL producers	-2	3,0,3	2.48±0.13	19.06	<0.001	0.477	0.034
ESBL carriage rate	Prevalence of infections by ESBL producing *K. pneumoniae*	-4	0,0,4	3.99±0.08	50.52	<0.001	0.114	0.052

ESBL carriage rate	Prevalence of infections by ESBL producing *E. coli*	-2	1,0,0	7.53±1.60	4.71	<0.001	0.939	0.420
ESBL carriage rate	Prevalence of infections by ESBL producers in adults	-2	0,0,3	2.49±0.16	16.01	<0.001	0.235	0.008
ESBL carriage rate	Prevalence of infections by ESBL producers in children	0; -7	0,0,3	2.60±0.45 ; 0.12±0.30 5	5.75; 3.70	<0.001; 0.002	0.152	0.741
Carriage rate of ESBL-producing *K. pneumoniae*	Prevalence of infections by ESBL-producers	-2	3,0,3	1.48±0.17	8.79	<0.001	0.375	0.062
Carriage rate of ESBL-producing *K. pneumoniae*	Prevalence of infections by ESBL-producer *K. pneumoniae* in adults	-4	1,0,1	2.13±0.29	7.25	<0.001	0.411	0.050
Carriage rate of ESBL-producing *K. pneumoniae*	Prevalence of infections by ESBL-producer *K. pneumoniae* in children	-6; -8	0,0,1	1.40±0.47 ; 1.22±0.45	3.00; 2.74	0.008; 0.014	0.466	0.891

Carriage rate of ESBL-producing *E. coli*	Prevalence of infections by ESBL-producers	-7	0,0,3	1.27±0.50	25.37	<0.001	0.570	0.096
Carriage rate of ESBL-producing *E. coli*	Prevalence of infections by ESBL-producer *E. coli* in adults	-1	0,0,3	3.56±0.13	26.50	<0.001	0.684	0.108
Carriage rate of ESBL-producing *E. coli*	Prevalence of infections by ESBL-producer *E. coli* in children	-2; -4; -5	0,0,3	2.40±0.83 ; 2.63±0.37 ; 2.25±0.72	2.88; 7.13; 3.14	0.009; <0.001; 0.005	0.885	0.015

Table 9. Results of the dynamic regression models with multiple regressors (paper II). Probabilities representing significant differences are shaded.

For *K. pneumoniae*, the effect of infections manifested with longer lags than for *E. coli*; in composite models only the effect of adults and *K. pneumoniae* infections was significant on ESBL carriage. *K. pneumoniae* infections predicted carriage in adults as well as in children, the effect of *E. coli* infections was only significant in adult carriage (Table 10).

Dependent variable(s)	Regressor(s)	Lag	ARIMA terms	coefficient	T-statistic	probability
ESBLcarriage rate	Prevalence of infections by ESBL producing E. coli	-2	0,0,4	0.88±1.26	0.69	0.495
	Prevalence of infections by ESBL producing K. pneumoniae	-4		3.52±0.54	6.46	<0.001
ESBL carriage rate	Prevalence of infections in adults	-3	0,0,3	1.88±0.43	4.35	<0.001
	Prevalence of infections in children	0		0.94±0.68	1.39	0.177
Carriage rate of ESBL-producing K. pneumoniae	Prevalence of infections by ESBL-producer K. pneumoniae in adults	0	0,0,3	1.00±0.15	6.80	<0.001
	Prevalence of infections by ESBL-producer K. pneumoniae in children	0		1.8±0.19	9.31	<0.001
Carriage rate of ESBL-producing E. coli	Prevalence of infections by ESBL-producer E. coli in adults	-1	0,0,3	2.93±0.31	9.42	<0.001
	Prevalence of infections by ESBL-producer E. coli in children	-4		1.08±0.61	1.77	0.091

Table 10. Results of the dynamic regression models with multiple regressors (paper II). Probabilities representing significant differences are shaded.

6. Discussion

ESBL producers rapidly spread worldwide since their discovery in the early 1980s. Several shifts in species and gene distribution occurred since the first discovery of these enzymes. Initially TEM and SHV type ESBLs were dominant enzymes produced mainly by *K.pneumoniae*, then the emergence of pandemic clones of *K. pneumoniae* producing CTX-M enzymes and now the emergence of CTX-M-15-producing *E. coli* is a major concern in both hospital and community settings, however epidemiological studies of ESBL producing bacteria may provide data for the evaluation, management and planning of services for the prevention, control and treatment as well.

There are many reports of asymptomatic carriage of ESBL-producers both in hospital patients (De Champs et al., 1989; Hollander et al., 2001; Schoevaerdts et al., 2012) and healthy individuals (Franiczek et al., 2003; Kader et al., 2007). It is obvious that the prevalence of ESBL-producers differs markedly between countries. The prevalence of faecal carriage in communities in Europe ranges from 0.6% to 11.6% (Woerther et al., 2013). The highest carriage rate (11.6%) observed among patients upon admission to a geriatric unit in Belgium (Schoevaerdts et al., 2012). At the time we began our studies, almost no data available on asymptomatic carriage of ESBLs in Hungary. In our first study (2009-10), we found that the prevalence of ESBL-producers among healthy individuals in the employment screening group was low (2.0%), which was comparable to other European studies conducted on healthy individuals (Geser et al., 2012b; Stromdahl et al., 2011). However, higher prevalence in the LTC group observed (7.2%), and the rate was closer to the prevalences reported in our hospitalized patients (7.4%) and in other studies examining hospitalized asymptomatic carriers (Rodriguez-Bano et al., 2008; Schoevaerdts et al., 2012). The higher rate in the LTC group is likely to reflect the effect of previous hospitalizations since the average age was higher in this group, and their application for long-term care indicates poor health in the

majority of them. This is also supported by the fact that ESBL carriage may last for several months (Birgand et al., 2013; Tham et al., 2012). The carriage rate in the employment screening group most probably corresponds to the actual rate in the community. Most of these individuals work in hospitals, food processing industries and day-care units, where there is a high transmission risk of pathogens. The high proportion of asymptomatic carriers in the community leads to an increase in number of carriers as a consequence of human-to-human transmission of resistant bacteria or through environment (e.g. food) (Geser et al., 2012a; Levin, 2001) and the admission of carriers harbouring resistant bacteria to hospitals increase the risk of nosocomial infection (Bonten et al., 1998; Harris et al., 2004). These colonized individuals may act as a source of ESBLs for clinical strains through horizontal gene transfer, or as direct source of ESBL-producers, consequently may become initiators of outbreak (e.g. a nurse in a hospital ward).

The employment screening group and the LTC group also differed regarding the ESBL-producing species carried as well as regarding the diversity of ESBL genes. We found high diversity of carried ESBL genes in the employment screening group, with a significant number of genes other than $bla_{\text{CTX-M-15}}$, while in both *E. coli* and *K. pneumoniae* isolates from the LTC group, $bla_{\text{CTX-M-15}}$ was dominant in 2009-2010. *K. pneumoniae*, which was absent in the employment screening group, showed higher co-resistance, as expected (Hansen et al., 2012). In *E. coli* from the LTC group co-resistance profile was more similar to that of *K. pneumoniae*, than to *E. coli* from the employment screening group. This was also reflected in distribution of the aminoglycoside modifying enzyme gene patterns with more frequent carriage of *aac(6')-Ib* gene associated with $bla_{\text{CTX-M-15}}$ in the LTC group, but the dominance of *ant(3')-Ia* in *E. coli* isolates from the employment screening group, which is in agreement with other studies which reported the *ant(3')-Ia* as a frequently found gene in the intestinal *E. coli* of healthy individuals and animals as well (Diarra et al., 2007; Gow et al., 2008; Skurnik

et al., 2005). The higher rate of co-resistance and higher frequency of *aac(6')-Ib* in *E. coli* isolates in the LTC group might be related to the dominance of phylogroup B2 (pathogenic phylogroup) which belonged to the *bla*$_{\text{CTX-M-15}}$ producer O25b:ST131 pandemic clone, commonly found as a cause for ESBL-related infection in the hospital as well as in the community. These results suggest the ESBL-producing isolates in the LTC group originate from the hospital microbiota, whereas the colonization of individuals in the employment screening group may also originate from different environmental sources. Besides the exportation of hospital-derived strains either through colonized patients after hospital discharge or through hospital workers, the source of *bla*$_{\text{SHV}}$ may be the horizontal gene transfer from exported *K. pneumoniae* isolates (Coque et al., 2002; Doi et al., 2012; Tellevik et al., 2007), whereas for *bla*$_{\text{CTX-M}}$ other than *bla*$_{\text{CTX-M-15,}}$ livestock and animal-derived food (Geser et al., 2012a; Hille et al., 2014; Toth et al., 2013), companion animals (Carattoli et al., 2005; Wieler et al., 2011), wild animals (Bonnedahl et al., 2009; Literak et al., 2010), surface water (Tacao et al., 2014), drinking water (De Boeck et al., 2012) are also potential sources, either by direct transfer of the resistant bacteria or by the horizontal transfer of resistance plasmids to human commensal *E. coli*. The assumption of diverse environmental sources for colonization with ESBL-producers in the employment screening group is supported by high frequency of *bla*$_{\text{CTX-M-1}}$ genes which is in accordance with high frequency of the same gene in *E. coli* isolates of animal origin in Hungary (Toth et al., 2013), in *E. coli* isolates of water birds in Poland (Literak et al., 2010) and in samples of food producing animals and raw milk in Switzerland (Geser et al., 2012a). Similarly, frequent occurrence of *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-14}}$ (belonging to *bla*$_{\text{CTX-M-9-group}}$) and *bla*$_{\text{CTX-M-32}}$ in *E. coli* isolates of food animals (Bortolaia et al., 2011; Ferreira et al., 2014; Liao et al., 2015; Toth et al., 2013), points to a possible zoonotic dissemination of certain ESBL-producing *E. coli* strains (Bonnedahl et al., 2009; Overdevest et al., 2011).

We hypothesized different sources of colonization with ESBL-producers in case of healthy individuals screened for employment purposes and for the LTC group in 2009-10. This hypothesis was also supported by the result of outpatients and screened medical students in our other study conducted in 2010-13. Carriage rates in these groups were comparable with the rate reported among healthy individuals.

The prevalence of faecal carriage of ESBL producers in the employment screening remained the same (2.0% vs. 2.3%) in the study repeated in 2013-14 in same population. However, in the LTC group we observed a slightly lower prevalence rate than our previous study (7.2% vs. 5.3%, statistically comparable). Though the change in the prevalence is small, significant differences were found in the gene distribution in the employment screening group. The high gene diversity in the employment screening group in 2009-10 disappeared in 2013-14 and all isolates were CTX-M producers, with a marked dominance of $bla_{\text{CTX-M-15}}$ in both groups. In the employment screening group the prevalence of $bla_{\text{CTX-M-15}}$ increased significantly, and almost all other $bla_{\text{CTX-M}}$ genes disappeared.

This change was paralleled by the high frequency of ciprofloxacin and amikacin resistance, and high carriage of *aac(6')-Ib* and *aac(3')-IIa*, similar to other studies where this co-resistance and these genes are commonly associated with $bla_{\text{CTX-M-15}}$ gene (Hansen et al., 2012; Pitout et al., 2004a). These results point to the emergence of $bla_{\text{CTX-15}}$ producing *E. coli* in asymptomatic individuals (i.e. individuals in the employment screening group), this suggests that ESBL-producers which were characterized by different colonization sources are being replaced by highly successful $bla_{\text{CTX-M-15}}$ producing strains. A possible explanation for this shift is that there is a high frequency of phylogroup B2 in the employment screening group and is in parallel with the dominance of $bla_{\text{CTX-M-15}}$ in hospitalized patients in 2010-13 both in *E. coli* and *K. pneumoniae* which points to an exportation of pathogenic ST131 clone carrying $bla_{\text{CTX-M-15}}$ from hospital to the community and its spread within the community.

This epidemiological shift closely mimics the change which took place in the UK a decade earlier (Woodford et al., 2004).

The total prevalence of faecal carriage of ESBL-producing bacteria in the different inpatient populations was comparable to other European studies (Miro et al., 2005; Woerther et al., 2013) and close to that of the LTC group of the other two batches. However, adult inpatients in 2010-13 showed significantly higher prevalence than applicants for LTC in both consecutive studies, and this may suggest that a proportion of colonization may be transient. There are few studies regarding the duration of faecal carriage of ESBL-producers, and based on these findings, some people are transiently colonized, while others may remain colonized from months to years (Alsterlund et al., 2012; Apisarnthanarak et al., 2008; Tham et al., 2012). These carriers may be a source of exportation of ESBLs from one ward to other wards or from the hospital to other hospitals through patient exchange as well as to the community with patients discharged and may also spread further within households (Valverde et al., 2008; Woerther et al., 2013). Among outpatients and screened medical students the prevalences were comparable to the employment screening groups, reflecting the less probable hospitalization history among outpatients and students (Geser et al., 2012b; Stromdahl et al., 2011). Higher prevalence of ESBL-producers was found in non-Hungarian students than in Hungarian students, who were mainly from African and Asian countries, which was in agreement with the findings from Asia and Africa (Luvsansharav et al., 2012; Tian et al., 2008; Woerther et al., 2013; Yoo et al., 2010). This again points out that differences must exist between colonization risks in different geographical regions.

Among inpatient groups the prevalences were highly different. Highest prevalence was found in the rehabilitation wards (27.1%), which was close to the studies conducted on patients in long-term care facilities (Lim et al., 2014; Tinelli et al., 2012; Yoo et al., 2010). As expected the prevalence in ICU patients was higher than in non-ICU patients. Among adults, carriers

were more frequent than among children, even children in ICU wards showed lower prevalence than adults in non-ICUs. This higher prevalence rate among patients in ICUs is not surprising, as patients in ICUs are likely to have more risk factors (e.g. higher use of invasive devices such as vascular and urinary catheters and broad-spectrum antimicrobials), therefore there is a higher chance of transmission of resistant bacteria among patients (Sader et al., 2014), and these reflecting that the risk of infection among patients in ICU wards is higher (Benner et al., 2014; Sader et al., 2014). The lower prevalence in children either in ICU or non-ICU wards may be explained by their lower age and lower probable frequency of previous hospitalization.

Characterization of the ESBL genes revealed different distribution of ESBL genes among inpatients especially between adult and pediatric patients. The differences in the distribution of ESBL genes among adults and children may be explained by different probable source of colonization. Most of the ESBL carriers from pediatrics represent patterns (higher diversity of ESBL genes and higher number of *E. coli*) similar to employment screening group in 2009-10, and they are more likely to be imported to the hospital rather than acquired in a hospital-based manner. It is a conceivable hypothesis that the ESBL producing isolates were already present in intestinal flora of children before admission into hospital and there is lower chance that they are nosocomially acquired than in case of adults. Childhood curiosity and occasional neglect of avoidance of dirt may be the cause that a proportion of ESBL producers carried by children seems to be linked to environmental sources. In adults most carriers seemed to be of hospital-based colonization, similar to ESBL colonization among the LTC group in 2009-10 and in 2013-14 (higher proportion *K. pneumoniae*, higher frequency of $bla_{\text{CTX-M-15}}$ and lower diversity of ESBL genes).

This was also supported by time-series analysis where infections Granger-caused colonization in case of adults but not in case of children. This assumption is supported by low

frequency of pathogenic phylogroups among *E. coli* from pediatric non-ICU in comparison with adult non-ICU, as was the case for healthy individuals in 2009-10. ESBL-producing *K. pneumoniae* are usually clonal and show higher co-resistance rate (Damjanova et al., 2008; Webster et al., 2011). *K. pneumoniae* is a typical nosocomial pathogen and colonization with these bacteria is a hospital-based acquisition. Based on time-series analysis, carriage rate of ESBL-producing *K. pneumoniae* is influenced more strongly by infection rates and the effect of infections manifested with longer time lags than in case of *E. coli*. Time-series analysis also demonstrated that adults carriage of ESBL-producers seems to be the consequence of infection, especially in ICUs (where *K. pneumoniae* were more frequently found). We found higher rate of integron carriage in *K. pneumoniae* isolates from adult patients which indicates that these integrons are widely present in ESBL-producers and may be considered as an important factor in development of multidrug resistant strains. Regarding species, *K. pneumoniae* carried integrons more frequently than *E. coli*, which is in parallel with a higher co-resistance rate and higher frequency of two aminoglycoside modifying enzyme genes, *aac(3')-IIa* and *aac(6')-Ib* in *K. pneumoniae*,. Beside this, carbapenem resistance and *rmtA* gene were only detected in *K. pneumoniae*.

These differences may suggest the different epidemiology of ESBL carriage not only in two species, but among adults and pediatric patients. In some studies it has been shown that the use of third generation cefalosporins and fluoroquinolones increases the infection incidence with ESBL-producers (Aldeyab et al., 2012; Kang et al., 2012; Toth et al., 2014); however, in our study the effect of antibiotic consumption was very limited on either colonization or infection with ESBL-producers. The reason for this may originate from the intensive usage of carbapenems and consequent deacresed prevalence of infections due to ESBL producers, in the period of our study. It is a tempting hypothesis that infection and consequent colonization driven by antibiotic consumption earlier is now may be replaced by a relatively stable

endemic-like presence of a few established strains. This would explain why this study did not demonstrate a strong link between antibiotic consumption and prevalence of either infections and colonization with ESBL producers.

In conslusion, a clear difference between dynamics of community-based and hospital-based colonization was found, as suggested by differences in carrier species, ESBL gene diversity and co-resistance patterns. The basis of carriage in the community seems to be exportation from the hospital, which is characterized by establishment of hospital-derived clones in the healthy carriers and replacement of the diverse environment-derived strains. Colonization of children seems to derive more from environmental sources, emphasizing the importance of community transmission from family members or from healthy individuals in daycare or in food processing especially in their case.

7. Summary

In summary, our results denote the importance of the intestinal tract as a reservoir for ESBL-producers both in the community and in the hospital settings. Besides being able to serve as outbreak sources directly, they may also act as resistance gene reservoirs. The study draws attention to the different epidemiology of colonization with ESBL-producers in both hospital and community. Sources of colonization in the employment screening group in 2009-10, outpatients, screened medical students and pediatric patients in 2010-13, seem to involve environment-based besides nosocomial sources. In contrast, it was shown that colonization precedes infection in case of adults and they are more likely to acquire colonizer strains in the hospital. Intestinal colonization by these organisms among pediatric patients, can be a source for influx of ESBL determinants into the hospital settings and can represent a risk factor for colonization as well as infection of other hospitalized patients and health workers. We found a marked decrease of diversity in ESBL genes among the employment screening group in 2013-14. This suggests that ESBL-producers originating from different sources in 2009-10, have been replaced by highly successful *bla*$_{\text{CTX-M-15}}$ producing *E. coli*. This was in line with our observation of the increase in the importance of *bla*$_{\text{CTX-M-15}}$ gene in both *E. coli* and *K. pneumoniae* in 2010-13. The reason might be related to the exportation of *bla*$_{\text{CTX-M-15}}$ producing *E. coli*, with majority belonged to phylogroup B2, from hospital to the community, and consequently further spread within the community.

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9. Keywords

Extended spectrum beta-lactamase, *E. coli*, *K. pneumonia*e, asymptomatic carriage, integron, faecal carriage, time-series analysis

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11. Appendices

UNIVERSITY OF DEBRECEN
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Candidate: Fatemeh Ebrahimi
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List of publications related to the dissertation

1. **Ebrahimi, F.**, Mózes, J., Mészáros, J., Juhász, Á., Majoros, L., Szarka, K., Kardos, G.: Asymptomatic faecal carriage of ESBL producing Enterobacteriaceae in Hungarian healthy individuals and in long-term care applicants: A shift towards CTX-M producers in the community.
Infect. Dis. "Accepted by Publisher" p. 1-12, p. 2016.
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IF:1.495 (2014)*

2. **Ebrahimi, F.**, Mózes, J., Mészáros, J., Juhász, Á., Kardos, G.: Carriage Rates and Characteristics of Enterobacteriaceae Producing Extended-Spectrum Beta-Lactamases in Healthy Individuals: Comparison of Applicants for Long-Term Care and Individuals Screened for Employment Purposes.
Chemotherapy. 60 (4), 239-249, 2015.
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List of other publications

3. Mózes, J., **Ebrahimi, F.**, Gorácz, O., Miszti, C., Kardos, G.: Effect of carbapenem consumption patterns on the molecular epidemiology and carbapenem resistance of Acinetobacter baumannii.
J. Med. Microbiol. 63, 1654-1662, 2014.
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IF:2.248

4. Mózes, J., Szűcs, I., Molnár, D., Jakab, P., **Ebrahimi, F.**, Szilasi, M., Majoros, L., Orosi, P.,Kardos, G.: A potential role of aminoglycoside resistance in endemic occurrence of Pseudomonas aeruginosa strains in lower airways of mechanically ventilated patients.
Diagn. Microbiol. Infect. Dis. 78 (1), 79-84, 2014.
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Total IF of journals (all publications): 7,488
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Other paper related to the dissertation

Ebrahimi F, Mózes J, Gorácz O, Fésűs A, Majoros L, Szarka K, Kardos G. Comparison of the faecal colonization rates with extended spectrum beta-lactamase producing enterobacteria among patients in different wards, outpatients and screened medical students. Microbiol Immunol (submitted, referees asked for a minor revision).

Conferences

Ebrahimi F, Mózes J, Kardos G. Changes in the distribution of asymptomatically carried ESBL-producing enterobacteria and their ESBL genes among healthy individuals. Hungarian Society for Microbiology, Budapest, Hungary (July 8-10, 2015).

Ebrahimi F, Mózes J, Kardos G. Prevalence and integron carriage in ESBL producers isolated from faecal samples of inpatients and outpatients. Hungarian Society for Microbiology, Keszthely, Hungary (October 15-17, 2014).

Ebrahimi F, Mózes J, Kardos G. Characterisation of ESBL-producing enterobacter in stool samples of individuals screened for enteric pathogens. 4th Central European Forum for Microbiology, Keszthely, Hungary (Oct 16-18, 2013).

Ebrahimi F, Mózes J, Kardos G. Prevalence of ESBL-producing enterobacteria in stool samples of asymptomatic individuals. Spring Wind Conference, Sopron, Hungary (May 31-June 2, 2013).

Ebrahimi F, Mózes J, Kardos G. Prevalence of ESBL-producing enterobacteria in patients with diarrhoea. European Medical Students' Conference, Debrecen, Hungary (Oct 19-22, 2012).