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Detection of plasmid families carrying ESBL genes in clinical and environmental *E. coli* and *K. pneumoniae* isolates

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Populärvetenskaplig sammanfattning

Extended Spectrum β-Lactamases (ESBLs) är den vanligaste och snabbast ökande typen av multiresistens. ESBLs är enzymer som bryter ner β-laktamantibiotika. Det är ett allvarligt problem då β-laktamantibiotika är en av världens mest använda grupper av antibiotika.

ESBLs produceras av bakteriefamiljen *Enterobacteriaceae*. Bakterierna i familjen förekommer i miljön men några arter är också några av våra vanligast förekommande tarmbakterier, som *E. coli* och *K. pneumoniae*. Dessa bakterier orsakar oftast inte infektion i tarmen, men när de hamnar utanför tarmen i urin eller blod kan de orsaka infektioner som urinvägsinfektion och blodförgiftning. Dessa infektioner kan bli allvarliga om bakterierna är svårbehandlade på grund av ESBL-multiresistens.

ESBLs sprids snabbt eftersom generna som kodar för ESBLs (s.k. *bla*-gener) ligger på plasmider. En bakterie kan ha hundratals plasmidkopior och kan snabbt sprida dessa plasmider till andra bakterier. Detta beror på att plasmider kopierar sig självständigt med en kopierande del av plasmiden som kallas replikon. Plasmider med samma replikonvariant tillhör samma plasmidfamilj.

Syftet med detta examensarbete var att undersöka plasmidfamiljer som bär olika typer av ESBL-gener i *E. coli* och *K. pneumoniae* som isolerats från patientprov och miljöprov. Vidare undersöktes vilka plasmidfamiljer som var vanligast generellt och för varje typ av ESBL.

Patientisolaten hade samlats in sedan tidigare och kom från patienter som bor i närheten av Kristianstad. Miljöisolaten kom från vattenprov som också hade samlats in tidigare från Helge Å i Kristianstad. Från bakterieisolaten utvanns bakteriernas plasmider. Metoden multiplex PCR (Polymerase Chain Reaction) användes för att detektera plasmidernas replikonvariant. Replikonen representerade de 27 plasmidfamiljerna i *Enterobacteriaceae* samt 3 nya replikon som inte delats in i plasmidfamiljer än. PCR-metoden kopierade upp replikonen till det antal som behövdes för att kunna detektera dem. Eftersom olika replikon har olika storlekar efter PCR identifierades vilket replikon det var och därmed vilken plasmidfamilj plasmiden med replikonet tillhörde.

Plasmidfamilj F var den vanligaste i båda arter isolerade från patientprov och miljöprov. Andra plasmidfamiljer som tidigare visats vara vanliga förekom knappt eller inte alls i denna studie. Det kan bero på att plasmidfamiljerna tidigare visats vanliga i

andra arter i bakteriefamiljen *Enterobacteriaceae* eller i bakterier som producerar andra typer av ESBL än de som undersöktes i detta examensarbete.

Plasmider i framför allt plasmidfamilj F bar gener för flera typer av ESBL på samma plasmid, vilket har visats i tidigare studier. Tidigare studier har kunnat förknippa vissa typer av ESBL med vissa plasmidfamiljer, men i detta arbete var det generellt svårt att förknippa en viss typ av ESBL med en viss plasmidfamilj, eftersom de flesta bakterier som undersöktes producerade flera typer av ESBL och hade flera plasmidfamiljer.

Slutligen måste plasmider som bär ESBLs fortsätta studeras för att spridningen ska kunna hindras, framför allt inom sjukvården på grund av de allvarliga konsekvenserna ESBLs kan medföra vid bakterieinfektioner.

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Sammanfattning
Extended Spectrum β-Lactamases (ESBLs) produceras av bakteriefamiljen *Enterobacteriaceae*, främst av *E. coli* och *K. pneumoniae*. Eftersom dessa arter är bland de vanligaste orsakerna till urinvägsinfektioner och sepsis är ESBL-produktion ett allvarligt problem.

ESBL är också oroande eftersom det sprids epidemiskt. Detta möjliggörs av att generna som kodar för ESBLs (s.k. *bla*-gener) ligger på plasmider, som replikerar och sprider de replikerade plasmidkopiorna självständigt. Plasmider replikeras som s.k. replikon. Plasmider med samma replikonvariant tillhör samma plasmidfamilj.

Syftet med detta arbete var att detektera plasmidfamiljer som bär *bla*-gener i *E. coli* och *K. pneumoniae* isolerade från kliniska prov (n = 6) och miljöprov (n = 22) från Helge Å. Plasmidfamiljernas prevalens undersöktes, liksom sambandet mellan plasmidfamiljer och *bla*-gener.

Plasmidfamiljerna detekterades med ett PBRT-kit (PCR Based Replicon Typing), ett multiplext PCR-kit som detekterade 30 replikon varav 27 replikon som representerar de 27 plasmidfamiljer som finns i *Enterobacteriaceae* och tre nya replikon.

Plasmidfamiljen IncF var vanligast förekommande i båda arter i både kliniska isolat och miljöisolat. IncF verkade förekomma för alla undersökta typer av ESBL, men det var generellt svårt att förknippa en *bla*-gen med en plasmidfamilj, eftersom de flesta isolaten bar flera *bla*-gener och flera plasmidfamiljer.

Abstract
Extended Spectrum β-Lactamases (ESBLs) are produced by the *Enterobacteriaceae* bacterial family, mainly by *E. coli* and *K. pneumoniae*. As these species are some of the main causes of urinary tract infections and sepsis, ESBL-production is of major concern.

Occurrence of ESBLs also gives rise to concern as it is increasing epidemically. This because the genes coding for ESBLs (i.e. *bla*-genes) are located on plasmids replicating and spreading the replicated copies independently. Plasmids replicate by replicons. Plasmids with the same replicon variant are grouped into the same plasmid family.

The aim of this study was to detect plasmid families carrying *bla*-genes in *E. coli* and *K. pneumoniae* from clinical (n = 6) and environmental water (n = 22) isolates. Plasmid family prevalence was examined. Association between plasmid families and *bla*-genes was also examined.

Plasmid families were detected by a PBRT kit (PCR Based Replicon Typing), a multiplex PCR kit that detected 30 replicons, whereof 27 replicons representing the 27 plasmid families in *Enterobacteriaceae*, and three novel replicons.

The IncF plasmid family was the most prevalent for both species in both clinical and environmental isolates. IncF seemed to be prevalent for all examined ESBLs, but it was difficult to associate one *bla*-gene with one plasmid family as most isolates carried several *bla*-genes and several plasmid families.

Ämnesord/Keywords
β-lactamases, ESBLs, *bla*-genes, Plasmid families, Incompatibility groups, Replicon typing, *E. coli*, *K. pneumoniae*

Contents

1. Background

The largest bacterial family is *Enterobacteriaceae*, which contains hundreds of species. Bacteria in *Enterobacteriaceae* are Gram-negative rods that can be found in the environment but also commonly in the mammalian gut, including human. The bacteria in the human gut microbiome are normally non-pathogenic, but they can cause opportunistic infections if they end up outside the gut - in wounds or supposedly sterile fluids like urine and blood. This makes *Enterobacteriaceae* species in the normal gut microbiome, especially *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* (*K. pneumoniae*), some of the main causes of urinary tract infections (UTIs) and sepsis (Public Health Agency of Sweden (PHAS), 2014).

Lately, *E. coli*, *K. pneumoniae* and other clinically important *Enterobacteriaceae* species have acquired Extended Spectrum β-Lactamases (ESBLs), mediating resistance against β-lactam antibiotics - one of the most used class of antibiotics globally. This, in combination with ESBLs being the fastest increasing kind of antibiotic resistance, makes ESBLs of major concern (PHAS, 2014).

1.1. β-lactam antibiotics

β-lactam antibiotics (i.e. penicillins, cephalosporins, carbapenems and monobactams) are bactericidal antibiotics that lyse the bacterial cell wall. All include a β-lactam ring structure in their molecules, which is similar with a structure in the peptidoglycan building blocks of the cell wall (Figure 1). During cell wall synthesis, the peptidoglycans are cross-linked with peptide bonds by enzymes called Penicillin Binding Proteins (PBPs). When β-lactam antibiotics are present, PBPs bind to the antibiotic instead of the peptidoglycans. This incident inactivates the PBP enzymes and thereby further cell wall synthesis. Furthermore, this inactivation leads to lysis of the cell wall, hence death of the bacterial cell (Drawz & Bonomo, 2010).

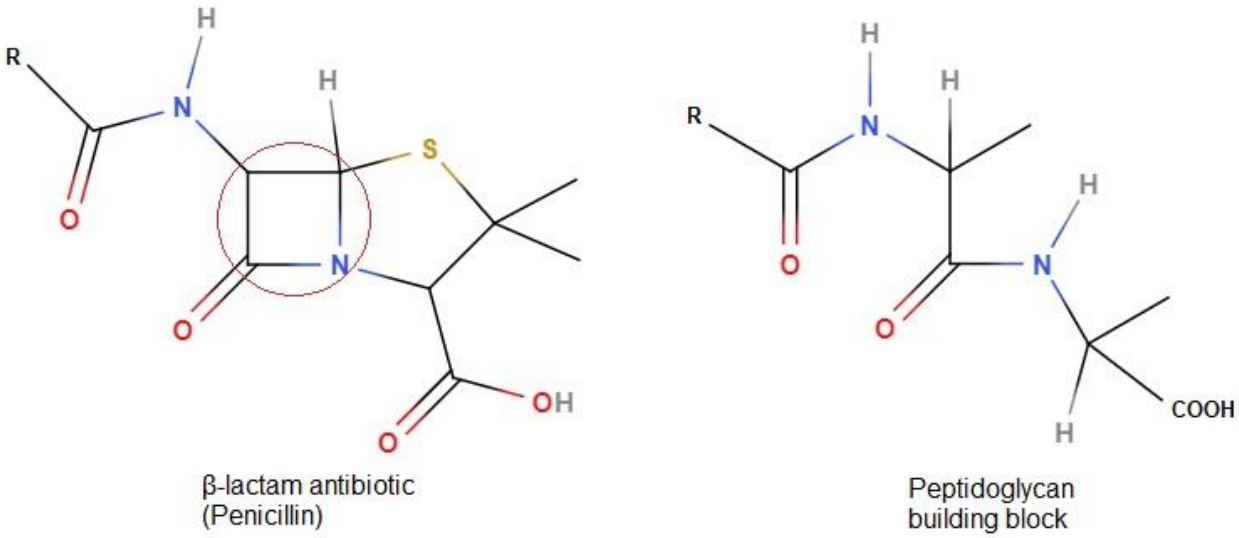

Figure 1. The figure, drawn by the author, shows the resemblance between the structure of β-lactam antibiotics and the peptidoglycan building blocks in bacterial cell wall synthesis. The β-lactam ring is marked with a circle.

1.2. β-lactamases

Bacteria can acquire β-lactam antibiotic resistance in various ways, but bacteria in *Enterobacteriaceae* usually acquire it by producing β-lactamases. β-lactamases are enzymes that use the β-lactam ring as a substrate. The interaction results in disruption of the β-lactam ring, which inhibits the β-lactam antibiotic (Figure 2). In this way, bacteria expressing the β-lactamase become resistant against β-lactam antibiotics (Drawz & Bonomo, 2010).

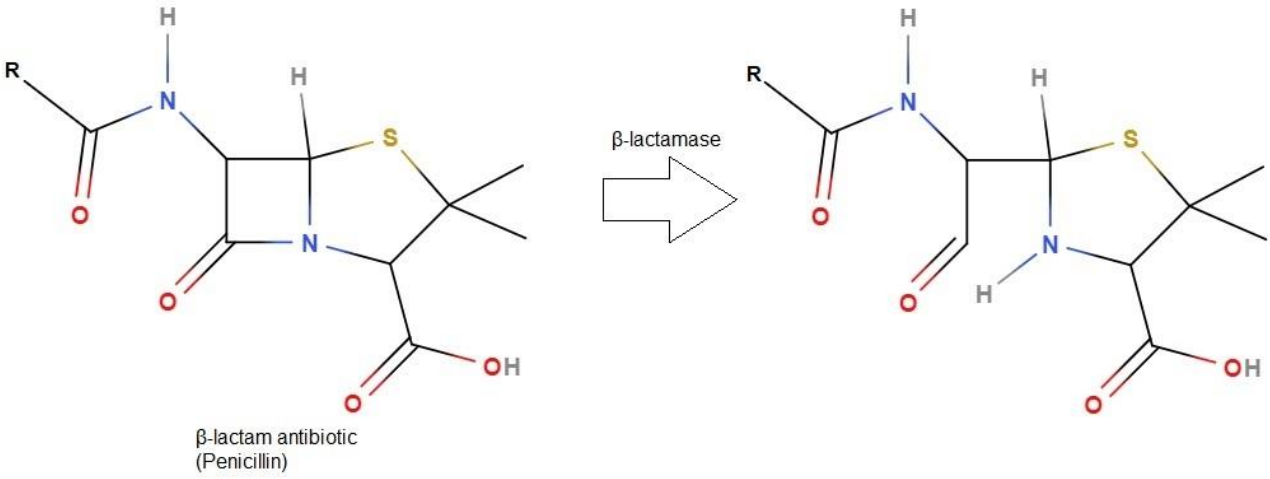

Figure 2. The figure, drawn by the author, shows how β-lactamases disrupt the β-lactam ring.

β-lactamases originated in the environment as a natural defence against naturally occurring β-lactam antibiotics in the same environment. From these original genes a large variety has evolved due to point mutations There are hundreds of β-lactamases and expressing one β-lactamase does not necessarily make the bacteria resistant against all β-lactam antibiotics. Some β-lactamases, for example penicillinases, cephalosporinases and carbapenemases, make the bacteria resistant against the corresponding kind of β-lactam antibiotic, while other, more recently found β-lactamases can make the bacteria resistant against several or all β-lactam antibiotics. These β-lactamases are ESBLs (Bush, 2018).

1.3. ESBLs

ESBLs emerged because extended spectrum cephalosporins were developed to treat infections caused by bacteria expressing the earlier β-lactamases. At first, this generation of extended spectrum cephalosporin antibiotics was an effective treatment against pathogenic β-lactamase producing bacteria, but because these antibiotics were widely used, a selection pressure occurred (Drawz & Bonomo, 2010).

Some ESBLs occurred in pathogenic bacteria because of point mutations in the earlier β-lactamase genes. This caused the occurrence of TEM ESBLs, originating from the *E. coli* TEM-1 β-lactamase, and SHV ESBLs, originating from the *K. pneumoniae* SHV-1 β-lactamase. Other ESBLs had already occurred as a natural defence in non-pathogenic bacteria, but the ESBL genes (i.e. *bla*-genes) were transferred from non-pathogenic bacteria to pathogenic. This caused the occurrence of CTX-M ESBLs, originally found in *Klyuviera spp*. (Bradford, 2001; Drawz & Bonomo, 2010).

Today, ESBL-production is the most common and fastest increasing mechanism for antibiotic resistance, with ESBLs being most commonly produced by *E. coli* followed by *K. pneumoniae* and with CTX-Ms being the most common ESBLs in both clinical and environmental isolates, increasing epidemically over SHV and TEM ESBLs (Couque et al., 2008; Swedres-Svarm, 2017). In Sweden as well as other European countries, CTX-Ms are prevalent in healthy and sick humans, wild animals, food producing animals, food and environments, especially environmental waters and wastewater before and after treatment in water treatment plants (WWTPs) (Coque et al., 2008a; Egervärn et al., 2013; Swedres-Svarm, 2017). The water coming out of WWTPs contains both antibiotics and antibiotic resistant bacteria from households, hospitals, farms and industries, and as these

bacteria mix with environmental bacteria in presence of antibiotics, selection pressure favours antibiotic resistance. This environment is thus favourable for spreading ESBLs (Lupo et al., 2012).

1.3.1. ESBL classification

ESBLs were initially classified by two main classification systems: The Ambler's system from 1969 classifying ESBLs by structure, and the Bush-Jacoby-Mederios' system from 1995 classifying ESBLs by function. Nowadays in Sweden, ESBLs are clinically classified by a system developed by Giske et al. (2008). This system classifies ESBLs depending on the antibiotic or antibiotics a bacterium is phenotypically resistant against and susceptible to. The classification system contains three classes: $ESBL_A$, $ESBL_M$ and $ESBL_{CARBA}$ (Table 1). $ESBL_A$ make the bacteria resistant against cefotaxime and/or ceftazidime but the bacteria are inhibited by clavulanic acid. $ESBL_M$ make the bacteria resistant against cefotaxime and/or ceftazidime and additionally against cefoxitin. Bacteria in this group are inhibited by cloxacillin. $ESBL_{CARBA}$ make the bacteria resistant against cephalosporins and/or carbapenems. Bacteria in this group are inhibited by boric acid, dipicolinic acid or not inhibited at all, depending on the $ESBL_{CARBA}$ subclass (PHAS, 2014).

The ESBL classes are furtherly divided into subclasses (Giske et al., 2008) (Table 1). $ESBL_A$ is divided into two subclasses: high prevalent $ESBL_A$ and low prevalent $ESBL_A$. High prevalent $ESBL_A$ are mainly CTX-M-, TEM- and SHV-ESBLs. $ESBL_M$ is also divided into two subclasses: C and D. $ESBL_{M-C}$ is plasmid mediated AmpC (Ampicillinase C). $ESBL_{M-D}$ is OXA-ESBLs. $ESBL_{CARBA}$ is divided into three subclasses: $ESBL_{CARBA-A}$, $ESBL_{CARBA-B}$, which is metallobetalactamases, and $ESBL_{CARBA-D}$, which is OXA-carbapenemases.

Table 1. ESBL classification based on the classification system by Giske et al. (2008).

ESBL classes	$ESBL_A$	$ESBL_M$	$ESBL_{CARBA}$
ESBL subclasses	High prevalent $ESBL_A$ e.g. CTX-Ms, TEM-ESBLs, SHV-ESBLs	$ESBL_{M-C}$ – AmpC e.g. CMY	$ESBL_{CARBA-A}$ e.g. KPC
	Low prevalent $ESBL_A$	$ESBL_{M-D}$ – OXA-ESBLs e.g. OXA-10s	$ESBL_{CARBA-B}$ – Metallobetalactamases
			$ESBL_{CARBA-D}$ – OXA-carbapenemases
Bacteria resistant against	Cefotaxime, ceftazidime	Cefotaxime, ceftazidime, cefoxitin	Cephalosporins and/or carbapenems
Bacteria inhibited by	Clavulanic acid	Cloxacillin	Boric acid, dipicolinic acid or not inhibited

The ESBLs in the subclasses are furtherly subclassified. Because of the CTX-M epidemic, the CTX-M subclassification is especially relevant. The CTX-M subclass 15 (CTX-M-1-like CTX-Ms) and CTX-M subclass 14 (CTX-M-9-like CTX-Ms) have the highest prevalence worldwide as well as in Sweden (Coque et al., 2008a; Swedres-Svarm, 2017; Zhao & Hu, 2013). Even though CTX-Ms originated from *Klyuviera spp*., they are prevalent in several *Enterobacteriaceae* species. Furthermore, *E. coli* is thought to be the cause of the epidemic spread of CTX-M-15 (Coque et al., 2008a; Zhao & Hu, 2013).

TEM-1 and TEM-2 are common β-lactamases in *E. coli* and other *Enterobacteriaceae* (Livermore, 1995). TEM ESBLs are still most commonly produced by *E. coli*, but also by *K. pneumoniae* and other *Enterobacteriaceae* and non-*Enterobacteriaceae* species. In Sweden, the TEM-3 subclass is the most prevalent of the TEM ESBLs in clinical *E. coli* (PHAS, 2014), but in other parts of Europe, TEM-3 is not associated with *E. coli* but with *K. pneumoniae* (Coque et al., 2008a). Furthermore, in other parts of Europe, the TEM-52 subclass has been found in clinical *E. coli* and is also the most common TEM subclass in food producing animals.

SHV ESBLs are mostly found in *K. pneumoniae,* but also in *E. coli*. In Europe, subclasses SHV-5 and SHV-12 are highly prevalent in *K. pneumoniae* and have also been found in *E. coli* (Coque et al., 2008a).

OXA ESBLs are mainly subclasses derived from OXA-10, originally found in *Pseudomonas aeruginosa* (*P. aeruginosa*). OXA-11 was the first OXA-10 derived subclass found, this also in *P. aeruginosa*. The OXA-1 β-lactamase has been found in

Enterobacteriaceae, but the prevalence of OXA ESBLs is yet poorly studied in *Enterobacteriaceae* (Cantón et al., 2008; Livermore, 1995; Naas et al., 2008).

1.4. Plasmids

The prevalence of ESBL-mediated antibiotic resistance has increased epidemically since the *bla*-genes coding for ESBLs (e.g. $bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV} and bla_{OXA}) occur on bacterial plasmids (Egervärn et al., 2013). While the chromosomal DNA harbours genes that are essential for the bacteria in general, the smaller plasmids contain genes that enhance bacterial survival, commonly antibiotic resistance genes against different kinds of antibiotics (Carattoli, 2013). However, both chromosomes and plasmids can contain genes coding for β-lactamases. While naturally occurring β-lactamase genes in environmental species are commonly found on chromosomes, ESBL genes are usually found on plasmids (Bradford, 2001). As plasmids are smaller and replicate faster and independent from the chromosomes, i.e. the chromosome commonly occurs in just one copy per cell, while plasmids can occur in up to a hundred copies, thus enabling a higher gene expression. Furthermore, plasmid genes are normally expressed at high levels due to strong promotors. Plasmid copies can transfer between bacteria, usually by conjugation, and if they carry *bla*-genes, the quick replication and transfer enables ESBL prevalence to increase epidemically (Thomas & Nielsen, 2005).

1.4.1. Plasmid replication

Plasmids carrying antibiotic resistance genes occurs in varying copy numbers, which the plasmid itself is controlling by regulating the plasmid replication. Plasmids replicate independently – they are replicons. A replicon is a genetic part of the plasmid that contains the origin of replication, where plasmid replication is initiated, and genes coding for proteins that initiate and regulate plasmid replication. The plasmid replication is regulated by higher copy numbers leading to slower or inhibited replication (Carattoli, 2013; Novick, 1987).

1.4.2. Plasmid families

Two plasmids containing the same replicon can't reside in the same bacterial cell if one of the plasmids is introduced to the bacterium by conjugation, transformation or transduction (Couturier et al., 1988). In conjugation, which is the main mechanism for

spreading *bla*-genes in *Enterobacteriaceae* (Carattoli, 2013), this means a non-conjugative bacterial cell can't receive a plasmid with a replicon that it's already carrying. Instead, the receiving bacteria will inhibit the sex pili or inhibit plasmid introduction into the bacterial cell (Schröder & Lanka, 2005). This phenomenon is called plasmid incompatibility. Plasmids with the same replicons are incompatible and plasmids with different replicons are compatible. Furthermore, plasmids with the same replicons belong to the same so-called incompatibility group (Inc.-group), or plasmid family (Couturier et al., 1988). Thus, plasmid families carry the name of the corresponding Inc.-group, named from A-Z. There are currently 27 prevalent plasmid families, including subgroups, in *Enterobacteriaceae*.

1.4.3. Detection of plasmid families

By detecting a representative replicon for each plasmid family, it's possible to detect plasmids containing replicons from one or more plasmid families. This is used in the multiplex PCR (Polymerase Chain Reaction) PBRT method (PCR-Based Replicon Typing), the standard method for detecting replicons that resistance plasmids in *Enterobacteriaceae* is known to contain (Carattoli, 2013). When it's known that the bacterium is an *Enterobacteriaceae* species (e.g. by using Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF)), produces ESBLs (e.g. by examining antibiotic disc susceptibility) and carries known *bla*-genes coding for the ESBL-production (e.g. by real time PCR detecting sequences from known *bla*-genes), the PBRT method can be used to detect the plasmids carrying the *bla*-genes. The PBRT method is based on multiplex PCR, meaning that several primer pairs specific for different replicons are used in the same PCR reaction, so that many replicons can be detected simultaneously. The PBRT method is based on conventional PCR, thus, the replicons are amplified through a cycle of three steps: denaturation of DNA at ~95°C, annealing of the primers at ~60°C and extension of the primer by *Taq* polymerase incorporating nucleotides at ~72°C, using the denaturated DNA strands as template. After ~30 cycles, the amplicons in the same multiplex PCR mix are separated from each other with respect to size by agarose gel electrophoresis. The agarose gel constitutes a meshwork that smaller amplicons can move through easier than larger ones. The amplicons move because of an electrical field, making the negatively charged DNA move

towards the positive electrode. As the agarose gel contains a stain that binds to DNA, the separated amplicons are detected.

1.5. Aim

This study aimed to detect plasmid families in *E. coli* and *K. pneumoniae* isolated from clinical and environmental samples, carrying *bla*$_{\text{CTX-M-14}}$, *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and/or *bla*$_{\text{OXA}}$. The issue was if one plasmid family was more frequent than others. The issue was also to examine if the carriage of *bla*-genes in clinical and environmental *E. coli* and *K. pneumoniae* could be associated with one or more plasmid family/families.

2. Materials & Methods

This study was performed at Kristianstad University (Kristianstad, Sweden).

2.1. Isolates

63 frozen environmental and clinical isolates, whereof 48 *E. coli* and 15 *K. pneumoniae* isolates, and a frozen control isolate, *E. coli* strain CCUG59351 carrying *bla*$_{\text{CTX-M-15}}$, were thawed. 28 *E. coli* and 11 *K. pneumoniae* isolates had previously been collected from the river Helge Å, Kristianstad, Skåne County, Sweden, upstream and downstream a WWTP. The upstream water was collected near the Tivoli park in Kristianstad and the downstream water was collected where water is pumped from the WWTP into Helge Å. Three isolates, whereof all *E. coli* isolates, of the 39 isolates had been collected near the Tivoli park and 35 isolates, whereof 25 *E. coli* and 11 *K. pneumoniae* isolates, near the WWTP.

The other 24 isolates, whereof 20 *E. coli* and four *K. pneumoniae* isolates, had originally been isolated from patients living near Kristianstad and were achieved from the Clinical Microbiology Laboratory, Laboratory Medicine, Lund University Hospital.

All isolates had previously been examined, including species, ESBL-production and *bla*-genes, i.e. *bla*$_{\text{CTX-M,}}$ *bla*$_{\text{TEM}}$, *bla*$_{\text{SHV}}$ and/or *bla*$_{\text{OXA}}$. For *bla*$_{\text{CTX-M}}$, the method included a subclassification into *bla*$_{\text{CTX-M-14}}$ or *bla*$_{\text{CTX-M-15}}$. This had however not previously been performed for all the isolates (see 2.4).

2.2. Culturing, confirming species and confirming resistance

The thawed isolates were cultured on Tryptone Glucose Extract (TGE) agar plates. After the isolates had been plated, an antibiotic disk of the cephalosporine antibiotic

ceftazidime (Oxoid, UK) was placed on each plate to confirm the bacterial ESBL-production. The agar plates were incubated at 37° C (Termaks, Norway) until next morning. The species identity was confirmed with MALDI-TOF (Matrix Assisted Laser Desorption/Ionization-Time of Flight) (Bruker, USA).

28 of the 63 isolates were chosen with respect to growth on the agar plate, confirmed species and ESBL-production and harboured *bla*-genes.

2.3. DNA extraction

Five well defined bacterial colonies of each chosen bacterial isolate were dissolved in 100 µl sterile, distilled water in sterile microtubes and heated for ~10 minutes at ~98°C in a heating block (Falc Instruments, Italy) to lyse the bacteria. The suspensions were centrifuged for 5 minutes at 15 000 x g in a microcentrifuge. The supernatants, containing plasmid DNA, were pipetted to sterile microtubes and frozen.

2.4. Detection of *bla*$_{\text{CTX-M}}$ genes

For the isolates of which CTX-M had not previously been subclassified into CTX-M-14 or CTX-M-15, real time PCR was performed. The real time PCR method was based on the method by Naas et al. (2007) and SYBRGreen and melt curve analysis were used to detect *bla*$_{\text{CTX-M-14}}$ or *bla*$_{\text{CTX-M-15}}$/*bla*CTX$_{\text{-M-55}}$, but not other subclasses. *bla*$_{\text{CTX-M-14}}$ was identified by a melt curve Tm at 84.5°±0.5 °C and *bla*$_{\text{CTX-M-15/}}$ *bla*$_{\text{CTX-M-55}}$ were identified by a melt curve Tm at 82°±0.5 °C. The reference *E. coli* strain CCUG59531 carrying *bla*$_{\text{CTX-M-15}}$ was used as positive control and sterile water in the reaction mix was used as negative control.

2.5. Detection of plasmid families

2.5.1. Multiplex PCR

The detection of plasmid families was made through multiplex PCR. The multiplex PCR was performed with a PBRT kit (Diatheva, Italy) (Carattoli, 2009), a kit for simultaneous detection of 30 replicons representing the 27 plasmid families in *Enterobacteriaceae* and three replicons that had not been classified into a plasmid family yet (Table 2). The kit included eight PCR mixes including primers for multiplex detection of the 30 replicons

(Table 2). The kit also included positive controls for each PCR mix, and *Taq* polymerase (5U/µl). The master mixes and *Taq* polymerase were thawed on ice.

Table 2. Replicons detected by primer pairs in each PCR mix, and the replicons' length.

PCR mix	Replicon	Length (bp)
1	HI1	534
	HI2	298-308
	I1a	159
2	M	741
	N	514
	I2	316
	B/O	159
3	FIB	683
	FIA	462
	P1	345
	W	242
4	L	854
	X3	284
	I1y	161
5	T	750
	A/C	418
	FIIS	259-260
	N2	177
6	U	843
	X1	370
	R	248
	FIIK	142-148
7	FIB KN	631
	X2	376
	FIB KQ	258
	K	190
8	HIB-M	570
	FIB-M	440
	FII	288-292
	X4	172

Each PCR mix was mixed with *Taq* polymerase in a 119:1 ratio, the volume depending on numbers of isolates. For each isolate, 24 µl mix (of which 23,8 µl PCR mix and 0,2 µl *Taq* polymerase) was added to a 96-plate well. For the eight positive controls, 24 µl mix was added to each one of eight wells. Negative controls were pooled in order to save material in the kit, thus, 6 µl of each mix 1-4 was added to one well and 6 µl of each mix 5-8 was added to another well.

Thereafter, 1 µl (unknown concentration) of DNA from each isolate or positive control was added to their respective wells. 1 µl sterile, distilled water was added to each negative control well. The 96-plate was covered and then ready for PCR. The PCR run started with one cycle at 95°C for 10 minutes. Secondly, 30 cycles including one step of 95°C for 60 s., one step of 60°C for 30 s. and one step 72°C for 60 s were run. Finally, one cycle at 72°C for 5 min. was run before a cooldown to 4°C.

The PCR was initially performed with undiluted and 10x diluted DNA (unknown concentration) from the *E. coli* CCUG59531 control isolate to examine which dilution gave the most distinct bands on the later gel electrophoresis. Thereafter, the method was performed with the 28 chosen isolates.

2.5.2. Agarose gel electrophoresis

Each agarose gel was made by dissolving 1,25 g agarose with 50 ml 1x TAE-buffer (ThermoFisher, Lithuania) diluted 50x (2,5% agarose gel). 0,5 µl GelRed (Biotium, USA) diluted 10000x was added to the solution. The solution was boiled and thereafter poured into the electrophoresis vessel. The solidified agarose gel was then covered with 1x TAE-buffer.

After PCR, 3 µl loading buffer (6x) was mixed with each amplicon in the 96-plate. 10 µl of each amplicon-loading buffer mix was then pipetted into their respective wells of the agarose gel. 5 µl of a 0,1 µg/µl 100 bp DNA-ladder (Thermo Fisher, Lithuania) was also pipetted into a well of the agarose gel. The gel electrophoresis was performed at 75 V for ~1 h.

2.6. Statistical analysis

The detected plasmid families' overall prevalence is presented as percentage, and the plasmid families' prevalence in each species and localisation is presented as percentage in bar charts. No further statistical analysis was made.

2.7. Ethical aspects

No ethical permission was needed for this study. There were no ethical aspects on the environmental isolates. However, origin of the clinical isolates had been decoded prior to the study, anonymizing the patients from which the samples had been collected.

2.8. Sustainability aspects

The PCR method is sensitive for contamination, which means it requires plastic materials that can't be reused, such as pipette tips, microcentrifuge tubes and 96-plates. Thus, it's sustainable to perform PCR on full 96-plates. It would also be sustainable to collect all used plastic materials (e.g. petri dishes for culturing and pipette tips, microcentrifuge tubes and 96-plates for PCR) for recycling.

The PCR method generally uses small volumes, meaning that microcentrifuge tubes are sometimes unnecessarily large. It is sustainable to prepare all PCR mixes in one microcentrifuge tube, instead of preparing new PCR mixes in new tubes for every PCR run.

The PCR mixes, *Taq* polymerase, DNA and DNA-ladders are biological and thus not hazardous for humans or environments. TGE agar and agarose are also of biological origin. TAE-buffer used in the agarose gel and for agarose gel electrophoresis is not hazardous according to the safety data sheet (ThermoFisher, Lithuania). Loading buffer used for agarose gel electrophoresis is neither hazardous according to the safety data sheet (Invitrogen, Lithuania). GelRed, used in the agarose gel is, in contrast to the earlier gel stain ethidium bromide, not hazardous for humans and the environment (Biotium, USA), making it more sustainable.

Even though the bacterial isolates are also biological, they must be handled carefully as the bacteria are alive, contagious and ESBL-producing. Thus, the bacteria must be killed by autoclavation after being studied to prevent spread of contagion to humans and animals, and to prevent spread of ESBLs to humans, animals and environments.

3. Results

The PCR was performed with 28 isolates (Table 3) that had been chosen with respect to growth on the agar plate, confirmed species and ESBL-production and harboured *bla*-genes. The PCR was performed with undiluted samples. The replicons detected by the agarose gel electrophoresis (example seen in Figure 3) are shown for each isolate together with the isolate number, species, localisation and produced ESBLs in Table 3.

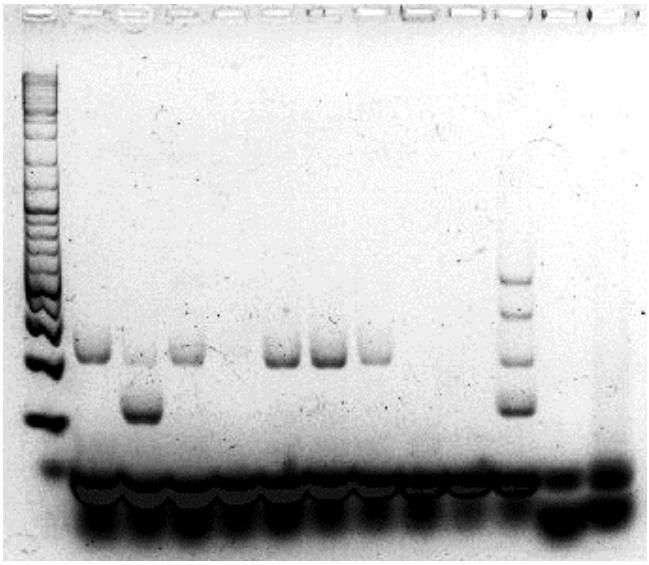

Figure 3. An example of detected replicons by one PCR mix by agarose gel electrophoresis. The DNA ladder is shown to the left, followed by 9 isolates, followed by a positive control for the PCR mix, followed by two negative controls for all PCR mixes.

Table 3. Detected replicons for each examined isolate.

Isolate number	Species	Localisation	ESBL/ESBLs	Replicons
1	*E. coli*	Clinic	CTX-M-15	FIB, FII, R
2	*E. coli*	Clinic	CTX-M-15, TEM	FIA, FIB, FII
3	*E. coli*	Clinic	CTX-M-15, TEM, OXA	FIB, FII, I1a, N
4	*E. coli*	Clinic	CTX-M-15, TEM, OXA	FIA, FII, N
5	*K. pneumoniae*	Clinic	CTX-M-15, SHV	A/C, FIIK, FIB KN
6	*K. pneumoniae*	Clinic	CTX-M-15, SHV, TEM, OXA	A/C, FIB-M, FIB KN
7	*E. coli*	Environment	CTX-M-15, TEM	FIB, FIB KN, FIB KQ, I1a, I1y
8	*E. coli*	Environment	CTX-M-15, TEM	FII, X1
9	*E. coli*	Environment	CTX-M-15, OXA	FIA, FIB, FII, I1a, X4
10	*E. coli*	Environment (Tivoli park)	CTX-M-15, OXA	FII, FIIS
11	*E. coli*	Environment	CTX-M-15, OXA	FIB, FII, FIIS, I1a
12	*E. coli*	Environment	CTX-M-15, TEM, OXA	FII, I1a, X4
13	*E. coli*	Environment	CTX-M-15, TEM, OXA	FIB, FII, I2
14	*E. coli*	Environment	CTX-M-15, TEM, OXA	FII, FIIS, I1a
15	*E. coli*	Environment	CTX-M-15, TEM, OXA	X1
16	*E. coli*	Environment (Tivoli park)	CTX-M-15, TEM, OXA	HI1
17	*E. coli*	Environment	CTX-M-15, TEM, OXA	FIB, FIB KN, I1a
18	*E. coli*	Environment	CTX-M-15, TEM, OXA	FII, FIIS
19	*E. coli*	Environment	CTX-M-14, TEM	FII, I1a, X1
20	*E. coli*	Environment	CTX-M-14, TEM, OXA	FIA, FIB, FII, FIIS, N
21	*E. coli*	Environment	CTX-M-15, SHV	FIB KN, I1a
22	*E. coli*	Environment	CTX-M-15, SHV	FIB KN, I1a
23	*E. coli*	Environment (Tivoli park)	SHV	I1a
24	*E. coli*	Environment	SHV, TEM	FII
25	*K. pneumoniae*	Environment	CTX-M-15, TEM	FIIK, N2
26	*K. pneumoniae*	Environment	CTX-M-15, TEM	FIIK
27	*K. pneumoniae*	Environment	CTX-M-15, SHV, TEM	FIB KN, I1a
28	*K. pneumoniae*	Environment	CTX-M-14, SHV	I1y

The IncF plasmid family, shown in shades of green, was the most prevalent plasmid family in both clinical and environmental isolates of both species (Figure 4), containing ~60% of all 73 detected replicons. IncFII was the most prevalent replicon in the IncF plasmid family, constituting 34% of the replicons in the IncF plasmid family. Most *E. coli* isolates carried IncFII or another IncFII replicons (FIIS, FIB KN or FIB KQ) together with FIA and/or FIB, but no FIA or FIB replicon occurred without a FII replicon (Table 3). FII, FIA and FIB did not occur in *K. pneumoniae* isolates, but instead another FII replicon – the FIIK replicon or the FIIK replicon FIB KN – occurred (Figure 4).

IncI, showed in shades of violet, was the second most common plasmid family, constituting ~21% of all detected plasmid families.

IncN, shown in shades of pink, was the third most common plasmid family, constituting ~5% of all detected plasmid families. IncN occurred in *E. coli* isolates and IncN2 occurred in *K. pneumoniae* isolates (Figure 4).

IncHI1 (light blue) occurred in ~2% of environmental *E. coli* isolates, while IncHI2 and IncL/M didn't occur in any isolate (Figure 4). IncX plasmids (IncX1 and IncX4, shown in shades of bluish green) were more prevalent (~10%) than both IncHI1 (~2%), IncHI2 (0%), IncN (~2%), IncL/M (0%) and IncA/C (0%) in environmental *E. coli* isolates, but only occurred in environmental *E. coli* isolates (Figure 4).

One R replicon (shown in yellow) was only found in clinical *E. coli* isolate (isolate 1, Table 3), constituting 8% of clinical *E. coli* isolates, while IncA/C (shown in red) and FIB-M (striped) were only found in clinical *K. pneumoniae* isolates, constituting ~33% respectively ~17% of clinical *K. pneumoniae* isolates (isolate 5 & 6, Table 3) (Figure 4).

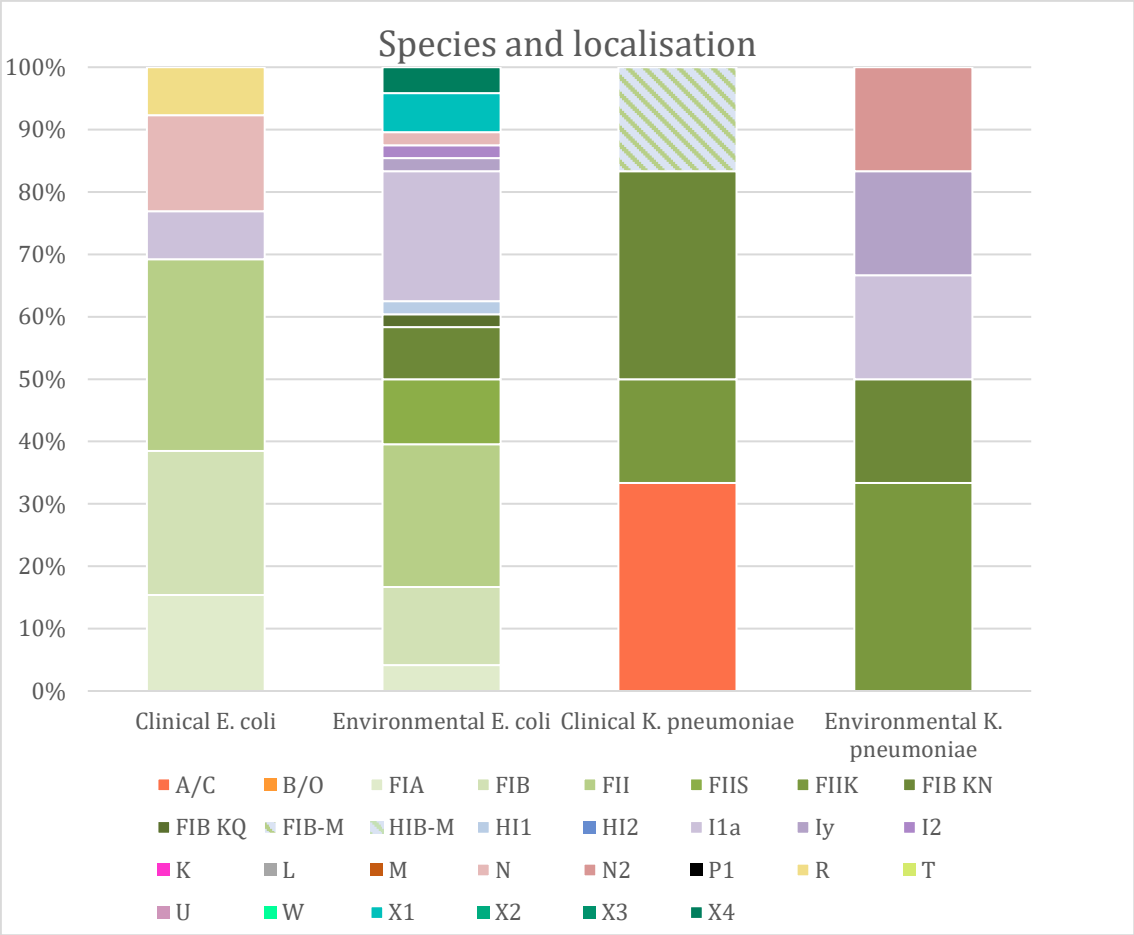

Figure 4. Distribution of the overall replicon prevalence in *E. coli* and *K. pneumoniae* from clinical ($n_{E.\ coli}$ = 4, $n_{K.\ pneumoniae}$ = 2) and environmental ($n_{E.\ coli}$ = 18, $n_{K.\ pneumoniae}$ = 4) isolates. Replicons in the same plasmid families are shown in shades of the same colour.

For clinical and environmental *E. coli* isolates, the replicon prevalence was similar for CTX-M-15, TEM and OXA (Figure 5 & 6), with a few exceptions. The same plasmid families were found with almost the same prevalence. In clinical *E. coli* isolates, the only exception was that the R replicon was prevalent in clinical isolates producing CTX-M-15, but no other ESBLs (Figure 5). In environmental *E. coli* isolates, the exception was for isolates producing CTX-M-15, where IncN was not found, CTX-M-14, where IncHI1 was not found, and SHV, where only IncF and IncI were found (Figure 5). In environmental *K. pneumoniae* isolates, the exception was also for isolates producing SHV, where only IncF and IncI were found, and for CTX-M-14, where only IncI was found (Figure 6, Table 3).

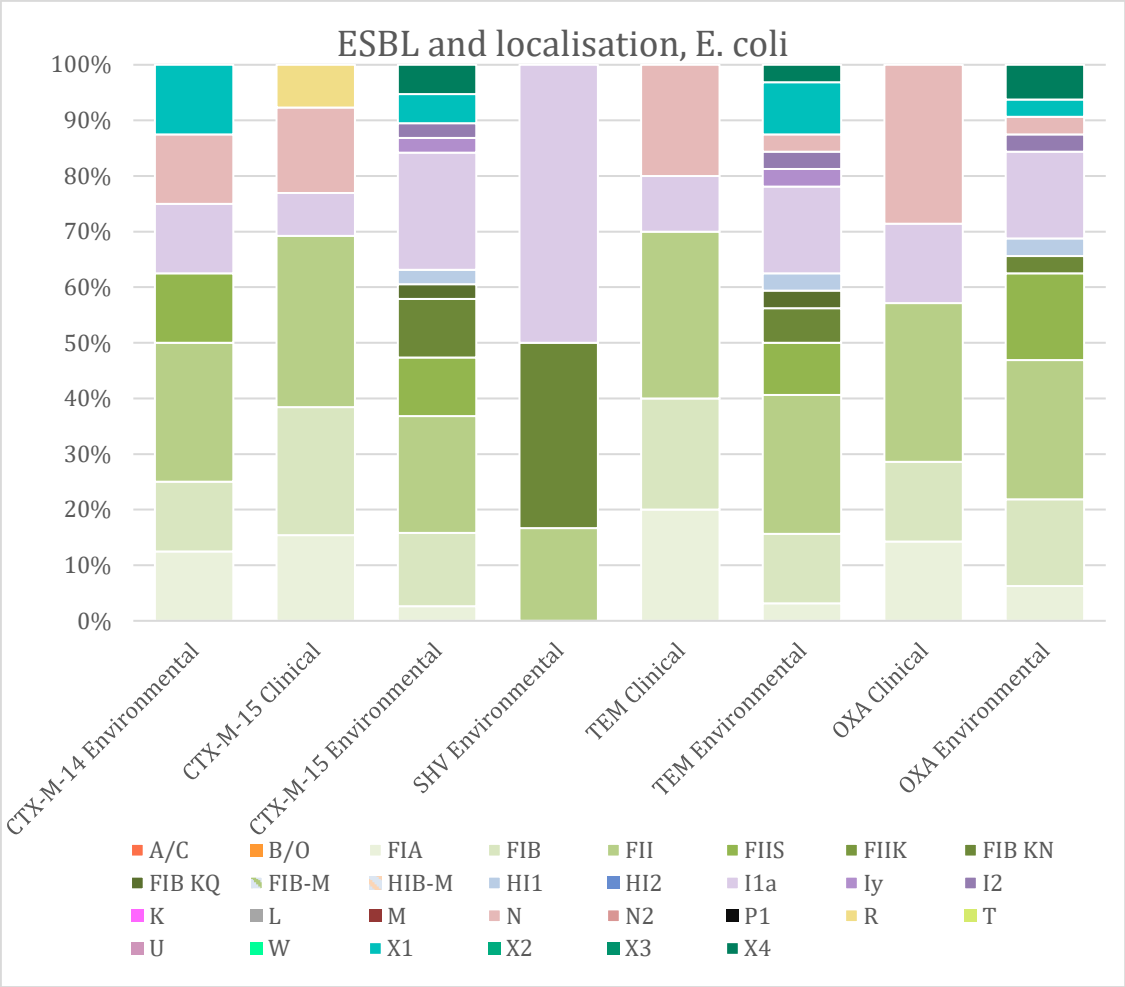

Figure 5. Distribution of the replicon prevalence in clinical and environmental *E. coli* producing CTX-M-14 ($n_{clinical}$ = 0, $n_{environmental}$ = 2), CTX-M-15 ($n_{clinical}$ = 4, $n_{environmental}$ = 14), SHV ($n_{clinical}$ = 0, $n_{environmental}$ = 4), TEM ($n_{clinical}$ = 3, $n_{environmental}$ = 12) and/or OXA ($n_{clinical}$ = 2, $n_{environmental}$ = 11) ESBLs. Replicons in the same plasmid families are shown in shades of the same colour.

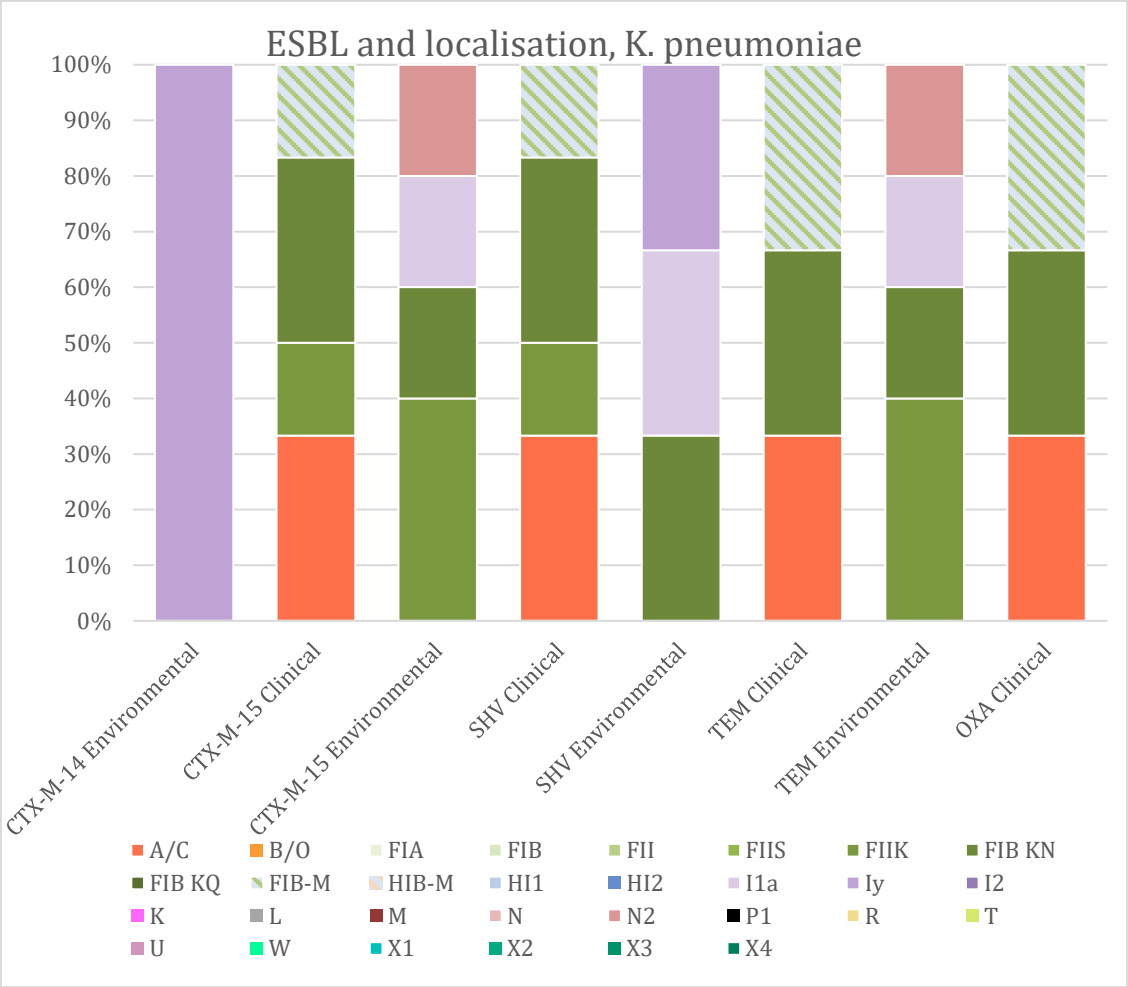

Figure 6. Distribution of the replicon prevalence in clinical and environmental *K. pneumoniae* producing CTX-M-14 ($n_{clinical}$ = 0, $n_{environmental}$ = 1), CTX-M-15 ($n_{clinical}$ = 2, $n_{environmental}$ = 3), SHV ($n_{clinical}$ = 2, $n_{environmental}$ = 2), TEM ($n_{clinical}$ = 1, $n_{environmental}$ = 3) and/or OXA ($n_{clinical}$ = 1, $n_{environmental}$ = 0) ESBLs. Replicons in the same plasmid families are shown in shades of the same colour.

All except two CTX-M-15 producing bacteria of both species and localisations (i.e. clinic or environment) carried IncF replicons. The two isolates that didn't carry any IncF replicons were both environmental *E. coli* isolates carrying $bla_{CTX\text{-}M\text{-}15}$, bla_{TEM} and bla_{OXA} together on IncX1 plasmids (isolate 15, Table 3) or IncHI1 plasmids (isolate 16, Table 3)

$bla_{CTX\text{-}M\text{-}14}$ was carried together with bla_{TEM} in one *E. coli* isolate (isolate 19, Table 3). This isolate carried replicons FII, I1a and X1. In the other *E. coli* isolate (isolate 20, Table 3), $bla_{CTX\text{-}M\text{-}14}$ was carried together with bla_{TEM} and bla_{OXA}. In *K. pneumoniae* isolates, $bla_{CTX\text{-}M\text{-}14}$ was carried together with SHV on an IncI1y plasmid (isolate 28, Table 3).

Environmental isolates of both species producing SHV harboured IncF and/or IncI1 plasmids (Figure 5 & 6). Clinical *K. pneumoniae* isolates producing SHV harboured IncF and IncA/C plasmids (isolate 5 & 6, Table 3).

4. Discussion

4.1. Discussion of results

IncF being the most prevalent plasmid family was expected as IncF has been shown to be the most prevalent plasmid family in earlier studies (Carattoli, 2011, 2013). Additionally, the prevalence of IncFII was expected as IncFII has been shown to be common in carrying *bla*-genes (Carattoli, 2011). IncFII replicons was in this study as well as earlier studies occurring alone but mostly together with FIA and/or FIB replicons (Carattoli, 2009; Hopkins et al., 2006; Zurfluh et al., 2015). This could be explained by the fact that a bacterium that already carries a plasmid can more easily receive other genes or replicons and is advantageous in carrying multireplicon plasmids due to plasmid incompatibility (Couturier et al., 1988).

IncFII didn't occur in *K. pneumoniae* isolates, but FIIK did, which was not unexpected as FIIK occurred in *K. pneumoniae* isolates instead and FIIK is a FII replicon associated with *K. pneumoniae* (García-Fernández et al., 2012), explaining why FII was not found in *K. pneumoniae* isolates and FIIK not found in *E. coli* isolates.

IncI and IncN was also expectedly prevalent. Interestingly, different IncN replicons occurred in *E. coli* (N) compared to *K. pneumoniae* (N2) isolates, but the same IncN replicon (N respectively N2) occurred in clinical and environmental isolates of respective species. Eikmeyer et al. (2012) have shown that IncN replicons are prevalent in *E. coli* and *K. pneumoniae*, but replicons in the IncN plasmid family are usually not transferred between species. This is in agreement with this study. Furthermore, Eikmeyer et al. (2012) showed that IncN plasmids from clinical and environmental isolates are similar, also in agreement with this study. This indicates that plasmids spread from the environment to clinic (Eikmeyer et al., 2012).

Unexpectedly, IncHI1, IncHI2, IncL/M and IncA/C, that has been shown to be common in *Enterobacteriaceae* and/or common carriers of *bla*-genes (Carattoli 2011, 2013), only occurred in low prevalence or not at all in both species isolated from both localisations (i.e. clinics or environments) in this study. One explanation to the low prevalence could be that plasmid families are more commonly found in other *Enterobacteriaceae* species. Thus, IncHI1 and IncHI2 has been found in *Salmonella* species (Holt et al. 2011; Hopkins et al., 2006) and IncHI2 has as well been found in

Enterobacter cloacae (*E. cloacae*) (Novais, 2006; Poirel, 2007). However, earlier studies have found IncHI1 in *E. coli*. *E. coli* carrying IncHI1 is highly associated with carrying CTX-M-1 in horses, both in Sweden and other European countries (Apostolakos et al., 2017; Dolejska et al., 2011; Lupo et al., 2018). Thus, it's possible that the *E. coli* isolate in this study that carried the IncHI1 plasmid originated from horses grazing near the river Helge Å, especially as the isolate came from water collected upstream of the WWTP.

Another explanation to the low prevalence of IncHI1, IncHI2, IncL/M and IncA/C could be that the plasmid families have been commonly found carrying other *bla*-genes in *E. coli* and *K. pneumoniae* than the ones examined in this study (IncL/M) (Markovska et al., 2013; Novais et al., 2007). A third explanation could simply be that the plasmid families are rare (e.g. IncB/O, IncK, IncT and IncX (Carattoli, 2013).

The IncX plasmid family being rather common in this study is in agreement with earlier studies finding X replicons at relatively high prevalence, hence, suggesting that they are not as rare as previously thought (Johnson et al., 2012; Lo et al., 2014). The prevalence of IncX in environmental isolates but absence of IncX in clinical isolates in this study is tentatively explained by IncX1 being found in *E. coli* from horses in the same study where IncHI1 was found (Dolejska et al., 2011). Additionally, IncX1 and IncX4 has been found in *E. coli* from food producing animals (Lo et al., 2014). Thus, likewise for IncHI1, horses and cows that are grazing near the river Helge Å may explain the prevalence of IncX plasmids in environmental isolates. However, high diversity of plasmid families is common in the environment.

In contrast to IncHI1 and IncX only occurring in environmental *E. coli* isolates, the R replicon only occurred in a clinical *E. coli* isolate. The R replicon origins from *K. pneumoniae* and is still an epidemic replicon in European *K. pneumoniae* (Rodrigues et al., 2014; Zurfluh et al., 2015). Furthermore, *K. pneumoniae* carrying a R replicon has been shown to be able to conjugate with *E. coli* (Rodrigues et al., 2014). Because of the prevalence abroad in combination with the absence of R plasmids in environmental isolates, I suggest that the R replicon was acquired from other countries' environments. Furthermore, the replicon could have been acquired if the patient has been travelling to countries where it's epidemical. Indeed, travelling abroad, and especially hospitalization abroad, are major risks for gaining ESBL-producing gut bacteria (Egervärn et al., 2013; PHAS, 2014).

FIB-M and IncA/C only occurrred in clinical *K. pneumoniae* isolates. FIB-M was originally found in *K. pneumoniae* (Villa et al., 2012), which could explain why it was only found in *K. pneumoniae* isolates in this study. FIB-M and Inc/AC occurring in clinical isolates may be explained by bacteria being acquired from other countries or imported food. IncA/C has been associated with food producing animals in both China and USA (Guo et al., 2014; Mulvey et al., 2009). In these countries, as well as in Europe, studied *E. coli* isolates have shown that IncA/C plasmids are common carriers of CMY ESBLs, a kind of AmpC (Hopkins et al., 2006).

The absence of IncA/C in *E. coli* isolates was unexpected as the A/C replicon has been shown to be a common carrier of several different *bla*-genes and occurring in *E. coli* (Carattoli 2009, 2013). Though, IncA/C, as well as FIA and FIB, occurred in the *E. coli* control isolate. The control isolate was known to be producing CTX-M-15, but plasmid type had not previously been examined. As FIA and FIB replicons are likely carried on the same plasmid and CTX-M-15 is more commonly coded by IncF plasmids than IncA/C plasmids (Caratolli, 2009), the control isolate is probably carrying other antibiotic resistance gene/s, e.g. CMY genes or other *bla*-genes. CMY belongs to the $ESBL_{M-C}$ class while CTX-M-15 belongs to the $ESBL_A$ class (Giske et al., 2008). No information regarding $ESBL_{M-C}$ was available for the control isolate reference strain, thus, it's possible that $ESBL_{M-C}$ had not been previously examined for the reference strain. This, as the classification is based on the antibiotic or antibiotics a bacterium is phenotypically resistant against or susceptible to. Both $ESBL_A$ and $ESBL_M$ make the bacteria resistant against cefotaxime and/or ceftazidime but $ESBL_M$ makes the bacteria additionally resistant against cefoxitin, which may not have been examined. Furthermore, bacteria producing $ESBL_A$ are normally inhibited by clavulanic acid and $ESBL_M$ by cloxacillin, which also may not have been examined.

In this study, association between plasmid families and ESBLs were examined for both species from clinical and environmental isolates. Generally, it was difficult to associate one *bla*-gene with one plasmid family, as most isolates carried more than one *bla*-gene and more than one plasmid family.

For clinical and environmental *E. coli* isolates, the replicon prevalence was similar for CTX-M-15, TEM and OXA, probably because most isolates produced all these ESBLs, leading to the same prevalence for these ESBLs for these isolates. Most CTX-M-15 producing bacteria of both species from both clinical and environmental origin carried

IncF replicons, showing that *bla*$_{\text{CTX-M-15}}$ being commonly carried on IncF plasmids. These findings are in agreement with Egervärn et al. (2013), finding *bla*$_{\text{CTX-M-15}}$ dominantly carried on IncF plasmids in clinical isolates in Sweden, Carattoli et al. (2008), Coque et al. (2008b), Gonullu et al. (2008), Hopkins et al. (2006) and Novais et al. (2007), associating the spread of CTX-M-15 with *bla*$_{\text{CTX-M-15}}$ being carried on IncF plasmids in clinical isolates in other European or non-European countries, and Zurfluh et al. (2015) finding *bla*$_{\text{CTX-M-15}}$ dominantly carried on IncF plasmids in isolates from European clinics and environmental waters.

Some clinical and environmental *E. coli* isolates carried *bla*$_{\text{CTX-M-15}}$ together with *bla*$_{\text{TEM}}$ or *bla*$_{\text{OXA}}$ on IncF plasmids, conforming earlier studies showing that *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM}}$ as well as *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{OXA}}$ with or without *bla*$_{\text{TEM}}$ are commonly carried on the same IncF plasmid (Carattoli, 2009, 2013; Doumith et al., 2012; Torres et al., 2016). Likewise, one environmental *K. pneumoniae* isolate carried *bla*$_{\text{CTX-M-15}}$ and *bla*$_{\text{TEM}}$ on the same IncF plasmid.

The isolates that didn't contain any IncF replicons were both environmental *E. coli* isolates which harboured *bla*$_{\text{CTX-M-15}}$, *bla*$_{\text{TEM}}$ and *bla*$_{\text{OXA}}$ together on an IncXI respectively IncHI1 plasmid, suggesting that these three *bla*-genes can be carried by other plasmid families than IncF.

CTX-M-14 has as well been associated with IncF plasmids in earlier studies (Carattoli, 2009, 2013). In this study, the CTX-M-14 producing *E. coli* isolates carried IncF plasmids, but also IncX1, IncI1a and/or IncN. The only CTX-M-14 producing *K. pneumoniae* isolate harboured *bla*$_{\text{CTX-M-14}}$ on an IncI1y plasmid, suggesting that *bla*$_{\text{CTX-M-14}}$ can be carried by other plasmid families than IncF. This *K. pneumoniae* isolate also produced SHV. In addition, one environmental *E. coli* isolate harboured *bla*$_{\text{SHV}}$ on an IncI1a plasmid. Other environmental *E. coli* isolates were shown to carry *bla*$_{\text{SHV}}$ on IncFII plasmids. Furthermore, the IncFII replicon FIB KN was relatively highly prevalent in both *E. coli* and *K. pneumoniae* isolates producing SHV. *E. coli* has been shown to carry both *bla*$_{\text{SHV-2}}$, *bla*$_{\text{SHV-5}}$ and *bla*$_{\text{SHV-12}}$ on IncF plasmid families, but Inc FIIK has only been found in *K. pneumoniae* producing SHV-12 (Carattoli, 2009, 2013), one of the most common SHV subclasses in European *K. pneumoniae* (Coque et al., 2008a). As the FIB KN replicon is an IncFIIK replicon commonly found in *K. pneumoniae*, and was also found in *K. pneumoniae* isolates in this study, the *E. coli* have suggestively received a

FIB KN replicon carrying *bla*$_{\text{SHV-12}}$ from a *K. pneumoniae*. Earlier studies have also found European *E. coli* producing SHV-12 (Coque et al., 2008a).

Even though FIB KN may be associated with *bla*$_{\text{SHV}}$, FIB KN also occurred in *E. coli* isolates not producing SHV. Furthermore, clinical *K. pneumoniae* isolates producing SHV also carried IncA/C, which has been associated with both *bla*$_{\text{SHV}}$ and other *bla*-genes (Carattoli, 2009, 2013). Thus, as for the other ESBLs, it was difficult to determinedly associate SHV with a specific plasmid family.

In future studies, it would be interesting to compare the plasmid family prevalence between *E. coli* and *K. pneumoniae* and between clinics and environments. In this study, there was an indication of *E. coli* being more diverse in plasmid family content than *K. pneumoniae* and environmental isolates being more diverse than clinical isolates. Earlier studies have found differences between *E. coli* and *K. pneumoniae*, where *E. coli* carried more plasmids and different plasmid families than *K. pneumoniae* (Sherley et al., 2003). Furthermore, *E. coli* has been shown to have mechanisms for stably containing multireplicon plasmids that *K. pneumoniae* doesn't have (Porse et al., 2016). Thus, *E. coli* could be more diverse than *K. pneumoniae*, but more isolates than in this study would need to be studied to make any conclusions.

Likewise, more isolates would need to be studied to make any conclusions about environmental isolates being more diverse than clinical. Earlier studies have found that environmental bacteria could constitute a gene pool of natural antibiotic resistance genes originating in the environment (Bush, 2018), and that environmental waters are advantageous reservoirs for dissemination of *bla*-genes (Lupo et al., 2018). Furthermore, as some *bla*-genes have been associated with some plasmid families (Carattoli, 2009, 2013), environmental bacteria could constitute a pool of plasmid families.

Finally, it would be interesting to examine how eventual environmental plasmids carrying *bla*-genes spread from the environmental pool of plasmids and genes to the clinics. Furthermore, it would be useful to prevent the spread of plasmids carrying *bla*-genes – both the eventual spread from environments to clinics, the spread in the environments, and finally the spread in the clinics. Studies have already been examining the prevention of plasmids spreading antibiotic resistance genes, e.g. by inhibiting plasmid conjugation (Cabezón et al., 2017) or by plasmid curing (Buckner et al., 2018). Either way, it is of major interest that plasmids carrying and spreading *bla*-genes are furtherly studied and prevented because of the concerns ESBLs entail.

4.2. Discussion of methods

The PBRT method is an effective method as it is multiplex, making it possible to rapidly detect several plasmid families in several isolates simultaneously. The method was easy to use as it didn't need any optimisation. The method could however be optimised if desired, as some primers seemed to be less specific than others, giving faint bands on the agarose gel that did not correspond to bands in the positive control for the same PCR mix. The method could also be even more rapid if desired, e.g. by being a real time PCR method, not using agarose gel electrophoresis for detection. The detection would then also not be visually interpreted as on an agarose gel, where the bands were sometimes hard to see or hard to interpret.

A limitation of the PBRT method is that it is unable to detect novel replicons, in the same way the real time PCR method is unable to detect *bla*-genes of other subclasses than the ones the primers are specific for. As some novel *bla*-genes develops by point mutations (Bradford, 2001; Drawz & Bonomo, 2010), it could be desirable to be able to detect novel *bla*-genes. It could also be desirable to be able to detect novel replicons and divide them into plasmid families (e.g. FIB-M, HIB-M and R). This could be solved by sequencing *bla*-genes and novel replicons (Carattoli, 2009). However, as some ESBLs are associated with some plasmid families, there is not necessarily a desire for detection of novel ones, depending on the aim of the study.

Another limitation of the PBRT method is that it was sometimes difficult to associate one *bla*-gene with one plasmid family, as most isolates carried more than one *bla*-gene and more than one plasmid family. However, for isolates only carrying one plasmid family, association with *bla*-genes was possible.

5. Conclusions

In conclusion, the IncF plasmid family was the most prevalent for both species in both clinical and environmental isolates of *E. coli* and *K. pneumoniae*. The IncF plasmid family seemed to be prevalent for all ESBLs, but it was difficult to associate one *bla*-genes with one plasmid family, as most isolates carried more than one *bla*-gene and more than one plasmid family.

Sequencing may be a better method for association of *bla*-genes and plasmid families. However, the PBRT method was in fact also able to associate *bla*-genes and plasmid

families in isolates only carrying one plasmid family. Lastly, the PBRT method was rapid and easy to use for detecting plasmid families.

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