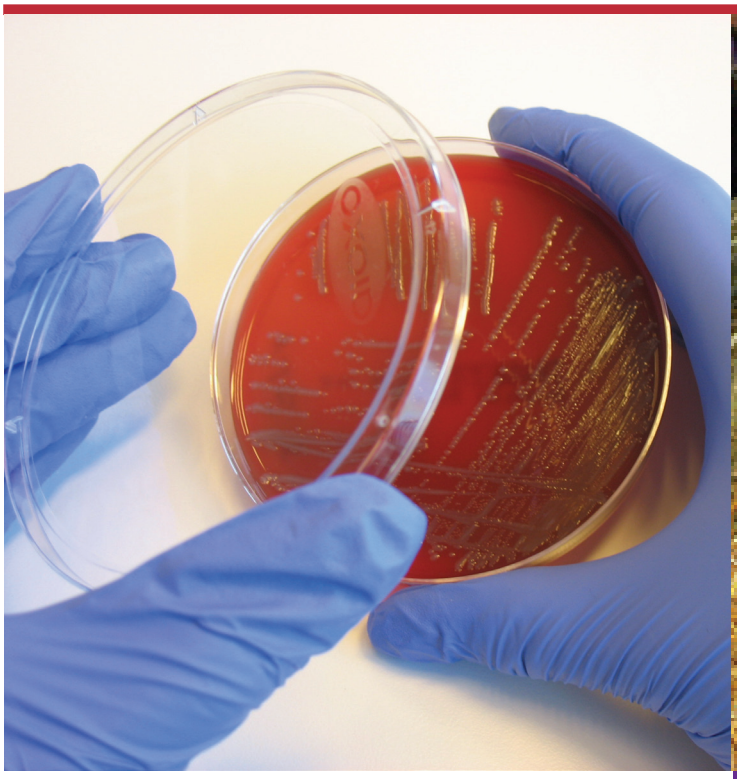
Diversity and epidemiology of plasmids from Enterobacteriaceae from human and non-human reservoirs



Eliza Maria Bielak
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Diversity and epidemiology of plasmids from Enterobacteriaceae from human and non-human reservoirs

PhD Thesis

by

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Preface

The work described in this PhD thesis was carried out at the Division for Epidemiology and Microbial Genomics (former Division of Microbiology and Risk Assessment), National Food Institute, Technical University of Denmark, from December 2008 to February 2012. Part of the work from February to March 2011was conducted at the Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy.

The project was supervised by the senior scientist Henrik Hasman and professor Frank Aarestrup and founded by the Danish Agency of Science, Technology and Innovation (grant number FøSu 2101-07-0046).

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Summary

The family of Enterobacteriaceae is comprised of Gram negative bacteria found in a variety of natural environments as well as in the gastrointestinal (GI) tracts of humans and many animals including diverse mammals, birds and reptiles. Three species of the enteric bacteria are largely responsible for causing infections both in humans and animals worldwide; these are *Escherichia coli*, *Salmonella enterica* and *Klebsiella pneumoniae*; β-lactams are antimicrobials commonly prescribed to treat uncomplicated as well as severe infections caused by these Enterobacteriaceae and other Gram negative and also Gram positive bacteria. In particular, aminopenicillins, cephalosporins and carbapenems found broad application in treatment of infections caused by the aforementioned enteric species. Recently however, increasing levels of resistance to β-lactams is observed in these key infectious agents as well as in many other previously susceptible species. This phenomenon has become a major public concern.

Antimicrobials including β-lactams have been often used in heavy amounts in farming, not only to treat the diagnosed infections in individual animals but also as prophylaxis, metaphylaxis and growth promotion. It is believed that these practices lead to the generation of reservoirs of antimicrobial resistance genes in the GI tracts of intensively reared food - production animals like pigs, poultry and cattle. Moreover, it has been previously shown that the *bla* genes (i.e. genes encoding resistance to β-lactams) could be transmitted between different bacteria on mobile genetic elements (MGEs) like plasmids and variety of transposons. Evidences were also published indicating that zoonotic bacteria like *E. coli* or *S. enterica* resistant to diverse antimicrobials and harbouring plasmids might have been transmitted from farm animals to humans (farm workers, animal caretakers etc.). It has been therefore speculated whether the plasmids with the *bla* genes found in Enterobacteriaceae in humans could actually originate from the animal sources.

The overall aim of this thesis was to verify if indeed related resistance plasmids can circulate between enteric bacteria from humans and food production animals; and if so, then which of these plasmid species are specifically associated with the epidemic types of bla_{TEM} genes in Enterobacteriaceae. Furthermore, the association of the plasmid encoded bla_{TEM} genes with transposable elements is also studied in order to get a broader perspective of which MGEs are involved in mobilization and spread of these *bla* genes in the diverse reservoirs. Finally, an attempt is made to encompass ecological aspects of plasmid driven transmission of resistance among the enteric bacteria.

In the first study the relationship between plasmids harbouring *bla*$_{\text{TEM-52}}$ genes isolated from humans, poultry and also meat products was examined. Twenty- two plasmids from a collection of *E. coli* and different serovars of *S. enterica* were characterized. The study delivered molecular evidences that epidemiologically related plasmids circulated in the diverse species of enteric bacteria and between humans and animals, and the possible transmission route could have been contaminated food products like meat. Two types of epidemic plasmids were detected in isolates of *E. coli* and *S. enterica*; namely undistinguishable IncI1 *bla*$_{\text{TEM-52}}$ plasmids were found in human and poultry isolates of *E. coli* and *S. enterica*; also undistinguishable IncX1 plasmids were isolated from *E. coli* and *S. enterica* from human infections, poultry and meat products (from poultry and broiler, beef). The strains harbouring these plasmids were confirmed not to be clonally related, hence indicating the transmission of the plasmids between the different bacteria from humans and animals rather than isolation of the same bacterial clones from the different reservoirs.

With relation to the study I, a range of other observations was made. On majority of the examined plasmids, the *bla*$_{\text{TEM-52}}$ genes resided on the Tn*3*-related transposons. Further analysis of the genetic environment of these *bla*$_{\text{TEM}}$ genes resulted in the conclusion, that it was a defined type of Tn*3*-like element that harboured the *bla*$_{\text{TEM-52}}$, namely the Tn*2* transposon. This knowledge was later used in the second study to design more discriminatory PCR method that would allow for distinction of which transposon types (Tn*1*, *2* or *3*) or insertion sequences (IS*26*) could be linked to the *bla*$_{\text{TEM}}$ genes of interest. Moreover, the initial typing of – as realized later the IncX1 –*bla*$_{\text{TEM-52}}$ plasmids with the use of available standard PCR-based methods for replicon typing (PBRT) was unsuccessful. In the course of this study the whole plasmid from *E. coli* 2161 was sequenced and deposited in GenBank as plasmid pE001. It became apparent that the standard PBRT method targeted another group of IncX family of replicons, namely the IncX2 plasmids, which so far have been rather rarely detected in humans or animals. The replicon of the pE001 was designated in the published Manuscript I as an IncX1A. The reason to that was the discovery of dissimilarities between the replicon of pE001 and the replicon of an IncX1 plasmid called pOLA52. The latter was published before pE001 and was considered to carry a classical IncX1 replicon. In the study I, an incompatibility assay for the pE001 and the pOLA52 (variant with the deletion in *bla*$_{\text{TEM}}$ gene) was performed. The two plasmids turned out to be compatible, which was surprising considering the high degree of overall similarities between the two sequenced scaffolds. Based on these results it was concluded that the standard incompatibility assays may in some cases give a false reflection of the real relatedness of the examined plasmids. Combining this

experience with the knowledge that the PBRT method is often sensitive to the sequence substitutions within the replicon scaffolds, another idea was generated. It was previously reported that the plasmids from *Klebsiella pneumoniae* often escaped the detection by the classical PBRT methods, which was originally designed based on *E. coli* replicons. Therefore in the third study, which will be described later in this summary, a novel method was elaborated for rapid detection and sub-classification of plasmids from this species.

The $bla_{\text{TEM-52}}$ genes that were the focus of the first sub-project are in fact evolutionary descendants of $bla_{\text{TEM-1}}$ genes. The second study aimed therefore at verifying on which plasmids scaffolds these bla_{TEM} predecessors are usually located in Enterobacteriaceae found in humans and food production animals like pigs, poultry and cattle. In this sub-project the focus was stated on the plasmids from *E. coli*, which is known to be either an indicator organism colonizing (as a commensal) both the human and animal GI tracts; or it may cause infections to its hosts. Evidences were found in the study II that either undistinguishable or similar $bla_{\text{TEM-1}}$ plasmids circulated in different *E. coli* from humans and from animal sources in Denmark. Possibly epidemic $bla_{\text{TEM-1}}$ IncI1 and IncB/O plasmids were found in humans and the diverse animals (pigs, poultry and cattle). Moreover, a larger variation of the transposable elements linked to the $bla_{\text{TEM-1}}$ genes was detected on plasmids in the second study compared to $bla_{\text{TEM-52}}$ plasmids. In the second study usually specific alleles of the $bla_{\text{TEM-1}}$ genes resided on either the Tn*2* ($bla_{\text{TEM-1b}}$ and $bla_{\text{TEM-1c}}$) or Tn*3* ($bla_{\text{TEM-1a}}$) transposons. In many cases the insertions of IS*26* elements upstream of the $bla_{\text{TEM-1}}$ genes were detected by PCR. These results gave important clues not only regarding which plasmids but also which specific transposons might have served as platforms for mobilization and evolution of the $bla_{\text{TEM-1}}$ to $bla_{\text{TEM-52}}$ genes.

In the third sub-project plasmids from *K. pneumoniae* from human infections and from surface waters (designated as environmental isolates) were typed. In this study the strains were not pre-selected based on the defined resistance markers. The results allowed for evaluating if there are differences in the replicons normally found on plasmids from humans and from the environment in this bacterial species. Additionally, these potentially host specific replicons could have been compared to the replicons of plasmids previously shown to be specifically associated with the resistance genes in the clinically relevant *K. pneumoniae*.

At the time when this project was initiated the standard PBRT method often failed to detect the replicons from *K. pneumoniae*. Therefore a novel multiplex PCR (mPCR) was designed for detection of these otherwise untypable plasmids. While this was pursued, updated

protocols for PBRT were published by other authors and many of the sequenced plasmids from *K. pneumoniae* used as references for designing of the mPCR turned out to be the $IncFII_k$ types. However, an interesting observation was made. Namely, in one of the previously sequenced *K. pneumoniae* strain MGH78578 apparently multiple plasmids with the similar incompatibility determinants $IncFII_k$ were detected; this was opposing to the theory that the plasmids from the same incompatibility groups typically would not be able to co-exist in the same bacterium. In fact, a similar pattern was seen in study III in some of the examined *K. pneumoniae* strains from human infections and from the environment, where also multiple $IncFII_k$ plasmids were present in the same isolates simultaneously, but these plasmids harboured diverse secondary replicons detected by the mPCR. The conclusion from the third study was that plasmids may acquire secondary replicons in order to persist in the given bacterium and to overcome the incompatibility phenomenon and this seems to be fairly common in *K. pneumoniae*. Analysis of literature data against the data from the study III resulted also in a conclusion, that the same replicons that are generally predominant in *K. pneumoniae* ($IncFII_k$, likely also IncR and yet unknown replicons) are often associated with a variety of the *bla* genes in the clinical strains of this species.

In summary, the combined data from the three studies suggested that often the bla_{TEM} plasmids are generally host specific to the species they were detected in. This is exemplified by IncFII, IncFI, IncB/O and IncI1 replicons in *E. coli*, IncI1 and likely IncX1 in *S. enterica* or $IncFII_k$ in *K. pneumoniae*. Many of the broad host range replicons (IncP, IncA/C, IncR, IncL/M, IncN) were found rather occasionally in these hosts. Although some exceptions were seen, namely the IncP were often found with bla_{TEM-1} genes in particular in cattle, while IncA/C were often associated with bla_{TEM} variants encoding Extended Spectrum β-Lactamases in the diverse reservoirs. Evidences presented above indicate that the transmission of plasmids between animal and human Enterobacteriaceae is possible and it is likely that in some cases the resistance plasmids might have been delivered from animal to human strains via food chain.

Further studies are needed to determine the chromosomal progenitors of the resistance genes like bla_{TEM-1}. Determination of the very origins of resistance genes is crucial if further mobilization of these genes from the given source is to be prevented. Plasmids undoubtedly play a major role in transmission of *bla* genes also across the reservoirs. Solutions like whole genome sequencing should be preferentially applied in the future in order to efficiently detect, classify and tract the epidemiology of resistance plasmids in populations of Enterobacteriaceae.

Dansk Sammendrag

Escherichia coli, *Salmonella enterica* og *Klebsiella pneumoniae* tilhører familien Enterobacteriaceae og er vid udstrækning skyld i infektioner hos både mennesker og dyr; β-lactamer og i særdeleshed aminopenicilliner, cephalosporiner og carbapenemer er antibiotika, som ofte bruges til behandling af denne type infektioner. I den senere tid har der været en bekymrende stigning i resistens over for β-lactamer, dels i infektionsgivende bakterier, og dels i tidligere følsomme bakterier. Tidligere studier indikerer, at zoonotiske bakterier resistente over for forskellige antimikrobielle stoffer, samt indeholdende mobile genetiske elementer (MGEer), som f.eks. plasmider, kan overføres fra husdyr til mennesker (landarbejdere, dyrepassere osv.). Det overvejes derfor, om plasmider med *bla*-gener (gener, som koder for β-lactam-resistens) i enterobakterier hos mennesker, muligvis kan stamme fra dyrekilder.

Målet med denne Ph.d. afhandling var, at se om nært beslægtede resistens plasmider kan cirkulere mellem enterobakterier fra mennesker og fra produktions dyr; og hvis det er tilfældet, hvilke af disse plasmidtyper der kan være associeret med epidemiologiske typer af bla_{TEM} gener i Enterobacteriaceae ($bla_{\text{TEM-1}}$ and $bla_{\text{TEM-52}}$). Endvidere er der i denne Ph.d. afhandling kigget på sammenhængen mellem bla_{TEM} gener og transposable elementer for at få et bredere perspektiv af hvilke MGEer, der er involveret i mobilisering og spredning af disse *bla*-gener i diverse reservoirer.

I det første delprojekt blev relationen mellem plasmider med $bla_{\text{TEM-52}}$ gener isoleret fra mennesker, fjerkræ og fødevarer undersøgt. Toogtyve plasmider fra en kollektion med *E. coli* samt forskellige serotyper af *S. enterica* blev karakteriseret. Der blev fundet to typer af epidemiologiske plasmider, nemlig de gængse IncI1 $bla_{\text{TEM-52}}$ plasmider fundet i *E. coli* og *S. enterica* isoleret fra mennesker og fjerkræ; samt de gængse IncX1 plasmider fundet i *E. coli* og *S. enterica* isoleret fra humane infektioner, fjerkræ samt fødevarer (fjerkræ, slagtekylling og oksekød)

Det andet delprojekt var rettet mod verificering af, på hvilket plasmidskelet (scaffold) bla_{TEM-1} sædvanligvis er lokaliseret i *E. coli* fra mennesker og produktions dyr såsom svin, fjerkræ og kvæg. Syvoghalvtreds $bla_{\text{TEM-1}}$ plasmider fra mennesker og mere end hundrede $bla_{\text{TEM-1}}$ fra dyr (svin, kylling og kvæg) blev undersøgt. Der blev fundet to typer af epidemiologiske plasmider, IncI1 og IncB/O, i flere isolater fra både mennesker og dyr.

I studie I og II blev bla_{TEM} genet ofte fundet på Tn*3*-familie transposoner, og Tn*2* transposoner var det mest udbredte.

I det tredje delprojekt blev plasmider fra halvtreds *K. pneumoniae* isolater fra humane infektioner samt fra overfladevand (betegnet som miljøprøver) typebestemt. Udvælgelsen af plasmiderne skete tilfældigt og var ikke baseret på definerede resistens markører. En ny multiplex PCR (mPCR) blev designet til typebestemmelse af replikaser af plasmiderne fra *K. pneumoniae*. Denne metode blev anvendt sammen med standard PCR-baseret replikon typning (PBRT). $IncFII_k$, IncR og den nye type replikon repIV blev oftest fundet. Generelt set fandt man forskelle i udbredelsen af de forskellige replikaser fundet med mPCR i mennesker i forhold til dem fra miljøprøver. Et højt antal af repII og repIV positive og ikke-typebare blev fundet i isolater fra blod, repIV og repV så ud til at være karakteristisk for plasmiderne fra miljøprøverne.

Den samlede data fra de tre studier tyder på, at bla_{TEM} ofte er placeret på et værtsspecifikt plasmid såsom IncFII, IncFI, IncB/O og IncI1 replikoner i *E. Coli*, IncI1 og IncX1 i *S. enterica* and $IncFII_k$ i *K. pneumonia*; eller i mindre grad på de mindre værtsspecifikke plasmider tilhørende replikoner såsom IncP, IncA/C, IncR, IncL/M og IncN. Overordnet set har denne Ph.d. afhandling givet vigtig evidens for, at relaterede bla_{TEM} plasmider kan cirkulere mellem enterobakterier fra både dyr og mennesker. Bla_{TEM} plasmider kan være udvekslet mellem bakterier fra mennesker og dyr enten *in vivo*, i mave-tarm kanalen eller i ydre omgiverlser.

Translated by Lisbeth Andersen with assistance of Berith E. Knudsen

List of abbreviations

ABC - ATP- Binding Cassette

APEC- Avian pathogenic EC

ATP - Adenosine Triphosphate

BHR- Broad host range

bla – genes coding for β- lactamases

CC- Clonal complex

CMT - complex mutant TEM β- lactamases

ctRNA- countertranscribed RNA

DAEC –Diffuse- adhering EC

DR - Direct repeats

EAEC –Entero-aggregative EC

EC – *Escherichia coli*

EEX- Entry exclusion

EIEC –Entero-invasive EC

EPEC – Entero-pathogenic EC

ESBLs- Extended Spectrum β-Lactamases

ETEC –Entero-toxinogenic EC

Ex-PEC – Extra-intestinal pathogenic EC

GI – Gastro intestinal (tract)

Hfr- High frequency recombination

HTG- Horizontal gene transfer

HUS- haemolitic uraemic syndrome

ICE- Integrative conjugative element

Inc – Incompatibility

IR - Inverted repeats

IRT- Inhibitor resistant TEM β-Lactamases

IS- Insertion sequence

MATE – Multidrug & Toxin Extrusion System

MBL- Metallo- β-Lactamase

MGE- Mobile genetic element

MHR-Moderate host range

MPF- Mating pair formation (region)

MSF - Major Facilitator Subfamily

NAG - N-acatyloglucosamine

NAM - N-acetylmuramic acid

NHR- Narrow host range

NT- non -typable

NTS - non- typhoidal *Salmonella*

PBP(s) - Penicillin Binding Proteins(s)

PBRT- PCR-based replicon typing

PCR – Polymerase chain reaction

pMLST- Plasmid Multilocus Sequence Typing

RC – Rolling circle

RND –Resistance/ Nodulation/ Cell Division

RST- Replicon sequence typing

SMR - Small Efflux Regulators

ssDNA – single stranded DNA

ST- Sequence type

STEC –Shiga-toxin producing EC

STEC –Shiga-toxin producing EC

TC- Transconjugant

TF- Transformant

UTI- Urinary tract infections

VTEC –Verotoxin producing EC

WT- Wild type

Objective of the study and outline of the thesis

Elaboration of the technique allowing for mass-production of penicillin in 1940-ties opened a new antibiotic era in the history of medicine and microbiology [120]. It became a common belief that bacteria causing infections could be easily eradicated with proper antimicrobials designed against them. However, it soon became obvious that bacteria are capable of developing various mechanisms to resist or overcome the actions of many antimicrobials.

Bacteria belonging to the family Enterobacteriacae can function both as true and opportunistic pathogens. The most common Enterobacteriaceae causing infections in humans and animals are *E.coli*, *K. pneumoniae* and serovars of *S. enterica*. The majority of these infections have so far been treated with β-lactam antimicrobials. This is believed to be the reason of selection for the *bla* genes in Enterobacteriaceae.

Antibiotics are produced by microorganisms present in the natural environments. It has been therefore suggested that the genes encoding also resistance to these natural compounds have been present in the environment long before the introduction of antibiotics in treatment.

Nowadays it is a known fact that the genes encoding resistance to antibiotics (and as explained later also to other antimicrobials) often reside on platforms composed of diverse mobile genetic elements. Resistance genes can be part of transposable elements and the latter might integrate onto a variety of self-transmissible or mobilizable plasmid scaffolds. In view of this it is not surprising that the selective pressure imposed by the use of antimicrobials might have stimulated mobilization of the resistance genes from the environment to the animal and human reservoirs. Gaining an inside knowledge about the molecular platforms driving that transmission is essential if one would like to predict the evolution and distribution pathways of the resistance genes. An ultimate goal is to apply the basic knowledge gained in studies like this one in order to control and possibly prevent the further spread of resistance among the key infectious bacteria.

This work aimed at verifying the diversity of resistance plasmids originating primarily from *E. coli* and also from *K. pnaumoniae* and *S. enterica*. The following questions were raised:

i) What is the diversity of and relationship between plasmids with bla_{TEM} genes circulating in Enterobacteriaceae, in particular *E. coli* from humans and from food production animals or meat products (Manuscripts I and II)

ii) Could the bla_{TEM} genes have been transmitted from the animal sources to Enterobacteriaceae causing infections in humans; or were the bla_{TEM} genes acquired from independent sources by plasmids endogenous to humans and animals (Manuscripts I and II)

iii) How plasmid host range, host specificity and incompatibility influence the potential of these MGEs to spread the bla_{TEM} genes among the key enteric species like *E. coli, S. enterica* and *K. pnaumoniae* (Manuscripts I, II and III)

iv) What is the diversity of transposable elements harbouring bla_{TEM} genes found on plasmids in *E. coli* and other key enteric bacteria in humans and animals (Manuscripts I and II)

The thesis is divided into three main sections. Section I gives an overview of β-lactams and their application against key infectious bacteria belonging to the Enterobacteriaceae family. The origins and spread of resistance to β-lactams and the role of mobile genetic elements in that spread are discussed. Details on the relevant mobile genetic elements involved in HGT and in the transmission of β-lactam resistance are introduced. Primarily, the attention is given to plasmids as the main actors in the resistance transmission (in particular resistance mediated by β-lactamases). Principles of plasmid biology, ecology and typing/classification methods are presented. Role of transposable elements in transmission of *bla* genes is accentuated. A large fraction of the theoretical background refers to *E. coli*, as this is a well studied model organism as well as a common pathogen of humans and animals. In section II the theories are plotted against the results obtained in this study. Evidences observed in this study suggesting plasmid transfer between animals and humans are highlighted. An attempt is made to point out which plasmid platforms -and why these - are particularly involved in the transmission of bla_{TEM} genes in Enterobacteriaceae from humans and from food production animals. The role

of plasmids in transmission and evolution of genes encoding the TEM type β-lactamases is discussed. Factors like plasmid host specificity and host range in relation to transmissible resistance and dynamics of microbial ecosystems is also commented. Section II is finalized with the overall conclusions from the study and the suggestions for the future studies. Section III is composed of the three manuscripts produced during this study:

I. **Bielak,E**., Bergenholtz,R.D., Jorgensen,M.S., Sorensen,S.J., Hansen,L.H., Hasman,H., 2011. Investigation of diversity of plasmids carrying the $bla_{\text{TEM-52}}$ gene. *J. Antimicrob. Chemother.*, 66, 2465-2474.

II. **Bielak, E.** ,Knudsen, B. E., Haugaard, E. , Andersen, L., Hammerum, A. M., Schønheyder, H. C., Porsbo, L. ans Hasman H. Characterization of plasmids carrying $bla_{\text{TEM-1}}$ gene from humans, poultry, cattle and pigs – manuscript in preparation

III. **Bielak, E**., Struve, C. and Hasman, H. Typing of plasmids from *Klebsiella pneumoniae* from human infections and from the environment with a novel multiplex PCR - manuscript in preparation

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SECTION I.

1. ANTIMICROBIALS AND ORIGINS OF ANTIMICROBIAL RESISTANCE IN ENTEROBACTERIACEAE

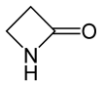
Bacterial infections due to opportunistic and true pathogens were the cause of morbidity and mortality in humans and animals probably since the very beginnings of their existence. However, many of the microorganisms like fungi and certain species of bacteria turned out to naturally produce small molecules that can kill or inhibit the growth of other bacteria. These natural compounds were termed antibiotics. All compounds with bacteriostatic or bactericidal properties including the semi- and fully synthetic compounds are termed antibacterials, or more generally antimicrobials [53].

Currently a whole range of antimicrobials is available for treatment of infections caused by both the Gram negative as well as Gram positive bacteria. Some of the major families of antimicrobials are β-lactams, aminoglycosides, chloramphenicols, ketolides, lincosamides, macrolides, oxazolidinones, streptogramins, tetracyclins, rifamycins, sulphonamides and trimethoprim, quinolones, nitrofurans and nitroimidazoles, glycopeptides, fosfomycin, bacitracin, polymyxins and recently recognized colistins [66]. The compounds within the given families share some common molecular structures and they usually act against the defined targets within the bacterial cells. These targets are usually the key steps of biochemical pathways in bacteria (nucleic acids replication, transcription and translation, protein synthesis, metabolism) or the key structural components (cell wall and cytoplasmic membranes). Owing to some structural differences between Gram positive and Gram negative bacteria and the diverse properties of individual species (fermenters *vs* non-fermenters, aerobic *vs* anaerobic, intracellular *vs* extracellular etc.) diverse antimicrobial families might be more or less effective against particular infectious agents. The different families of antimicrobials, their mode of action and specificity against given bacteria are reviewed elsewhere [66]. In this study the attention is given to β-lactams as they are one of the most commonly prescribed antimicrobials in human and animal chemotherapy in the world.

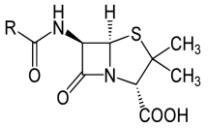
1.1 Structure and mechanism of action of β-lactams

In terms of organic chemistry a lactam is a cyclic amide and its name originates from the combination of *lact*one and *am*ide. Prefixes in a form of Greek alphabet letters (β, γ, δ etc.) in front of the lactam's name inform about the number of carbon atoms present in the lactam ring in addition to the carbonyl moiety. The β-lactam is a ring composed of two carbon atoms

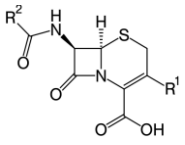
in addition to the third carbon in the carbonyl group. The simplest example of such β-lactam is presented on the Figure 1a. The β-lactam family of antimicrobials is comprised of several groups of compounds, namely penicillins (covering also a subgroup of aminopenicillins), penems and carbapenems, cephalosporins and cephamycins often collectively named as cephems, monobactams and β-lactam inhibitors. All of these compounds contain the β-lactam ring in their core structures (Figures 1b -f) [66].



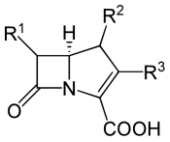
a) β-lactam



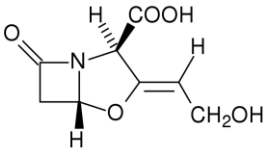
b) penicillins



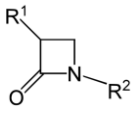
c) cephalosporins



d) carbapenems



e) clavulanic acid



f) monobactams

Figure 1. Chemical structures of the main groups of β-lactam family of antimicrobials; a- the β-lactam ring; b- general structure of penicillins; c- general structure of cephalosporins; d- general structure of carbapenems; e- example of β-lactamase inhibitor (clavulanic acid); f- core structure of monobactams

The targets for β-lactam antimicrobials are located within the bacterial cell walls. The organisation of the cell envelopes in Gram negative bacteria differs from the one found in Gram positive bacteria and this will be discussed further in this section (Figure 2). Nevertheless, in both cases the cell walls are composed of peptidoglycan layers (murein) [177]. To understand the mechanism of action of β-lactams it is first necessary to look at the composition of the peptidoglycan and how the latter is synthesised in bacteria. Each peptidoglycan layer is composed of two types of disaccharides, the N-acatyloglucosamine (NAG) and the N-acetylmuramic acid (NAM) [177]. An individual peptidoglycan layer can be imagined as a set of parallel glycan chains that are cross linked to each other. The individual chains are made up of NAG and NAM connected via amide bonds in a 'head-to-tail' fashion. These amide bonds are formed in a transglycosylation reaction catalysed by enzymes called penicillin binding proteins (PBPs). The cross- linkage of the parallel glycan chains occurs as a result transpeptidation reaction which is another reaction catalysed by the PBPs enzymes. Each of the NAM is also associated with a pentapeptide chain; these pentapeptides are termed stem peptides. In the transpeptidation reaction the stem peptides attached to the NAM

moieties belonging to the neighbour glycan chains are made to form bridges. In *E. coli* before any processing (e.g. the transpeptidation) the five stem peptides are usually the following: L-Ala$_{(1)}$–DGlu$_{(2)}$–*m*-A2pm$_{(3)}$–D-Ala$_{(4)}$–D-Ala$_{(5)}$, where the numbers in the subscripts indicate the position on the pentapeptide counting from the NAM [177]. In this case the bridge is formed between the D-Ala$_{(4)}$ of one of the stem chain with *m*-A2pm$_{(3)}$ of another stem chain attached to the NAM on the neighbor glycan chain[177] (Figure 3). In this way a layer of crosslinked glycan chains can be formed. The chemistry of crosslinking in some bacteria may differ from the one described here for *E. coli* [177]. However, in any case the multiple peptidoglycan layers form the final peptidoglycan grid called the cell wall.

PBPs own their names due to the ability to bind penicillin and as it has been shown later they also have affinity to other β-lactam antimicrobials. There exists a range of different PBP enzymes in the different microorganisms and usually several types of PBPs are simultaneously present in the given bacterium [177]. The β-lactam antimicrobials resemble structurally the chemical substrates utilized by the PBPs enzymes in the transpeptidation reaction. The mechanism of action of the β-lactam antimicrobials is as follows: the PBPs bind the β-lactam antimicrobials instead of their own substrates. However, the bond formed has a covalent nature resulting in inactivation of the PBPs. The cell wall synthesis is stalled and bacterium undergoes destruction during its growth or upon the cell division. This means also, that β-lactams are bactericidal only to these growing or dividing cells [177]. Efficacy of the given β-lactam against the bacterium depends on the ability of the compound to reach its target PBPs. In the Gram positive bacteria, the cell envelope is composed of only one plasma membrane and the thick outer cell wall comprised of many layers of peptidoglycan, hence the PBPs are more directly accessible for β-lactam antimicrobials (Figure 2). In Gram negative bacteria (among others Enterobacteriaceae) the cell envelope consists of the plasma membrane, the cell wall made up of a thinner peptidoglycan matrix and then the outer cellular membrane (Figure 2).

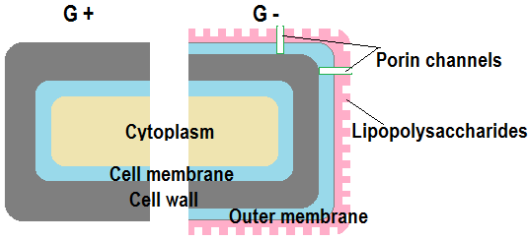


Figure 2. Schematic comparison of the cell envelopes in Gram positive and Gram negative bacteria. For explanation please refere to the text above.

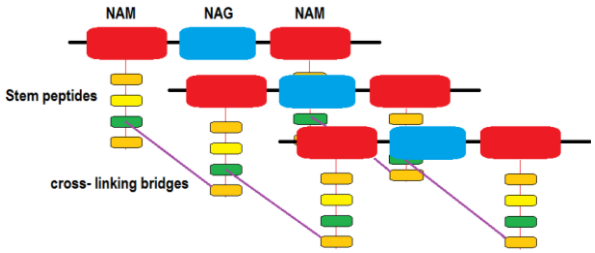
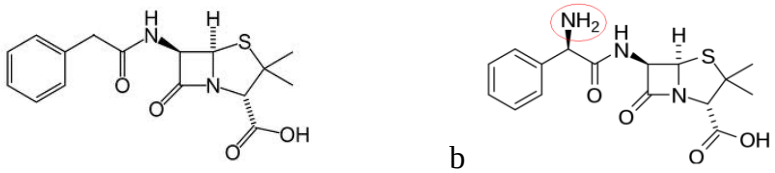


Figure 3. Schematic overview of the arrangement of cross-linked peptidoglycan chains within the bacterial cell wall.

The classical penicillins G and V are are not active against Gram negative bacteria due to the natural impermeability (or rather very low permeability) of the outer membranes in these [180]. Addition of an amine group to the classical penicillin G and synthesis of aminopenicillins made the latter compounds able to penetrate through the outer membrane of the Gram negative bacteria (Figure 4). This phenomenon is related to the porins that are the proteins forming channels located in the outer membranes of Gram negative bacteria [180]. The porin channels might be composed of a variety of protein subunits (monomers or multimers) and might allow for transport of only certain types of molecules to the inside of the bacterial cells (by diffusion). Semi-synthetic descendants of penicillin G, namely the aminoampicillins as well as many cephalosporins and other β –lactams readily traverse through the porin channels and can therefore be used in chemotherapy against many of the Gram negatives like *E. coli*, *K pneumoniae*, *S. enterica* and others not listed. The ability of the β –lactams to penetrate the pores was shown to be related to the size of the molecule (smaller molecules penetrate more easily), the charge of the given β –lactam (positive charge increase the penetration rate of the β –lactams through the certain types of porin channels) and the hydrophobicity of the given β-lactams (hydrophilic compounds penetrate faster through the pores) [180].



a b

Figure 4. Structure of penicillin G (a) and example of aminopenicillin (ampicillin, b); in the circle indicated is the amino group (b).

1. 2 General applications of β-lactams in human and animal chemotherapy and implications in mobilization and spread of *bla* genes in Enterobacteriaceae

1.2.1 Enteric bacteria -their significance for human and animal health and zoonotic potential

Enterobacteriaceae is a family of Gram negative bacteria covering large number of genera found in humans, animals, insects as well as different environmental niche including plants,

soil, surface waters etc.[97] Among others the three most clinically significant Enterobacteriaceae are *E. coli, S. enterica* and *K. pneumoniae* [79]; *E. coli* and *K. pneumoniae* are largely responsible for nosocomial infections as well as community acquired infections in humans. *S. enterica* is one of the most common food-borne pathogens of humans especially in the developing countries [77]. Among others in humans these enteric bacteria typically cause UTI (*K. pneumoniae* and *E. coli*), gastroenteritis (*E. coli, S. enterica*), typhoidal fevers (*S. enterica*) and pneumonia (*K. pneumoniae*) [77 ;79].

Zoonotic bacteria are those that can be transmitted from animals to humans and *vice versa* and can cause infection to the recipient organism [42]. *E. coli* and *S.enterica* are known for their zoonotic potential. In fact, both are particularly problematic agents causing infections in many of the food production animals like poultry, cattle and pigs [10;65;91].

In this thesis a bacterium that can live in natural environments will be termed 'free living'.

E.coli is not a free living bacterium and is almost exclusively associated with human or animal GI tract, which is the reason why this species is a suitable indicator of possible faecal contaminations of waters or food products [96]. *E. coli* can be either normally found as a commensal in the human and animal GI tracts, where it may occasionally cause infections in immuno-compromised individuals or when the inoculums size is large. Commensal GI flora of humans and animals was shown to be composed of resident and transient populations of *E. coli* strains [96]. The first ones may persist even up to years; the diversity of the transient strains may vary over the day. Some of the *E. coli* strains may function as true pathogens and these are generally classified as diarrheagenic (or intestinal pathogenic) or as extraintestinal pathogenic strains (Ex-PEC)[28]; the latter may cause infections in variety of sites in the affected organisms [99]. Six types of the diarrheagenic *E. coli* (EC) were recognized based on the detected virulence factors, serotypes and symptoms of the infections, namely enteropathogenic (EPEC), enteroinvasive (EIEC), entrotoxigenic (ETEC), Shiga toxin producing (STEC; variants of the latter are the enterohemorrhagic- EHEC and verocytotoxigenic- VTEC strains), enteroaggregative (EAEC) and diffuse adhering (DAEC)[96].

Well described zoonotic agent is *E. coli* O157:H7 that is known to be often shed by cattle and may cause EHEC type infections in humans (actually antimicrobials are not part of the treatment of EHEC diseases as this may increase the risk of subsequent development of life threateing haemolitic uraemic syndrome- HUS) [10]. It has also been suggested that the UTIs in humans due the *E. coli* can be in some cases zoonotic infections [55;94;95]. Jacobsen *et al.*[94;95] found clonally related *E. coli* isolated from meat and from the UTI in humans. In another

study *E. coli* isolates from UTIs in humans were shown to share similar patterns of resistance genes with isolates from pigs, broiler chickens and meat products from these two types of animals [79]. Food production animals may be reservoirs of Ex-PEC strains that may cause infections in humans [89]. Although, in some cases it can be difficult to unequivocally determine the clonal relationship of the Ex-PEC isolates from the given animal or meat sample with the particular isolate causing infection in human. This is due to the frequent remodelling of the genomes in *E.coli* and horizontal acquisitions of genetic elements from other bacteria [10].

S. enterica is not considered as a part of normal flora in humans although some serovars (Typhi and Paratyphi) may specifically colonize and infect humans[77]; non- typhoidal *Salmonella* (NTS) serovars are found in GI tracts of diverse animals, in particular birds and even reptiles often without causing infection to these hosts [77;80]. NTS are known to cause zoonoses in humans and the classical example of such NTS is the *S.*Typhimurium DT104 which is the most frequently detected serovar in animals and humans [128].

K. pneumoniae is not a classical zoonotic bacterium; however, it is a common inhabitant of soil, surface waters and plants and can be also found in GI tracts of mammals [159;178]. It produces number of virulence factors but is considered an opportunistic pathogen of humans and animals [159;178;217].

Because of the ability of the above described enteric bacteria to circulate between humans and animals and also in the environmental niches they are considered as vehicles potentially transmitting resistance genes between these reservoirs. As discussed in further sections the virulence of and resistance to antimicrobials in these key infectious Enterobacteriaceae is often related to the presence of plasmids [99].

1.2.2 β-lactams in human and animal chemotherapy and implications in mobilization and spread of *bla* genes on plasmids in Enterobacteriaceae

Often the same or similar classes of compounds are used in human and animal chemotherapy to treat infections caused by the same species of bacteria. Namely, amplicillin, amoxicillin (more often amoxicillin combined with clavulanate) and first generation cephalosporins are commonly prescribed to humans and companion animals to treat among others UTI (typically caused by Enterobacriaceae like *E. coli*, *K. pneumoniae* and *Proteus* species), salmonellosis and diverse types of diarrhoea, also infections caused by other than the enteric bacteria, examples being lyme disease caused by *Borrelia burgdorferi* (borreliosis; often treated with amoxycillin), infections caused by *Haemophilus influenzae* (one of the most

common agents causing otitis media) and *Listeria monocytogenes* (listeriosis), *Neisseria gonorhoe* (diversity of infection types), skin infections etc.[9;209] In animal food production aminopenicillins have found application in treatment of the common diseases like bovine respiratory disease, mastitis and footrot in cattle, infections common for farrowing pigs (metritis, agalactia), collibacillosis and salmonellosis (in particular in poultry and pigs) [126].

Currently four classes of cephalosporins are available on the market. 1st and some of the 2nd generation cephalosporins have similar spectrum of activity to aminopenicillins [9]; the 3rd and higher generations of cephalosporins are valuable drugs with broad application in treatment of severe, often hospital acquired infections in humans; 4th generation cephalosporins are the drugs of choice in treatment of infections caused by Enterobacteriaceae resistant to other β-lactams and importantly, they are the first group of cephalosporins active against *Pseudomonas aeruginosa* [9]. The classical 3rd and 4th generation cephalosporins used in humans are cefotaxime, ceftazidime (3rd) and cefepime (4th). Rather limited range of cephalosporins is approved to be used in food production animals. The 3rd generation cephalosporin designed strictly for veterinary use is ceftiofur [90]. This compound has different pharmacokinetic properties compared to other cephalosporins [90]. Carbapenems and monobactams are reserved mainly for human chemotherapy to treat severe infections caused by Gram negatives like *K. pneumoniae* and *E. coli* resistant to other classes of β-lactams [166;210].

As illustrated above, β-lactams have broad application in human and animal chemotherapy. Intense and often inappropriate usages of antimicrobials lead to the development of resistance in bacteria [126]. Antimicrobials are -or rather should be - prescribed to humans in the cases when a bacterial infection was diagnosed or as prevention when a high risk of bacterial infection exists, for example prior or after surgeries in hospitals [115]. In acute cases antimicrobials can be prescribed before the laboratory results arrive to the physician, if there are indications of bacterial infection [115]. In animals antimicrobials typically are administered to treat diagnosed infections but also as prophylaxis, metaphylaxis and as growth promoters (no longer in Europe, discussed below) [167;181;185]. Because of the practical reasons the treatment of livestock often involves administration of the antimicrobials in feed or water given to the whole herd of animals. This is especially valid for intensively reared animals like pigs and poultry [181]. It is believed that mainly these practices lead to the generation of reservoirs of resistance genes in food- production animals [181] .

There are various hypotheses explaining how the resistance was acquired by previously susceptible Enterobacteriaceae. The occurrence of the new resistance genes (or new variants

of existing resistance genes) may be related to the temporary increase in mutation rates in bacteria exposed to stress conditions; these bacteria were called transient hypermutators [3]. Another hypotheses points out on so called persister cells present in bacterial populations [119]. Persisters may enter a dormant stage during which they do not grow or divide compared to the remaining population (which would be especially effective way to avoid the action of antimicrobials like β-lactams) [98]; in exchange they may survive the temporary higher concentrations of antimicrobials and they may reverse later to the normal growth stage. If exposed to sub-inhibitory levels after antimicrobial treatment round or to repetitive usage of antimicrobials these persister bacteria might have developed mutations or acquired resistance genes on the MGEs like plasmids [119]. Persisters have been previously described in *E. coli* and in *P. aeruginosa* [98;119]. It is actually a common phenomenon that resistance is detected in individuals re-currently treated with antimicrobials or treated with low doses for a prolonged time [126;156;216]. The latter especially applies to usage of antimicrobials as growth promoters in animals [126]. Because of these reasons the use of antimicrobials as growth promoters is no longer allowed in Europe [37;62].

In practice resistance to each of the above mentioned classes of β-lactams have been observed in Enterobactertiaceae [154]. In majority of reported cases the most problematic resistances resided on self-transmissible or mobilizable MGEs like plasmids[33;154] .

1.2.3 Evidences of transmission of antimicrobial resistance from animals to humans

The main point of discussion is currently whether the resistance bacteria and hence resistance plasmids could have been transmitted to humans from animals [32;126;181;188]. Introduction of the antimicrobials in livestock production was previously shown to be followed by the occurrence of the resistant bacteria in the treated animals as well as in humans (caretakers, their family members etc.). Evidences exist that the transmission of resistance might have taken place from animals to humans. Many of these evidences were not direct and were based on the similarities between the resistance profiles of bacteria (among others indicatory *E. coli*) isolated from animals (poultry, pigs, cattle) and from humans having contact with these animals (farm workers, animal caretakers, their family members) [2;117;168;203;203]. Direct evidences have been based on the molecular methods allowing for detection of either clonally related, resistant zoonotic bacteria in humans and animals[108] or related resistance plasmids isolated from humans and animals [126]. Studies dated back to 1970s and 1980s reported the following cases: transmission of the same resistant *E. coli* (in fact harbouring a plasmid known currently as IncFII) was demonstrated from chickens to farm workers and their

family members by Levy *et al.*[118]; similar plasmids conferring resistance to ampicillin and tetracycline were described by Holmberg *et al.* in diverse *S. enterica* from beef and from infected humans with diagnosed salmonellosis [89]; similar plasmids encoding resistance to aminoglycoside- streptothricin were detected in different *E. coli* from pigs fed with this antimicrobial and also in farm workers and their families in the study of Hummel *et al.* [93]. In 1990s a range of similar plasmids conferring resistance to gentamycin and apramycin was detected in *E. coli* and *S. enterica* from cattle and *E. coli* from diseased humans in Belgium[38]. More than a decade later Cloeckaert *et al.* described an epidemic IncI1 $bla_{\text{TEM-52}}$ plasmid (now known to be ST5/CC5[71;100]) in various serovars of *S. enterica* from human (France) and poultry (Belgium)[43]. Recently, a range of *E. coli* isolates from humans and from poultry harbouring the same IncI1 $bla_{\text{CTX-M-1}}$ (mainly ST7/CC5) and IncI1 $bla_{\text{TEM-52}}$ (ST10, ST36/ CC5) plasmids were reported in the Netherlands by Leverstein-van Hall *et al.*[116]

Madec *at al.* [124] demonstrated that the IncFII (F31:A4:B1[205] /IncFII and F2:A-:B-/IncFII) plasmids harbouring the $bla_{\text{CTX-M-15}}$ genes and IncI1 (CC31) circulated between diverse clones of *E. coli* from humans and animals in France.

1.2.4 Routes of resistance transmission between animals and humans and evidences of *in vivo* plasmid transfer between Enterobacteriaceae in the GI tract

Transmission of the resistance may occur either via direct contact with the animal harbouring the resistant bacteria or via food chain (consumption of contaminated food or drinking water) [126]. It has been suggested that the resistant bacteria may reside on the particles suspended in the air which may then be deposited on the skin of or inhaled by the exposed individuals [86]. Contamination of agricultural products, in particular sprouts, with coli form bacteria resistant to multiple antimicrobials was also reported [17]. In passing, Shiga-toxin producing *E. coli* causing an outbreak in Germany in May 2011 resulting in fifty reported deaths was shown to harbour a large $bla_{\text{CTX-M-15}}$ plasmid [169]; the probable source of this bacterium were sprouts likely contaminated with faeces (curiously, as indicated later $bla_{\text{CTX-M-15}}$ gene is considered to be typically associated with humans rather than animals) [24]. Contamination of meat products was described among others by Wu *et al.*[214;215] (tetracycline and sulphonamide resistant *E. coli* were detected in pig carcasses in Denmark) and Zhao *et al.*[218] (*E. coli* resistant to divers antimicrobials were detected in retail meat in USA). Bortolaia *et al.* isolated CTX-M producing *E. coli* from chicken egg in Denmark, in this case interestingly the samples originated from organic farms with no history of antimicrobial usage [18].

Resistant bacteria transmitted from animals to humans or *vice versa* may either cause infections (zoonoses) or the resistance genes may transfer to bacteria colonizing the gut of the recipient host organism. There exist experimental evidences that the transmission of plasmids between diverse Enterobacteriaceae may take place *in vivo* in the gut of animals and humans. Transmission of an IncR bla_{TEM-1} pKPN5 plasmid from *K. pneumoniae* MGH78578 (human isolate) to *E. coli* recipient in the gut of mice[179] and transmission of plasmids between diverse *E. coli* isolates in the guts of human infants treated with antimicrobials [107, 15] was previously demonstrated. Trobos *et al*. demonstrated transfer of sulphonamide and ampicillin resistance on a plasmid from animal *E. coli* isolate to another *E. coli* recipient in the intestines of adult humans [201]; in her study the transfer was not stimulated by the presence of selective pressure (e.i. the human volunteers participating in that study were not ingesting antimicrobials for a month prior and then during the study).

Resistance transmission most probably occurs also outside the human reservoirs. Resistant bacteria originating from animal faeces[109] may contaminate fruits and vegetables (possibly via faecals-containing fertilizers), soils and the surface waters and from these sources may be picked up by other animals transmitting the resistance genes further (likely on plasmids) [1;5]. Enterobacteriaceae resistant to the ESBL-antimicrobials were detected in wild birds on the most remote inhabited island in the world, The Easter Island where the usage of antimicrobials on this island is suppose to be limited mainly to the local hospitals[1]. Increased attention recently was given also to the travellers who are suspected of transmitting the resistant bacteria from so called high prevalence countries to the countries where the occurrence of resistance in humans has been so far low (like Denmark or generally Scandinavian countries) [211]. Swedish study from 2010 demonstrated that 24 of 100 human volunteers previously not colonized by *E. coli* producing ESBLs came back from their travels outside the northern Europe and they were colonized by *E. coli* producing CTX-M, TEM or SHV ESBLs [196].

In summary to the above sections, isolates with the same resistance patters (in some cases confirmed to be clonally related) were reported in humans and in animals, typically following the introduction of an antimicrobial to the livestock production. Moreover, the same resistance plasmids were found in the diverse isolates from animals and humans; some of these plasmids were self-transmissible. Transmission of plasmids between enteric bacteria in human an animal guts was demonstrated. Considering these data, it seems likely that resistance plasmids found in humans may originate from animal sources.

1.3 Mechanisms of resistance to β-lactams in Enterobacteriaceae

There are four general mechanisms that bacteria utilize to resist the action of antimicrobials. These mechanisms are i) alteration of the outer-membrane permeability ii) over-expression of efflux pumps iii) antimicrobial target alteration and iv) enzymatic deactivation of the antimicrobials [154].

They will be briefly introduced below. More details will be described regarding the origins of and resistance mechanisms by β-lactamases as this is the mechanism typically utilized by *E. coli*, *K. pneumoniae*, *S. enterica* and also many of the other Enterobacteriaceae. Particular attention will be given to the TEM- type β-lactamases that are plasmid encoded enzymes dominating in ampicillin resistant *E. coli* of humans and animals [22;52;146;157;160].

1.3.1 Impermeability to antimicrobials

Alteration of the permeability refers mainly to mechanisms found in Gram negative bacteria due to the presence of the outer lipopolisaccharide membrane in these microorganisms. As indicated above, the efficacy of the given antimicrobial against the bacterium largely depends on the ability of the compound to reach the proper target. While in Gram positive bacteria the PBPs are readily available targets for the β -lactam antimicrobials, it has been shown that the diverse β -lactams present different efficacy in penetrating the outer membranes of the Gram negatives [180].The resistance mechanism via the membrane permeability alteration is based on the regulation of the expression of the specific porins and this influences the uptake of the β -lactams from the outside of the cell to the periplasmic space in the Gram negative bacteria. In some cases the porins specific for the bacterium can be even lost thus preventing the uptake of the given β –lactam [208]. The genes coding for the major porin proteins and their regulators are typically located on the chromosomes in bacteria [67]. Resistance to higher generations of cephalosporins and carbapenems via porin deficiency was observed in number of bacteria including *K. pneumoniae* and *E. coli* [162].

1.3.2 Resistance due to the active efflux systems

Once the antimicrobial reached the periplasmic space or the cytosol it is still possible for some bacteria to actively pump out the compound via the efflux pumps systems. An active efflux pump requires an energy for its action and therefore the ejection of the antimicrobial from the cell is coupled either with the hydrogen proton movement into the cell or with the hydrolysis of the ATP. There are five major classes of efflux systems in bacteria. These families are the Major Facilitator Subfamily (MSF, widely distributed in bacteria and often

plasmid and further transposon encoded; often involved in resistance to antiseptics), the Small Efflux Regulators (SMR), Resistance/ Nodulation/ Cell Division system (RND, very efficient efflux system widely distributed on chromosomes of Gram negative bacteria), ATP-Binding Cassette (ABC system, often chromosomally encoded by the antibiotic producing bacteria) and the Multidrug and Toxin Extrusion system (MATE) [162]. Typically the resistance problem in Gram negative bacteria is associated with the mutations causing the over-expression of the efflux systems, thus allowing the bacterium to efficiently pump out a wide range of the small molecules including antimicrobials like β-lactams [162]. In *E. coli* and also in *N. gonorhoeae* and *P. aeruginosa* mainly RND type systems were described conferring the β -lactam resistance [138].

1.3.3 Resistance by target alteration

In terms of β -lactam resistance the target alteration could be the modification of PBPs in a way that they lose the affinity to these antimicrobials. This was achieved in many Gram positive bacteria due to chromosomal mutations within the genes encoding the PBPs [176]. Currently, methicillin and oxacillin resistant *S. aureus* (MRSA/ORSA) are the common problem not only in the health care units but also in the community. In Enterobacteriaceae mutations in the bla_{TEM} genes causing the resistance of the encoded TEM enzymes to β-lactamase inhibitors could be considered as target alteration mechanism (author's comment).

1.3.4 Resistance due to enzymatic modification of the antimicrobials

Bacteria can produce enzymes capable of deactivating some of the antimicrobials. The enzymes may catalyze different reactions to chemically modify antimicrobials; examples of these reactions are acetylation, phorsphorylation, nucleotidylation and many others that are studied in details elsewhere [213]. The enzymatic hydrolysis is the mechanisms utilized by enzymes β-lactamases to degrade the β-lactam compounds both in Gram positive and Gram negative bacteria (usually β-lactamases are found in Gram negative bacteria). In Gram positive bacteria β-lactamases are often extracullar, while in Gram negative they are typically retained in the periplasmic space [121]. β-lactamases and their mechanism of action will be explained in more details below.

1.3.4.1 β- lactamases in Enterobacteriaceae- classification and mechanism of action

β- lactamases are probably the most common reason of resistance to β- lactams in Gram negative bacteria. There exist a range of different enzymes β- lactamases. Following the Ambler structural classification it is possible to group the majority of these enzymes found in

Enterobacteriaceae into four classes, A, B, C and D [170]. A, C and D classes have serine in their active sites. Otherwise, these three groups do not seem to share common primary structures. This indicates that they are probably distantly related with each other (which may be a result of convergent evolution) [170]. The B class enzymes have the metal ion in the active centre, although the general mechanism of hydrolysis of the β- lactam ring is similar to the one utilized by A, C and D enzymes.

β- lactamases can also be classified based on their substrate specificity. Bush and Jacoby [26] proposed an updated classification scheme combining the substrate specificities and structural similarities of the diverse β- lactamases. According to the Bush classification class A and D of β- lactamases belong to the group 2 of serine β- lactamases, class C enzymes correspond to group 1 cephalosporinases and class B correspond to group 3 metallo- β- lactamases (MBLs).

In clinical settings and also in animals the most commonly encountered in Enterobacteriaceae are class A enzymes: TEM, SHV and CTX-M [20]. Class A comprises β- lactamases like penicilinases, Extended Spectrum β-lactamases (ESBLs) or inhibitor resistant β- lactamases. The first ones hydrolyze penicillins and also some of the early generations of cephalosporins. TEM-1 and its derivative TEM-2 and also SHV-1 are the most commonly detected penicillinases in *E. coli*. Overall, in *E. coli* class A β-lactamases are almost exclusively located on transferable plasmids.

ESBLs are defined as β-lactamases capable of hydrolysing oxyimino-cephalosporins and that that are inhibited by clavulanic acid [27;122]. Oxyimino-cephalosporins are β-lactams harbouring oxyimino-aminothiazoyl side chains. Oxyimino groups are found on the third and fourth generation cephalosporins and the monocyclic β-lactams (for example azerotram). The SHV and TEM ESBLs are believed to be evolutionary descendants of the TEM-1 and SHV-1 penicillinases. The first ESBLs of SHV and TEM types have been described in *E. coli* and *K. pneumoniae* already in 1980s [122]. Since then almost 200 variants of TEM enzymes and more than 180 variants of SHV-type enzymes have been described. Many if not a majority of these are ESBLs. In the mid-1990s the TEM-52 ESBLs was first detected in a *K. pneumoniae* isolate [163]. Due to its stability and spectrum of activity TEM-52 became one of the most commonly detected TEM type ESBLs in *E. coli*, *S. enterica* and *K. pneumoniae* [147]. The most commonly detected SHV types ESBLs are SHV-2 and SHV-5 [50].

In 1990s the CTX-M ESBLs began to infiltrate the clinically relevant Enterobacteriaceae [137]. Possibly due to their higher efficacy against β- lactams like cefotaxime (this antimicrobial have high speed of penetration of the bacterial outer membranes[51]) the divers CTX-M in practice replaced the TEM and SHV β- lactamases. Nevertheless, the two latter enzymes are

still considered to significantly contribute to the resistance in the Enterobacteriaceae. Currently five major groups of CTX-M β -lactamases have been recognized, namely CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9 and CTX-M-25 [29]. Interestingly, the different groups are often highly prevalent in the specific geographical locations. CTX-M-14 (CTX-M-9 group) was shown to be most prevalent in Spain, while CTX-M-15 (CTX-M-1 group) was shown to be most prevalent in United Kingdome [29]. There seem to be also a reservoir bias, namely $bla_{\text{CTX-M-15}}$ genes are more frequently detected in humans [124;137;152] while $bla_{\text{CTX-M-1}}$ are more frequently detected in animals [137].

Some of the class A TEM and SHV β- lactamases evolved resistance to the inhibitors [20]. The TEM inhibitor resistant β- lactamases (IRTs) are often detected in *E. coli* isolates from urinary tract infections (UTIs). This is probably the side effect of the common application of amoxicillin-clavulanate in chemotherapy against these infections [30].

The B class covers the Metalo- β- lactamases (MBLs) and these enzymes have usually a zinc ion at their active site, although it is not always the case. Classical examples of MBLs are VIM and IMP type enzymes. [140] Class C β- lactamases is generally comprised of enzymes termed AmpCs which are typically encoded chromosomally; recently however increasing occurrence of plasmid encoded AmpC enzymes (class C) is being reported [155]. Class D is comprised of so called OXA –type enzymes. The class B- MBLs and class D- OXA enzymes are currently the major cause of resistance to caprbapenems mediated by β-lactamases in clinically relevant Enterobacteriaceae and also in *P. aeruginosa* and *Acinetobacter baumanii*, in which these enzymes are also typically associated with plasmids [140;154].

Reference sequences of the diverse variants of β- lactamases mentioned above are collected in the Lahey Clinic database at http://www.lahey.org/Studies/.

The mechanism of action is similar for all the β- lactamases [63;213]. These enzymes hydrolyse one of the bonds within the core β-lactam ring between the carbon and the nitrogen atom. Once the β- lactam ring is broken the molecule loses its activity as it is no longer recognized as a substrate by the bacterial housekeeping PBPs. The following steps are included in the chemistry of the β- lactam hydrolysis. The active serine residue of the A, C or D class enzymes nucleophilically attacks the carbonyl atom of the β- lactam ring. This generates a covalent but unstable enzyme- β- lactam intermediate. The covalent bond between the serine and the β- lactam is rapidly cleaved at the presence of water. As a result the enzyme is regenerated while the β- lactam ring is left degraded [63;213]. MBLs employ a different mechanism to open the β- lactam rings. They use the metal ion (typically zinc ion) to activate

the water molecule. A hydroxide ion is generated that attacks the β- lactam ring. Again the hydroxyl- β- lactam intermediate is unstable and falls apart producing the inactivated antimicrobial [63;213].

β- lactamases inhibitors are compounds having the β- lactam ring but with some exceptions they do not inactivate the PBPs in bacteria like the usual β- lactam antimicrobials [63]. Instead inhibitors inactivate β- lactamases and when combined with other β- lactam antimicrobials, the later are free to interact with their targets on PBPs. As indicated above some β -lactamases became or are resistant also to the inhibitor compounds [20;63].

1.3.4.2 Origins of the β- lactamases

The first reports of the enzymes penicillinases in *E. coli* are dated back to 1940-s, which corresponded to the start up of production of penicillin on a commercial scale [63;120]. The majority of the known β- lactamases probably evolved from the PBPs. PBPs own their names to the ability to bind penicillin and -as it has been shown later- they also had affinity to other β-lactam antimicrobials. There exists a range of PBP enzymes and usually several of these enzymes are present in diverse organisms [81;190]. They are found both as membrane-bound enzymes and as periplasmic proteins. Purified enzymes have been shown to function among others as D-alanine carboxypeptidases, peptidoglycan transpeptidases, and peptidoglycan endopeptidases. In all bacteria in which PBPs have been studied, these enzymes have been shown to catalyze more than one of the above reactions [190]. It has been shown the PBPs have the penicillin-insensitive transglycosylase domain at their N-terminal (involved in formation of linear glycan chains) and a penicillin-sensitive transpeptidase domain at the C-terminal (involved in cross-linking of the stem peptides) [8] . The serine is the key amino acid at the active site of transpeptidase domains and it is conserved in all members of the PBPs family [8]. This has implications into understanding of the occurrence and evolution of the resistance to β -lactams mediated by β-lactamases. The exact details on the structures of the different β-lactamases will not be given here. However, the A, C and D β-lactamases despite some overall differences at the amino acid sequence levels they all share the serine in their active sites. They could thus have evolved from the different PBPs. Moreover, it has been deduced that the substitution of one of the amino groups in the active centre of PBPs to water molecule changes the enzyme function from transpeptidase (PBPs) to a hydrolase (β-lactamases) [110;111]. Only the class B metalo- β-lactamases use another mechanism for hydrolysis of their β –lactam substrates, namely the metal ion instead of the serine in the

active site, as described above. Hall *et al*. suggested that MBLs had their very origins in Archea [82].

The PBPs are encoded by chromosomal genes in bacteria. In contrast, currently the most clinically problematic are the β-lactamases encoded by genes located on mobile elements like plasmids and transposons. On the other hand, it is now known that a number of these plasmid located *bla* genes have their analogs located on the bacterial chromosomes. Many of the Gram negative bacteria naturally produce the chromosomally encoded β-lactamases [20;155]. The most obvious evidence of the chromosomal 'very origins' of some of the plasmid encoded β-lactamases are illustrated by the cases of the AmpC β-lactamases (class C), the CTX-M type (class A) and the SHV (class A) β-lactamases. Species like *Enterobacter cloacae*, *Citrobacter freundii*, *Serratia marcescens*, and *Pseudomonas aeruginosa* were shown to be able to overproduce their chromosomal AmpC as a result of mutations in the promoter region of the gene [155]. However, over time some these 'foreign'AmpC β-lactamases were detected in *Klebsiella pneumoniae*, *Escherichia coli*, *Salmonella* and in other species and they were shown to be encoded from plasmids [155]. The bla_{SHV} are chromosomally encoded in *K. pneumonia*, but are currenly found on plasmids in other species. These examples clearly indicate the mobilization of the *amp*C genes (bla_{AmpC}) and bla_{SHV} from the bacterial chromosomes onto plasmids. Conversly, the CTX-M β-lactamases (class A) have been observed on plasmids in the clinical isolates of diverse Enterobacteriaceae but have been later shown to have their equivalents chromosomally encoded in *Kluyvera* species.In fact, the specific types of $bla_{\text{CTX-M}}$ genes found on plasmids originated from the different *Kluyvera* species. The $bla_{\text{CTX-M -9 \& 25}}$ probably originated from *K. georgiana* [161], while $bla_{\text{CTX-M-1 \& 2}}$ from *K. ascorbata* [92;139].

1.3.4.3 Origins and evolution of the TEM type β-lactamases

The chromosomal progenitor to the TEM β-lactamases was not yet revealed. The first record on TEM-1 β-lactamase is dated back to1963, when an *E. coli* strain harbouring a plasmid encoding β-lactamase was isolates from a Greek patient Temoniera, hence the abbreviation TEM [20;175]. TEM-1 is currently the most dominant transferable β-lactamase found worldwide mainly in *E. coli*, but also in other Enterobacteriaceae, in *P. aeruginosa*, *Haemophilus influenzae* and *N. gonorrhoeae* [20]. The $bla_{\text{TEM-1}}$ genes were the predecessors for numerous variants that encode the TEM type β-lactamases presenting diverse spectrum of activity against the different β-lactam antimicrobials. The variants of TEMs described in the Lahey reference data base (www.lahey.org/Studies/temtable.asp) correspond to proteins differing at the amino acid level between each other. These variants resulted from the miss-sense

mutations (mutations within the nucleotide codon sequence resulting in the change of the encoded amino acid)[3]. However, also silent point mutations are observed within the given *bla*$_{TEM}$ genes coding for the specific TEM variants. Until now nine alleles of the *bla*$_{TEM-1}$ gene have been detected on plasmids deposited in the public databases (GenBank). These alleles are designated with one letter suffixes, currently from *bla*$_{TEM-1a}$ to *bla*$_{TEM-1j}$ and they differ from each other by one to several nucleotide base pairs, although they all encode the same TEM-1 protein (the allele 'i' was not officially annotated on previously published sequences in GenBank, however the *bla*$_{TEM-1}$ gene found on pHCM1 - AL513383 probably constitute the tenth 'i' allele according to anlaysis performed in this study). In some cases the *bla*$_{TEM-1}$ alleles were defined not only for the *bla*$_{TEM-1}$ open reading frames, but also the mutations in the promoter regions were considered (e.g. *bla*$_{TEM-1d}$ and *bla*$_{TEM-1f}$)[199]. So far three alleles (a, b and –c) of the *bla*$_{TEM-52}$ variant of the gene encoding the TEM-52 β-lactamase were observed. Both the mis-sense and the silent point mutations are useful in tracing the pathways of evolution of the *bla*$_{TEM}$ genes.

Initially, the TEM-1 β-lactamases did not seem to present a major treat from a clinical point of view, as it should have been possible to use other classes of β-lactams to treat the infections caused by ampicillin resistance bacteria. However, it has been deduced that in some cases only one nucleotide substitutionin the *bla*$_{TEM-1}$ may result in an enzyme product called Extended Spectrum β- Lactamase (ESBLs)[20]. This is exemplified by *bla*$_{TEM-135}$ whcih differs by one nucleotide compared to *bla*$_{TEM-1b}$. Higher generations of cephalosporins harbouring the large side chains were initially simply too big to enter the active centres of the early TEM β-lactamases (as well of the early variants of SHV β-lactamases). Extensive usage of higher generations of cephalosporins most probably influenced the mutations and recombination frequency in some of the *bla*$_{TEM}$ genes[3;88;135;193]. As a result the active centre of the encoded enzymes underwent enlargement enabling the entrance of the different, more complex β-lactam substrates resulting in production of ESBLs TEM enzymes.

Certain amino acids within the TEM enzyme sequence play a critical role for the activity and stability of TEM β-lactamases enzymes [20;175]. Substitution of these amino acids was shown to result in either the ESBLs or the inhibitor resistance (IR) phenotypes conferred by the enzymes. The list and positions within the TEM-1 sequence of the amino acids whose substitution results in the ESBLs or IR phenotypes is given in the review of Bradford *et al.*[20]

Among the numerous TEM-type ESBLs observed in bacteria one of the most predominant types became the TEM-52 enzymes. Three substitutions are present in the TEM-52 compared to the TEM-1. Namely the glutamic acid at position 104 was substituted by lysine (E104K),

methionine at 182 was changed to threonine (M182T) and glycin at 238 was substituted by serine (G238S)[147]. It is suspected, that the substitutions E104K and G238S occurred as first ones resulting in the enlargement of the TEM active centre[147]. This is observed on the TEM-15 enzymes that differ from TEM-1 by the two substitutions, E104K and G238S. The last substitution M182T conferred the increased stability of the final TEM-52 structure [147]. It would actually be enough for the TEM-1 enzyme to receive a substitution at position G238 to significantly increase the size of the active centre and allow the enzyme to bind and hydrolyze the 3rd generation cephalosporine like cefotaxime [175]. The important lesson from the studies on the TEMs β-lactamases evolution was that the degree of 'success' of this evolution is the resultant of the enzyme stability and hydrolytic activity, like in the case of TEM-52. It is possible that the novel enzyme products may have increased activity against the higher classes of β-lactams but due to the instability of the protein structure they may in fact not be capable of conferring their function in the bacterium; or opposite, further mutations in the enzyme may be detrimental for the ESBL phenotype [147]. On the other hand the evolutionary potential of the TEMs as well as other classes of β-lactamases have not yet been fully utilized and there is still space for occurrence of new variants [147]. Interestingly, for many years no enzyme has been observed that would display the inhibitor resistance and ESBL phenotype simultaneously. Recently however, complex mutant TEM β- lactamases (CMTs) were described [30]. These enzymes combine the mutations typical for IRTs and ESBLs.

It is highly possible that the recombination between different alleles of the *bla*$_{TEM}$ genes that may be present in the same cell or on the same plasmid (possibly due to gene duplication[88]) contributes to the increasing variety of these genes. It has been previously suggested that gene conversion and homologous recombination might have played a critical role in the *bla*$_{TEM}$ evolution [88;135]. Evidences exist that allelic recombination most probably shaped the evolution of the *bla*$_{SHV}$ genes as well [7].

2. HORIZONTAL GENE TRANSFER, MOBILE GENETIC ELEMENTS AND SPREAD OF ANTIMIROBIAL RESISTANCE

Attention has been given above to the origins of the β-lactamases in bacteria. It has been pointed out that the *bla* genes are often associated with mobile platforms, which is the probable reason for their worldwide distribution. This section will focus on the basic biology of plasmids from Enterobacteriaceae (in particular *E. coli*) and their role in the transmission of antimicrobial resistance. Also other MGEs and the mechanisms of HGT will be discussed.

2.1 Modes of acquisition of resistance genes in bacteria- HGT

Some bacterial species are naturally insensitive or in other words intrinsically resistant to certain antimicrobials. This may be due to the lack of the antimicrobial target in the given species or due to the natural impermeability of the outer membrane to the given antimicrobial [197]. For example Gram negative bacteria like *E. coli* or *K. pneumoniae* are intrinsically resistant to penicillin G due to their low permeability to this antibiotic.

Acquired resistance refers to the resistance that appeared in the previously susceptible bacterial population as a result of mutation or as a result of acquisition of genes from exogenous DNA in the process of HGT [197]. There are three generally recognized mechanisms of HTG: transduction, transformation and conjugation. An accessory mechanism termed conduction has been recently described that is linked with conjugative transfer of DNA [74].

2.1.1 Transformation

Transformation is an uptake of the linear or circular double- stranded DNA by the bacterium directly from its surroundings [83]. Some bacteria are naturally competent which means they are naturally capable of uptake of DNA in this mode. *Bacillus subtilis* and *Streptococcus pneumoniae* are classical models of natural competence in Gram positive bacteria; the natural competence was described also in Gram negative bacteria like *H. influenzae* and *Neisseria* species [40].

2.1.2 Transduction

Transduction is a process of DNA transfer between bacteria mediated by bacteriophages (bacteria specific viruses) [83]. Upon infection the bectrophage first attaches to the receptors found on the bacterial surface (these receptors could be lipopolisaccharides, teichoic acids, pili and other surface antigens). Subsequently the bacteriophage injects its DNA to the bacterium. At this stage the bacteriophage can either undergo the lytic state or the lysogenic state. During the lytic pathway bacteriophage replicates itself and this is usually finalized by breakage of the bacterial cell and releasing the multiple new copies of the bactriophage [83]. If the bacteriphage enters the lysogenic state, it integrates into the bacterial chromosome as an inactive prophage. The prophage can be again activated and then enter the lytic pathway when induced by different stress factors, for example by means of UV light or other DNA damaging agents.

Two types of transduction mechanism have been described, the generalized and the specialized transduction. The generalized transduction refers to the situation, when only the

bacterial DNA is by a mistake packed into the phage capsid instead of the viral DNA [83]. In generalized transduction it is also possible that a whole plasmid, plasmid elements or a transposon DNA can be packed to the viral envelope. Upon the subsequent infection and injection of the DNA content to another host, the new genes can be inserted into the new host DNA by means of homologous recombination or transposition, if the transposon was initially packed. If a plasmid replicon was packed it can re-establish itself in the new host [83]. Specialized transduction refers to the situation, when the genes in the close proximity of the phage integration site on the bacterial DNA are accidentally excised and packed together with the viral DNA upon activation of the prophage. If the DNA incorporated into the phage is stable, the virus can replicate and infect other bacteria. Otherwise, it is also possible that the DNA fragment mobilized by the phage recombines with the DNA of the new host [83].

There are some evidences that the transduction might have been evolved in the transmission of the *bla* genes in Enterobacteriaceae. Study of Muniesa and co-workers showed that bacteriophages harbouring bla_{OXA} and bla_{PSE} type genes were present in sewage [136]. Colomer-Lluch *et al*. detected $bla_{\text{CTX-M}}$ and high prevalence of bla_{TEM} on DNA of bacteriophages isolated from faecal wastes from poultry, pigs and cattle [48; 49]. A sequence of a Phage 7 is deposited in GenBank (AF503408 from an unpublished study). According to the description available in GenBank this phage was isolated from human faeces and according to an *in silico* analysis (this study) it represents IncY group of replicons [130]. Its sequence contains the transposon Tn*2* linked with $bla_{\text{TEM-1b}}$ gene (this study). Nevertheless, the literature data regarding the impact of transduction in the transmission of *bla* genes is still scarce.

2.1.3 Conjugation

Conjugation and the related mechanisms are believed to be main contributors to the spread of antimicrobial resistance in Enterobacteriaceae .Conjugation is the direct transfer of one of the strands of a plasmid DNA (or another conjugative DNA molecule) from one bacterium to another [83]. The transfer is followed by the synthesis of the complementary DNA strands in the donor and in the recipient bacteria. Conjugation of integrative conjugative elements and conjugative transposons would be followed by the integration of such elements into host chromosome as these elements cannot self replicate. The transfer usually requires establishment of cell to cell contact and the expression of a conjugative channel through which the DNA could traverse from the donor bacterium [83].

There are in general two types of transferable plasmids: conjugative (self-transferable) plasmids and mobilizable plasmids [76]. The latter do not encode the full machinery required

for conjugation but they harbour as a minimum requirement their own conjugal transfer origin (*ori*T). Thus they can utilize for transfer the remaining protein machinery encoded by other plasmids present in bacterium. This phenomenon is called mobilization [76].

Conjugation was generally better studied in Gram negative bacteria due to the difficulties with establishing which factors are responsible for achiving the cell to cell contact in Gram positives; in Gram negatives this contact is mediated by conjugative pili [78]. The majority of the conjugative plasmids from Enterobacteriaceae encode the transfer systems that resemble the bacterial type IV secretion system (T4SS) [113]. Similar conjugation systems are also encoded by mobile elements like ICEs or conjugative transposons. The transfer regions of the self- conjugative plasmids (or ICEs) are comprised of three main elements encoding the relaxases, mating pair formation components (MPF) and the coupling protein [113]. Relaxases is a collective name describing family of enzymes catalysing site and strand specific nicking of a double- stranded DNA. In plasmids there are two types of relaxases, the replication initiatior proteins (Rep proteins) and the transfer initiator proteins (MOB proteins) [59;76]. In conjugation or mobilization the specific MOB protein binds to its specific site within the plasmid *ori*T [113]. MOB relaxase catalyses the nicking of one of the DNA strands at the *nic* site and initiates the transfer. A range of helper proteins is involved in the initiation of the transfer. Together with the MOB relaxase they bind to plasmid DNA at the initiation site and the resultant nucleoprotein complex is termed relaxosome [113]. The model explaining the conjugation mechanism of plasmids suggests that the relaxosome contacts the coupling protein that is linked to the channel formed within the donor cell envelope. The outer extension of the channel is termed a sex pilus and it contacts the recipient bacterium. The channel and the pilus are part of the transfer apparatus encoded by the MPF region on the plasmid. The coupling protein directs the relaxosome to the channel and the single stranded DNA is pushed through the channel. Experimental evidences suggest that the MOB relaxase traverses to the recipient together with the plasmid singe stranded DNA (*ss*DNA)[59;113]. When the ssDNA is fully transported to the recipient probably the MOB relaxase catalyses the recircularization and the host factors initiate the replication of the complementary plasmid DNA strand in the recipient[113]. The latter is then called transconjugant. The complementary strand of the plasmid DNA in the donor is being synthesized already while the transfer of the other strand is in progress.

Importantly, conjugative plasmids in Enterobacteriaceae can be either liquid maters capable of conjugation in liquids (typically with the T4SS like conjugation systems) or surface maters that require solid surfaces to conjugate[59]. The first type is represented by the IncF plasmids,

the example of latter type are IncW plasmids [173]. Regions allowing for conjugation both on the solid surfaces and in liquids were described on IncI1 plasmids [100].
Conjugative plasmids may also form co-integrates with another plasmids. If the co-integrate is not resolved prior the conjugation the two plasmids would be transported together to the recipient cell. This process is termed conduction [74].

2.2 Mobile genetic elements participating in the transmission of antibiotic resistance

The vehicles of the HGT in bacteria can be phages, plasmids, ICEs, conjugative transposons and transposable elements like insertion sequences (IS), transposons, integrons and other variants of these elements**.** Resistance genes might be associated with any of these elements and often the smaller transposable elements (IS-es, transposons) are integrated onto larger self-transmissible platforms like the ICEs, plasmids and finally chromosomes [189]. Below a broader introduction will be given to plasmids and in particular plasmids found in the family of Enterobacteriaceae. Also the MGEs specifically related with bla_{TEM} genes will be described with more details.

2.2.1 Plasmids - basic biology, classification and diversity in Enterobacteriaceae.

Plasmids are usually double- stranded circular DNA molecules found in a wide range of Prokaryotes (Bacteria and Archaea) and to lesser extend in some Eukaryotes. The linear plasmids have been described among others in *Streptomyces* and *Borrelia* species and some fungi [105]. The key property of plasmids is that they can replicate autonomously from their host chromosomes [34]. Similar to viruses, plasmids require the host proteins and substrates in order to express their own genes. Often the term *replicon* is used when describing a plasmid. In genetics, a replicon refers to a DNA or RNA molecule (or a region on that molecule) that can replicate from a single origin of replication. In case of a plasmid the term replicon refers to the minimal region that is indispensable for plasmids to replicate. In this thesis the replicon will be used to collectively describe the minimal region required for plasmid to replicate and to maintain itself at a characteristic copy number per host cell (replication, control and partitioning functions) [34]. Plasmids might be simple structures whose only functions seem to be their own replication. Such plasmids are designated as cryptic; or they can be sophisticated and complex systems encoding a variety of accessory functions in bacteria. Such complex systems are believed to be products of a continuous interplay between plasmid DNA acting like a selfish molecular parasite and selective pressure imposed by the changing external environment [189]. Perceiving a plasmid as a form of 'living' molecule makes it easier to

understand, that the evolution and spread of resistance to antimicrobials discussed further in the text is not only a matter of survival for bacteria exposed to those antimicrobials. It is actually a matter of survival for plasmids which in a way resemble leaving creatures willing to persist and replicate in a given niche and whenever possible to colonize other niches in order to increase their chances for survival [189]. Possibly as a result of this strive for survival the simple cryptic plasmids acquired some genes via DNA rearrangements (insertions, deletions) and homologous recombination events. Products of these genes either enabled plasmids to transfer to other bacterial hosts (plasmids developed transfer region), or offered the host bacterium some selective advantages to be able to persist in or colonize otherwise unavailable niche (evolution of accessory functions region) [189].

It has been deduced by researchers that acquiring more functions by plasmids and thus increasing in size might have become too much of an energetic burden for the host bacteria [164;204]. The latter might have therefore been more prone to lose such large plasmids upon the cell division, especially if the plasmid did not confer any selective advantage at the given time point. This imposed a need for plasmids for developing more stringent systems for control of their own replication and copy number (to diminish the energy requirements for the hosts)[204] and then active partitioning to daughter cells upon host replication (to assure stable inheritance on the cost of lower copy number)[19]. In some cases plasmids developed addiction systems assuring themselves that the bacteria would not lose the plasmid even if the function conferred by it is no longer essential for host survival [171]. Usually such addition systems are based on toxins and anti-toxins that at the presence of plasmid encoding both of them neutralize each other; upon loosing the plasmid the more stable and longer lasting toxin would kill the host [171].

Plasmids can be seen as the resultant products of the selective forces acting upon them and leading to DNA rearrangements. Acquisition of the new features and clustering of the core functions shaped by the plasmid-host- environment interactions resulted in modularity of plasmids structures [189]. Three basic modules were proposed for plasmids: the replication module (containing the control of replication elements), the transfer module and the accessory functions module. One could add to this list also the partitioning and maintenance units (covering respectively the active partitioning systems and the host addition systems) as well as host adaptation module related to the host range [189] .

2.2.2 Plasmid replication

Three modes of replication have been described in circular plasmids: the theta replication, strand displacement and rolling circle replication (RC) [60]. In the RC mode the replication initiation protein catalyses the nicking of the leading strand of the double-stranded plasmid DNA. The leading strand is then unwound while in the same time the new complementary leading strand is being synthesized on the lagging strand template. The unwound leading ssDNA is circularized and serves as template for the new complementary lagging strand [60]. RC plasmids were so far described primarily in Gram positive bacteria.

Strand displacement mechanism was described with most details in IncQ family of plasmids. This mode of replication involves three plasmid encoded replication proteins RepA (helicase), RepB (primase) and RepC (replication initiator). The region on the plasmid where the initiation of replication occurs is composed of the series of iterons followed by the GC rich region and then the AT rich region, downstream of the latter there are found two single stranded origins of replication, *ssi*A and *ssi*B located on the opposite DNA strands. The strands are termed respectively L and R. The RepC proteins bind to the iterons and together with RepA probably induce the opening of the two DNA strands downstream of the AT rich region. This results in exposition of the two *ssi* origins that are now available for priming by the RepB. The replication is conducted by the host polymerase and may proceed in one direction from one of the *ssi*-s or in both directions from both *ssi*-s simultaneously [58;123] (Figure 5) .The DNA strand that is not serving as the template for replication is being displaced by the newly synthesized one, thus the name of this mechanism of replication [60]. The lack of Okazaki fragments during the DNA replication is the unique feature of the strand displacement mechanism described in the IncQ replicons [123].

E. coli chromosome as well as many replicons of the large plasmids found in Enterobacteriaceae replicate via theta mode. In plasmids the theta replication may be initiated from one or from multiple origins and can be uni- or bi-directional, although in most cases the replication is unidirectional [60]. In the majority of plasmid families the initiation of the theta replication involves the replication initiator Rep proteins. The plasmid encoded replication initiator protein as well as a number of host and plasmid encoded helper proteins are assembled at the origin. This enables the opening of the doublestranded DNA. Similar to the strand displacement, the primase protein is synthesizing a short primer at the exposed single strand origin for the leading strand synthesis. The helicase protein is unwinding the doublestranded DNA at the newly initiated replication forks. However, contrary to the strand displacement, in theta replication also the laggings strand is synthesized simultaneously with

the leading strand following the same replication forks (Figure 6). The lagging strand is synthesized in a discontinuous fashion from the Okazaki fragments. During the plasmid theta replication a product resembling the Greek letter θ can have been observed under the electron microscope, which gave the origin to the name of this mechanism [60]. In some plasmids like those with ColE1 type replicons replication initiator protein is not required. Instead, a long RNA pre-primer designated as RNA II is constitutively transcribed by the host RNA polymerases [206]. The pre-primer is processed to achieve proper conformation and cleaved by host encoded RNaseH in the maturation process; after cleavage the pre-primer fragment that remains hybridized with the template plasmid DNA serves as the primer for plasmid DNA synthesis by host polymerases.

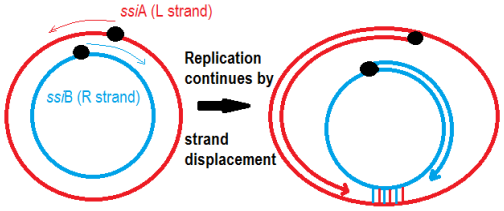


Figure 5. Replication by strand displacement (replication can occur from one or from both *ssi* origins; the example is not drawn to scale)

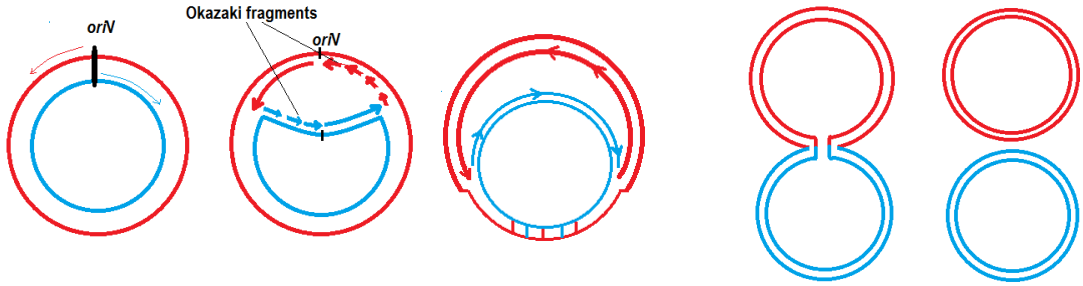


Figure 6. Replication by θ mechanism (example for bi-directional replication; uni-directional replication would occur in a similar fashion only with one replication forks proceeding in one direction)

2.2.3 Control of plasmid replication and its implications in plasmids incompatibility

A plasmid is thought to be an energetic burden for a bacterium as it requires the building blocks and assembly of the protein machinery for expression of its genes [69;164]. Therefore in the absence of selective pressure the bacterium may lose the plasmid during cell division. Plasmids developed strategies to assure themselves progression to daughter cells upon bacterial division. They may rely on random distribution to the daughter cells but are then usually present in higher copy numbers (such plasmids tend to be smaller as larger plasmids present in high copy are expected also to become unstable in bacterial cells) [69]. This increases the chances that at least one plasmid copy will be distributed further upon the cell division. On another hand, there is always a probability that when the host bacterium divides some of

the daughter cells will not receive the copy of the plasmid, while some cells will receive many copies [204]. This inequality in the distribution of plasmids to the daughter cells may lead to the increasing number of plasmid free cells in the populations and thus losses of even high copy number plasmids at the absence of selection against them [204]. It is proposed that an alternative strategy is active partitioning utilized by many plasmids. This mechanism is assuring them more stable inheritance to daughter cells. These plasmids are often present in lower copy number but are larger due to the additional DNA load [164].

Regardless the assumed partitioning strategy, plasmids need to be able to control their replication in order to assure that there is always a proper number of copies available for partitioning and also to be able to increase the replication rate if too low number of copies was received by the daughter cell after division [204]. Conversely, uncontrolled runaway replication would also lead to plasmid instability in the host [69]. Control of replication and copy number is thus essential for a plasmid stable inheritance. That implies plasmids need to be able to 'count themselves' and to control and adjust their replication frequency in order to achieve the desired copy number. Every plasmid replicon in the given host (and physiological conditions) has its characteristic, inherent copy number [206] . The copy numbers for different plasmids can vary from just one up to several hundreds [144]. The actual number of plasmid copies per cell can also fluctuate over time in the population in bacteria. This is normally corrected by the plasmid replication control systems. The copy counting can be imagined as counting of defined targets detected upon the plasmid replication initiation events. Finally, the control of plasmid replication is usually by one of two mechanisms based on the negative feedback loops, namely the iteron binding or by inhibitor-target circuit [206].

2.2.3.1 Replication control by iterons

Replicons controlled by this mode have a series of tandem repeats called iterons present within or/and in the close proximity of their origin of replication. Inverted repeats are often also present upstream the promoter for the *rep* gene encoding the Rep initiator protein [39;60]. These inverted repeats as well as the iterons contain a sequence 'code' recognized by the Rep protein. Some models indicated that Rep proteins can either be present as active monomers which can bind to the iterons; or Rep can be present in a form of dimers which, with some exceptions, are not capable of binding to iterons but they can bind to the inverted repeats inhibiting the transcription of Rep mRNA from its promoter. In the current models explaining the control by iterons, the Rep monomers bind to the origin iterons in the proximity of AT rich region and promote replication initiation. In some plasmids iterons are only present in

the origin region, while in other plasmids there exists a second set of regulatory iterons present further away from the key *ori*V. In the latter case the Rep monomers bind both to the origin iterons and to the regulatory iterons located away from the origin iterons. The origin iterons can thus participate both in the initiation of replication and the copy control. It is believed that the main copy control mechanism involves handcuffing of two plasmids via iterons to which the Rep proteins are bound. The iterons-Rep-iterons nucleoprotein complexes located on the two individual copies of plasmids can interact by locking the two origins together. The *ori*V-iterons-Rep complexes can also be locked together with the Rep bound to the regulatory iterons located further away from the *ori*V iterons. In the latter case the locking of the *ori*V-iterons with the other regulatory iterons mediated by the Rep complexes can occur within the same molecule or as handcuffing of individual plasmid molecules. Handcuffing of the origins prevents the initiation of replication. The process is reversible allowing for restoring of the replication when the copy numbers are too low. Classical examples of iteron controlled replicons in *E. coli* are among others IncP family plasmids, IncFIA and IncFIB replicons [39].

2.2.3.2 Replication control by inhibitory countertranscribed RNAs (ctRNAs)

Replication of the plasmid can be controlled by binding of an inhibitory molecule to a defined target on the replicon. Inhibition of the target results in the decrease of the replication rate [144]. One of such examples is control by antisense RNAs termed also ctRNAs. In plasmids whose replication is initiated by the Rep proteins, antisense RNAs are transcribed from the same DNA segment as the mRNA for the Rep proteins but in an opposite direction than the Rep mRNA. Antisense RNA has a short half life and it is always present in excess compared to its target [141;206]. This is due to the transcription of the ctRNA proceeds from a constitutive promoter, while the Rep mRNA promoter is normally under a transcriptional control. Antisense RNAs are complementary to the 5' end of the Rep- mRNAs and the two molecules interact with each other to form duplexes [141;206].

The initial contact is made via loops (hair-pin loops) which are structures formed on both the mRNA and the ctRNA [164]. After the initial complex is made, further duplex formation occurs and it prevents the translation of the Rep proteins from their mRNAs which results in the decrease of the initiator protein concentration [141]. This is followed by the decrease in the initiation of plasmid replication rate. In Enterobacteriaceae such control mechanism were described in IncFII , IncI- complex (IncI1, IncB/O, IncK , IncZ) and ColE1 replicons [164]. The details of how the duplex formation prevents the translation of the Rep protein may differ

between the various types of replicons. The general mechanism is similar to all of the aforementioned replicons; the key elements in these types control systems are the base pairs located in the loops of ctRNA and the loops of Rep mRNA that are interacting with each other and where the formation of the duplexes is initiated. Also the secondary structures called bulge-loops present on the ctRNA and the Rep mRNA (in addition to the aforementioned hair-pin loops) are of the key importance, as the formation of the inhibitory duplex is not only a matter of the exact complementary [141]. It is also necessary for the two molecules to properly align in the space (topologically) with respect to each other. Initially only the single stranded loop regions of the two RNAs interact. Then the target and inhibitor molecules need to be located in space in a way that the remaining complementary sequences on these two RNA molecules are also made to form further duplexes [141;144;206].

In ColE1 replicons which do not require the Rep protein for replication, the ctRNA forms duplex with the pre-primer RNAII preventing it from maturation and thus inhibiting the initiation of plasmid DNA replication[206].

Mutations in the DNA segment encoding the ctRNA and the Rep mRNA will not affect the complementarily of the two RNA molecules. They may however, affect the formation of secondary structures assumed by the transcribed RNA molecules as well as the composition of the key base pairs located in the interacting loops [141]. This in turn may result in decrease of the interaction strength between the target and the inhibitory loops, or decrease in speed of formation of the duplex after the initiation step. Overall, mutations may decrease the inhibition by the ctRNA of its corresponding target mRNA and as explained later, this may lead to the increases in copy numbers of such replicons. This may also influence the incompatibility properties of replicons with mutations affecting the ctRNA encoding region [144;164].

Certain proteins can play accessory roles in the control of plasmids replication driven by the ctRNAs. In a plasmid R1 which belongs to the IncFII family of replicons, the Rep protein can by translated from two types of mRNA transcribed from two promoters. In wild type conditions a long mRNA transcript containing the *cop*B and *rep* genes is constitutively expressed from the *cop*B promoter [60]. The Rep is normally translated from this long mRNA. There is a second promoter downstream of the *cop*B from which only the Rep mRNA can also be transcribed. The second promoter is usually suppressed by the CopB protein product that binds to this promoter. This promoter is released in emergency situations at very low copy levels detected [206]. The main control of Rep translation is still by the ctRNA that is complementary to the 5' *rep* regions on mRNAs transcribed from both types of promoters.

Although important, the control by CopB plays the secondary role [144]. In ColE1 plasmids the Rom (or Rop) protein was shown to influence the replication rate by stabilizing the initial complex between the ctRNA and its target on the pre-primer. In this case the Rop (Rom) proteins also have an accessory role in control [206].

2.2.3.3 Incompatibility of plasmids

If two plasmid replicon types cannot be stably maintained and replicated in the same cell line at the absence of selective pressure to maintain both plasmids, they are said to be incompatible [141;144]. Incompatibility (Inc) is therefore considered a measurement of plasmids relatedness. This property was used as a basis for typing and classification of plasmids to Inc groups (described in the next section) [36;54]. Incompatibility is a complex phenomenon and it may be either a result of a random selection of only one type of the two similar plasmids for replication and partitioning during bacterial division, which may lead to inequalities in replicons distribution and later dominance of cells harbouring only one type of plasmids in bacteria population (partition incompatibility [19]); or it may be due to the expression by plasmids of certain incompatibility functions (for example copy number control factors) that cause the instability and finally loss from the bacterial population of the other similar plasmids. The later scenario will be highlighted with more details further in this subsection in order to explain i) the basics behind plasmid classification to incompatibility families and ii) what are the shortcomings of grouping of replicons based on the incompatibility assays [54;144].

As underlined above replication control is critical for plasmid maintenance and inheritance. Plasmid replication and stability may be influenced by presence of other types of replicons in the same cells. In practice, three simplified scenarios are possible [144]. In the first scenario an external plasmid p1 with resistance marker r1 ($p1^{r1}$) may arise to a bacterium that already harbours a plasmid with an identical replicon to p1, but different resistance marker r2 ($p1^{r2}$). The loci encoding the replication and control functions of the two replicons are undistinguishable so their inherent copy numbers are also the same. In such situation the two plasmids would ‘see’ each other as additional copies of the same replicon p1. They would be able to ‘count’ each other as additional copies and the replication of both plasmids would be set up to adjust to the overall copy number equal to the (one) inherent copy number of either of the replicons. The loss of one of the plasmids at the absence of selection for any of them would be due to a random selection for replication and partitioning [144;206].

Second scenario assumes that two different replicons would arise to the same cell. If the difference would be enough for plasmids not to ‘see’ each other’s replicons for counting,

replication control machineries of the two different replicons would not recognize the foreign targets for copy 'counting'. The two plasmids would probably not influence each other's control circuits and be able to co-exist with each other. They would be compatible [141;145].

The third possible situation would be, when two plasmids with similar but not identical replicons would arise in the same cell. Although the two plasmids would have their own replication and control circuits it would be possible that they would be somehow sensitive to the similar replication control functions expressed by the other co-residing replicons (due to the similarities in the targets being under the control of these controlling factors). It is probable that one of the replicons could be more sensitive to the replication inhibitors (iterons, ctRNAs and possibly other factors) expressed by the other replicon type. In this situation the replication rate of the (more) sensitive plasmid would be more strongly repressed. As there are always fluctuations in the total plasmid copy numbers obtained by the daughter cells, it is possible that at some point the bacterium would receive less copies of the more strongly repressed plasmid. Since the replication of this plasmid would be continuously repressed by the presence of the additional control factors expressed by the second similar replicon, this plasmid would not be able to correct its copy numbers during the next rounds of bacteria division [141]. Such plasmid would become unstable and lost from the population. In this case the two plasmids would be incompatible because of the similarity in their copy control elements.

It needs to be underlined that the above cases are simplifications and the details behind the mechanisms of plasmids incompatibility are not yet fully elucidated [19;144]. Any element of the plasmid replicon participating in the replication, copy number control and also in partitioning may in fact contribute to the incompatibility properties displayed by the given plasmid [19;144].

2.2.3.4 Drawbacks of incompatibility assays and development of replicon typing methods

In 1970s Datta and Hedges proposed classification scheme for plasmid based on their incompatibility properties [54]. The incompatibility assay requires introduction of two plasmids with different selective markers into the bacterium. Subsequently the double transformant selected initially to contain both replicons needs to be propagated for a number of generations in selection free conditions. Finally the transformants are plated out onto media containing the individual selections for the respective plasmids and also on the media with selections for both plasmids. The ability of plasmids to co-exist is assessed based on the frequency of

occurrence of the transformants harbouring the two plasmids. Development of incompatibility assays was a step forward in studies on plasmids classification and hence epidemiology. The major Inc groups in Enterobacteriaceae, i. e. IncFII, IncFI, IncI1(IncI1=Iα=Iβ) and IncIε (IncIγ was established later[195]), IncX, IncO (= Inc B/O), IncN, IncA (=IncA/C), IncT, IncW, IncP and IncL (=IncL/M), have been defined in early 1970ies by this method [85]. However, the procedure is time consuming [144]. Also, the obstacles here are that many wild type plasmids harbours more than one replicon on the same scaffolds and they can utilize the different replicons if needed. This makes it possible for them to overcome the incompatibility problem. Therefore preferably a vector containing only the replicon of interest and the selective marker should be produced and used in such incompatibility assays. Moreover, the output of the assay may not always give the real overview on the relatedness of two replicon sequences. This is especially truth for the ctRNA controlled replicons [141;144]. Few bases pair mutations in the region encoding the key inhibitor may in this case result a new incompatibility group compared to the wild type replicon. Although the two replicons would be closely related, they might give a compatible phenotype [144]. Partial incompatibility (or partial compatibility) and one-side incompatibility might also be observed which makes the results of the assays difficult to interpret in a straightforward manner [144].

Finally, phenomenon's like surface and entry exclusion were shown to interfere with the interpretation of the incompatibility assays [75]. Some of the F-like plasmids described in *E. coli* were shown to encode surface proteins that prevented formation of stable co-aggregates between the mating cells already harbouring the similar F-like plasmids. This phenomenon was called surface exclusion. It was also shown that plasmids sharing the similarities in their transfer regions may also exclude each other's entry to the given recipient cell harbouring such plasmids even if the mating co-aggregate required for conjugation was formed between the donor and the recipient cell. In this case another factor was shown to prevent the entry of the plasmid from the donor to the recipient cell with the similar plasmid. Namely, it was proposed that a host plasmid encoded protein which is located in the periplasm of the recipient cell (harbouring this plasmid) probably prevents setting-up of the initiation machinery for donor plasmid DNA transfer at the recipient inner membrane. This was termed entry exclusion (EEX) [75]. The EEX functions were described for the variety of conjugative (IncF-family, IncI, IncP, IncN, IncW, IncX, IncHI) as well as mobilizable (ColE1) plasmids and even ICE's [75]. Surface and entry exclusion may result in miss-interpretation that the two replicons examined in incompatibility assay are incompatible, despite in this case the actual feature being tested would be the transfer module.

At the end of 1980ies the hybridization probes have been designed by Couturier an co-workers [54]. The probes corresponded to the known or putative incompatibility loci of nineteen plasmid replicons from *E. coli*, namely repFIA, -FIB, -FIC, FIIA, -9 (of com9 Inc group),-I1, -B/O, -K, -HI1 and -HI2,-L/M, -N, -P, -O, -T, -U, -W- X and –Y. The method was a breakthrough as it allowed for relatively rapid detection and classification of plasmid replicons to Inc groups. This methodology was still laborious if large sample number was to be screened. Over a decade later, in 2005 a rapid RCR based replicon typing method (PBRT) was published by Carattoli *et al*.[35] Again the targets for the PBRT were preferentially the key incompatibility determinants found on the diverse replicon sequences. Eighteen of the aforementioned rep groups (further termed Inc groups) were targeted by the PBRT (the rep9 was not included as target in the PBRT). This method was originally designed based primarily on replicons of plasmids described in *E. coli*. Later in time owing to the increasing number of sequences available in public databases replicon variants characteristic for other species, like *Salmonella*, *K. pneumoniae* and *Yersinia* were added to the PBRT scheme [72;205].

2.3 Classification of plasmids

Classification of plasmids is essential if one would like to be able to study their epidemiological relationships [56]. The reference point for classification of plasmids can be any of the modules described in the previous sections. In this section the relevant classification and typing schemes of plasmids in Enterobacteriaceae will be described.

2.3.1 Classification of replicons to Inc families

Currently there are 27 Inc groups recognized in Enterobacteriaceae by Plasmid Section of the National Collection Type Culture, Colindale, London [34;54]. The groups are designated with alphabet letters from IncA to IncZ [189]. In practice more than 30 replicon groups were described in the literature and also subgroups were recognized in this family of bacteria. This number will probably change along with the increasing number of replicon sequences available through next-generation sequencing. The terminology may sometimes be confusing. Overall, an assumption can be made that Inc families are comprised of Inc groups and the latter may be further divided into Inc subgroups (family>group>subgroup). As indicated below, plasmids within the given family and group may in some cases be compatible with each other.

The first Inc group (that later became a family) was described in 1960ies; this family is known today as IncF and is composed of IncFII, IncFIA, -B and –C replicons ans well as other F-like replicons which have been rather rarely detected in *E. coli* [56]. Within the

F- family IncFIC group -although similar to- is compatible with the IncFII replicons [13]. In many cases the different groups within the same families were distinguished based on the DNA sequence comparison, although the representatives of these different groups were still incompatible with each other. This is exemplified by the IncX family, where IncX1 R485 plasmid was shown to be incompatible with IncX2 R6K plasmid, despite an appreciable amount of differences in the two replicon sequences and also in the remaining key plasmids components [104]. Within the IncI-complex family of replicons, the IncB/O replicons are incompatible with the IncZ replicons, but both of these replicons are compatible with the family members IncI1, IncIγ and IncK replicons (the three latter replicons are incompatible with each other) [164;165].

Currently available PBRT methods target a selected range of the classical Inc groups listed in the above section [36]. The novel types of replicons like IncFII host specific variants ($FII_{k,}$ $FII_{s,}$ FII_{y}) and IncFIB *Salmonella* –specific variant ($IncFIB_{S}$), IncR, IncQ, IncU and IncX1 can be detected by the updated PCR protocols as described by Garcia-Fernandez *et al.*[72], Villa *et al.* [205] and in Manuscript I in this study [16] (here two PCRs were described targeting the replicator protein of IncX1A plasmid pE001 and the *rep*A of IncN-like plamids).

Some of the Inc groups are not 'real' plasmids; IncY group covers the bacteriophages that in the lysogenic cells are circular and can self replicate just like plasmids do [130]. The IncJ group covers the Integrative Conjugative Elements (ICEs) related to SXT/R391 [212]. These elements were initially miss-interpreted as plasmids. As mentioned elsewhere in the text, ICEs can conjugate but cannot self –replicate [212].

Furthermore, some of the Inc groups from Enterobacteriaceae overlap with the plasmid Inc groups of *Pseudomonas* (designated IncP- with a number). Namely, IncA/C group corresponds to IncP-3, IncU corresponds to IncP-6, IncP corresponds to IncP1 and IncQ corresponds to IncP-4, respectively [189].

2.3.2 Classification of plasmids based on the characteristics of transfer module

The plasmid encoded pili may serve as attachments sites for bacteriophages [44-47]. Phages can attach to the shafts on a pilus (these are usually very specific phages), or to the tip of the pilus (less specific filamentous and tailed phages) [186]. In the early history of plasmid classification in *E. coli* it was shown that certain bacteriophages could bind specifically to the conjugative pili of plasmids from the particular Inc families. The C type phages bound specifically to pili encoded by the IncC family plasmids (later this family was renamed to IncA/C-complex)[186]; the M bacteriophages were shown to be specific for pili of IncM (later IncL/M) plasmids [46],

X bacteriophages were shown to attach to pili of IncX plasmids[44], also phages specific for pili of IncI1α and IncI2 plasmids were described [45]. In 1980s it was further shown that these conjugative pili encoded by plasmids from the diverse Inc groups determined for *E. coli* could be grouped to three morphological types: thin flexible, thick flexible and rigid pili. The thin flexible pili were encoded by the I- complex plasmids, the thick flexible pili were encoded by IncC (= IncA/C), IncD, IncF, IncHI1 and IncHI1, IncJ, IncT, IncV and IncX plasmids; the third type was encoded by IncM (=IncL/M), IncN, IncP and IncW plasmids [21]. In fact, in some of the literature sources it has been proposed to group plasmids into four major incompatibility groups based on their genetic relatedness and structures of pili. These major groups were IncF (covering IncF, -S, -C, -D and –J replicons and elements), IncI (covering the IncI, -B/O and –K replicons), Ti-plasmid group (IncX, -H, -N and –T) and the IncP (IncP, -M, -W and –U) [31;207].

Recent studies of Francia *et al*. and Garcillan-Barcia *et al*. pointed out that both the conjugative and mobilizable plasmids could actually be classified based on other than the above mentioned characteristic (i. e. pilus) of their transfer regions [68;74;76]. Garcillian-Barcia and co-workers proposed an alternative classification scheme for transferable plasmids based on the similarities of the key MOB relaxases encoded by both the self-conjugative and the mobilizable plasmids [74;76]. Available sequences of plasmids from the public data bases (GenBank) were grouped to seven MOB families. Six of these were major MOB families MOB_F, MOB_H, MOB_C, MOB_Q, MOB_P and MOB_V [74;76]. Phylogenetic trees have been produced for each of the MOB groups using the available sequenced plasmids as references. It appeared that plasmids were generally grouped into branches of the MOB trees according to the Inc groups of their replicons and also according to the genes encoding coupling proteins. In other words, plasmids from the same Inc group usually belonged to the same branches within the given MOB family tree[59]. This implied that there is a strong correlation between the replicon and the transfer region on the plasmids. Possibly in the early evolutionary history plasmids had more conserved backbones composed of one type of the replicon and the specific transfer system. Later acquisition of new replicon modules and rearrangements in the plasmid scaffolds resulted in the larger replicons diversity observed to date. Overall, the *mob* genes seemed to be more conserved than the replicons on plasmids [59;74]. Diagnostic signatures representing the conserved and characteristic motifs of the MOB relaxases have been deduced for each of the MOB families [59;74]. The proposed method for screening and classification assumes an amplification of the *mob* targets with the use of degenerate primers, sequencing and then allocation of the sequences into the MOB

phylogenetic trees. Based on the position of the obtained sequence within the tree and its clustering pattern with the reference plasmids, further information could be deduced regarding the plasmid transfer system and also the corresponding replicon. Multireplicon transferable plasmids harbour typically only one transfer region[59]. Thus if other replicons than the one deduced from the tree would be detected on the multireplicon plasmid, it could be suspected that the other replicons have been acquired later in the evolution of such plasmid. Swapping of the whole transfer module between plasmids is also possible, although it is believed to be rather a rare event [59;74].

The *mob* genes seem to be suitable targets for typing species for screening and possibly epidemiological purposes. The method is still being elaborated and suffers from drawbacks as well. The main one being that some plasmids may not harbour the MOB relaxases at all; or the plasmid may encode relaxase that was previously not characterised thus it may be omitted by the detection based on signatures designed initially only for the known relaxases [74]. Although the MOB based method have undobtedely a good potential, it is currently not used in the classical plasmid screening and characterisation studies. Replicon typing continues to be the most commonly applied method.

2.3.3 Classification of plasmids based on function

Based on the accessory region, termed also an adaptation module, plasmids can be designated as i) resistance plasmids, when they encode resistance to antimicrobials ii) the ColE plasmids encoding colicins [87]; the latter being compounds with killing activity against other bacteria iii) degradative or catabolic plasmids, when they harbour genes for degradation of organic compounds or toxins and iv) virulence plasmids [189]. The different groups will not be described with details here. Worth mentioning is however, that many of the virulence plasmids found in Enterobacteriaceae have been shown to be associated with the resistance genes. Especially IncF plasmids and also IncI1 plasmids usually harbour the virulence genes and they are also among the predominant types associated with the resistance genes among others in *E. coli*, *S. enterica*, *K.pneumoniae* and other Enterobacteriaceae [33;34;99]. The virulence plasmids described in the literature typically encoded colonization factors (CF) like adhesins (plasmid encoded pili and fimbriae), aggregation factors (auto-aggregative adherence factors of EAEC), toxins (enterotoxins in ETEC strains), haemolysins (plasmids in majority of STEC and EHEC strains), iron sequestering systems (encoded by diverse virulence plasmids), invasion of the host cells factors (encoded by plasmids in EIEC and *Shigella*), colicins and necrotizing factors (exemplified by cytotoxic necrotizing factor CTN encoded by Vir plasmids in *E. coli*) [99].

2.3.4 Plasmids Multilocus Sequence Typing (pMLST)

The classical method for analysing the epidemiological relationship between plasmids is the RFLP [198]. This fingerprint-based method is not a classification or detection procedure for plasmids, however it allows for direct comparison of plasmids and their relatedness. Recent advances in sequencing methods allowed for development of another typing/classification method for plasmids that simultaneously allows for tracking down the epidemiology of these MGEs, namely the pMLST. The general procedure involves PCR amplification of the selected 'housekeeping' genes present on the backbones of plasmids belonging the given family or group; sequencing of these selected genes and then blasting the sequences against the corresponding reference data base. Reference allele types are pre-defined for the specific genes in the corresponding reference databases based on previously sequenced and annotated plasmids [158]. Sequence types are then defined for each of the genes on the analysed plasmid and combination of these STs defines the plasmid species (pMLST type) and hence its relationship to other plasmids from the same family. pMLST is currently available for IncF family of plasmids (in this case only the target locus or loci on the replicon/s are required for sequencing thus the abbreviation RST- replicon sequence typing), IncN, IncI1 and IncHI1 and -HI2 (here the term double locus sequence typing was applied by the authors) [70;71;73;205]. In the near future such method should also be available for IncX family of plasmids (personal communication with Alessandra Carattoli, Istituto Superiore di Sanità, Rome).

The drawback of these kinds of PCR /sequencing based methods is a large workload related to handling of multiple samples. Simplex PCR amplifications of multiple genes per one plasmid need to be initially set up. Subsequent purifications of the PCR products and sequencing of each sample is still a costly procedure when large number of samples needs to be analyzed. Generation of an excessive number of new STs due to the occurrence of novel alleles might also become a problem. On the other hand the main advantage of pMLST is obtaining valuable information regarding the plasmids backbones without necessity for sequencing of the whole plasmid. Single nucleotides polymorphisms can be tracked down for the selected genes. Rapid comparison of sequences from the distant geographical locations is also possible without the need for sending out the DNA preparations or the whole strains.

2.4 Other MGEs involved in the transmission of resistance genes

Plasmids may serve as mobile platforms onto which other MGEs can integrate or be excised from. Mobile DNA elements which movement is catalysed by so called transposases are collectively termed as transposable elements. There exists a whole variety of such mobile

elements and some of them like ICEs can in fact encode their own transfer systems to move between bacteria just like conjugative plasmids do. Unlike plasmids, these MGEs do not replicate autonomously [212].

2.4.1 Insertion sequences (IS) and composite transposons

The simplest in structure are class I transposons and this group in practice is comprised of IS elements [189]. They are composed of the genes encoding the transposases typically flanked by the characteristic end sequences, usually the inverted repeats (IRs)[189]. These end sequences are specifically recognized by the transposase upon the initiation of transposition. ISs usually utilize the mechanism termed 'cut and paste' to transfer to different DNA locations. This means that the IS is excised from the donor DNA strand and inserted into the recipient DNA strand. The hallmarks of such insertion are direct repeat sequences (DRs) at the target DNA flanking the incorporated IS [189]. Two IS sequences can also flank a region containing the gene(s) encoding resistance to antimicrobials. Such structures are called composite transposons. Often the genes encoding resistance to kanamycin, tetracycline and streptomycin are contained on the composite transposons. Classical examples are Tn*5* and Tn*10*. Composite transposons can be translocated either as a whole unit, or only one of the IS parts can transpose [189].

IS elements are believed to play essential role in integration of some of the plasmids into the bacterial chromosomes in the high frequency recombination cells (Hfr cells) [83]. Especially IncF plasmids harbouring the IS-es homologous to the targets on the bacterial chromosomes have been reported be able to recombine with and integrate into the chromosomal locations (in such case termed Hfr -plasmids) [133]. This phenomenon may lead to mobilization of chromosomal genes upon conjugation of such integrated plasmid to another host [83].

2.4.2 Class II transposons

Class II family transposons are generally composed of a transposase gene *tnp*A, a resolvase gene *tnp*R and are flanked by the IRs. Class II transposons often harbour additional genes encoding traits like antimicrobial resistance or degradation of organic compounds. According to Sota[189] Class II elements are currently grouped into five families, namely IS*1071*, Tn*3*-types, Tn*21*-types, Tn*4651*-types and Tn5393-types. The terminology used in the literature may be confusing; some authors collectively term the class II as Tn*3*-like transposons. However, in the following section the Tn*3*-like or Tn*3*-type will refer specifically to only one of the five families of the class II elements. Transposons from the Tn*3*- family have been shown to be predominant carriers of the bla_{TEM} genes in Enterobacteriaceae [6;151]. Three

subgroups were defined within the Tn*3* -family, namely Tn*1*, Tn*2* and Tn*3* transposons. Tn*3*-family of transposons display transposition immunity. That means that once the Tn*3*-type transposon is integrated into the plasmid scaffold, the plasmid becomes immune to the subsequent insertions of the similar Tn*3*-related element. The characteristic end sequences (IRs) are believed to confer the immunity to the reinsertion of the Tn*3* -like transposons[106]. It is common however, that other transposable elements like IS-es paste themselves into regions within the Tn*3*-like transposons. Typically IS*26* elements inserted within the *tnp*A regions of Tn*3* –related transposons were previously detected [6;152].

Class II elements typically transpose by the replicative mechanism [189]. In this process enzymes resolvases catalyse resolution of cointegrates at the *res* sites. In some cases resolvases may be substituted by integrases that catalyse not only the cointegrate resolution, but also the intra- and intermolecular recombination between the defined *att* attachment sites [189] .These may generally lead to an increase recombination rates for example between plasmids harbouring resistance genes located on the transposable elements.

Examples of other classes of transposons are Tn*7*-like and Tn*402*-like transposons [189]. It is probable that when more sequences will be characterized more classes and families will be defined.

2.4.3 Integrons and ICEs

Integrons constitute another individual class of transposable elements. They consist of the *int*I gene and the gene cassette or sometimes number of gene cassettes. IntI integrase catalyses site specific recombination of integrons. Upstream the *int*I there is typically located an *att*I recombination site, while gene cassettes harbour the *att*C site at their 3' end. Integrons can accept multiple insertions of other gene cassettes. The gene cassettes can encode resistance to a whole range of antimicrobials, including β-lactams. The cassettes can be transposed onto plasmids or onto the chromosomal DNA [189]. Another group of transposable elements are conjugative transposons discussed typically together with the ICEs group. ICEs of the SXT (conferring sulfamethoxazole-trimethoprim resistance) type were initially detected mainly in *Vibrio cholera* [25]. Recently it was proposed that some genetic elements initially classified as genomic islands and found in *Salmonella* (SPI-7) and other Enterobacteriaceae may in fact be a class of ICEs [183].

SECTION II.

3. SUMMARY OF THE RESULTS AND DISCUSSION

E. coli, *K. pneumoniae* and *S. enterica* are the most common Enterobacteriaceae causing infections in humans and animals[153]. Increasing occurrence of plasmid encoded resistance to β-lactams is being reported in these bacteria [33;34;36;126]. Plasmid encoded TEM β-lactamases were shown to be particularly prevalent in *E. coli*; bla_{TEM} genes were previously detected not only in pathogenic strains but also in normal faecal flora of humans and animals[33;55;149;184]. In this study the diversity of plasmids with bla_{TEM} genes (Manuscripts I and II) as well plasmids not selected based on the resistance (Manuscript III) was examined. The overall aim was to study the possible connection between plasmids from human and non-human sources like livestock animals, meat or natural environment (Manuscripts I, II and III). In manuscripts I and II the relationship between plasmids harbouring bla_{TEM} genes from humans and from animals or food products from animals is examined. The plasmids originated primarily from *E. coli* (Manuscript I and II) and also from *S. enterica* (Manuscript I). Relationship between transposable elements harbouring bla_{TEM} genes residing on the plasmids backbones is also discussed. In Manuscript III plasmids from *K. pneumoniae* are examined, namely plasmids from human infections are compared to plasmids from environmental samples (surface waters). A novel plasmid typing method was developed in this study for typing of plasmids in *K. pneumoniae* and the utility of this method as well as other available methods applied to study epidemiology of plasmids are discussed in relation to Manuscript III. Influence of plasmids host specificity and host range on the evolution and transmission of bla_{TEM} genes is discussed with relation to all three monographs.

3.1 Limited range of Inc types is found on bla_{TEM} plasmids in Enterobacteriaceae from humans and from animals

More than 27 Inc groups have been described in Enterobacteriaceae [54;72;205]. Resistance plasmids with bla_{TEM} genes (bla_{TEM-1}, bla_{TEM-30}, bla_{TEM-40}, $bla_{TEM-135}$, bla_{TEM-52}) examined in this study represented only a limited range of these Inc groups, namely IncF-complex (-FI and -FII), IncI1, IncX1 variant called IncX1A, IncA/C, IncL/M, IncK, IncHI1, IncY, IncP, IncR and the non-typable replicons (Manuscripts I-II; summarized below in Table 1). As explained in Manuscript I the bla_{TEM-52} IncX1A plasmid pE001 overall shared many similarities with other IncX1 scaffolds, therefore these plasmids from study I will be generally designated IncX1 in the following sections. In a previous study of Bergenholtz and

Jørgensen[11] replicons IncI1, IncA/C and NT were also detected on *bla*$_{\text{TEM-15,}}$ *bla*$_{\text{TEM-19}}$, *bla*$_{\text{TEM-20}}$ and *bla*$_{\text{TEM-63}}$ plasmids.

Similar replicons to the aforementioned range were previously described in the literature on plasmids encoding the TEM-1 enzymes[33;85;129;172]; additionally, plasmids with IncS, IncT, IncW (mainly in *Providencia* spp[83].) and also ColE replicons were shown by other authors to harbour genes encoding TEM-1 β-lactamases in diverse enteric hosts [33;85;129]. Combining data available in the literature with this study it can be concluded that the above listed replicons associated with the *bla*$_{\text{TEM}}$ genes are also frequently found with other epidemic types of *bla* genes encoding class A β-lactamases, namely *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{SHV}}$ [172;174].

This raises the question whether the aforementioned replicons are generally specific for the examined entero-bacterial hosts and therefore are found in the various reservoirs with or without the resistance genes; or some of these are host/ reservoir 'non-specific' replicons acquired from external reservoirs (natural environments, other bacteria families etc.) and accommodated in *E. coli* and other examined Enterobacteriaceae (*K. pneumoaniea* and *S. enterica*) because of the selective pressure for the resistance traits of these particular plasmids. This question will be further reviewed below.

3.2 Different Inc types are typically associated with *bla*$_{\text{TEM-1}}$ compared to *bla*$_{\text{TEM}}$ variants encoding ESBLs

Based on the data collected in Manuscript I and –II it can be concluded that plasmids with *bla*$_{\text{TEM-1}}$ and other *bla*$_{\text{TEM}}$ variants encoding IRTs (*bla*$_{\text{TEM-30}}$, *bla*$_{\text{TEM-40}}$) typically belonged to IncF-family (FII, FIB, FIA), IncI1, IncB/O or had non-typable (NT) replicons (Table 1 below). In contrast, IncB/O and IncF-family replicons were not found on plasmids encoding TEM-ESBLs (plasmids with *bla*$_{\text{TEM-135,}}$ *bla*$_{\text{TEM-52}}$; Manuscripts I and –II). In the latter case mainly replicons IncI1 and IncX1 were detected in *E. coli* and *S. enterica* and also less frequently IncA/C, IncL/M and IncN-related. In the studies of Bergenholtz and Jørgensen[11] and Carattoli *et al*.[33] the *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM}}$ encoding IRTs typically resided on IncF- family, IncB/O, IncI1 or NT replicons, while genes encoding ESBLs (*bla*$_{\text{TEM-15,}}$ *bla*$_{\text{TEM-19}}$, *bla*$_{\text{TEM-20}}$, *bla*$_{\text{TEM-63}}$, *bla*$_{\text{TEM-3}}$, *bla*$_{\text{TEM-10}}$, *bla*$_{\text{TEM-21}}$, *bla*$_{\text{TEM-24}}$, *bla*$_{\text{TEM-52}}$) were localized mainly on IncA/C and IncI1 replicons. Apparently there are differences in the replicon distribution among plasmids encoding TEM-1 genes compared to plasmids encoding TEM-ESBLs (Figure 7).

Table 1. Summary of the replicons detected in study I and II on the *bla*$_{TEM}$ plasmids

bla TEM variant	*bla* TEM-1		*bla* TEM-30 or *bla* TEM-40 (IRT encoding)		*bla* TEM-135, *bla* TEM-52, *bla* TEM-15, -19, -20, -63 [a] (ESBLs encoding)[b]		
Reservoir	Human	Animal	Human	Animal	Human	Animal	Meat
Individual plasmids from the specified reservoir	57	92	2	2	14	9[c]	4
Replicon(s) detected on the plasmids							
IncA/C					1 *bla* TEM-52, 1 *bla* TEM-19, 1 *bla* TEM-63		
IncB/O	10	10	1 *bla* TEM-30				
IncB/O & IncP	3	3					
IncFIB		2	1 *bla* TEM-40				
IncFII	7	13		1 *bla* TEM-30			
IncFII & IncFIB	14	15					
IncFII & IncFIB & IncFIA	2	6					
IncFIB & IncFIA		1					
IncFII & IncFIB & IncP	2	5					
IncFII & IncP		1					
IncFIB & IncY	1						
IncI1	3	18			1 *bla* TEM-135, 5 *bla* TEM-52	2 *bla* TEM-52, 1 *bla* TEM-20	
IncI1 & IncP	1	2					
IncI1 & IncFIB &IncFIC		1					
IncHI1		1					
IncK	1						
IncL/M					2 *bla* TEM-52		
IncN or IncN-related	1	2				2 *bla* TEM-52	
IncP		2					
IncR		1 [IncFII &IncR]			1 *bla* TEM-52		
IncX1A	1				1 *bla* TEM-52	4 *bla* TEM-52	4 *bla* TEM-52
NT	11	9		1 *bla* TEM-30	1 *bla* TEM-52		

[a]- plasmids with these *bla*$_{TEM}$ alleles originated from the study of (Bergenholtz and Jørgensen, 2008) and they were part of the collection covering also the *bla*$_{TEM-52}$ plasmids characterized in Manuscript I; these additional data was included here in order to obtain a broader overview of the replicons associated with *bla*$_{TEM}$-ESBLs encoding variants; [b]-TEM-135 is not a true ESBL enzyme, however it has the ESBL-characteristic substitution M182T and hence it was included to ESBLs group; [c]- Cloeckaert TC was considered as poultry isolate; IRT- Inhibitor resistant TEM- β- lactamase.

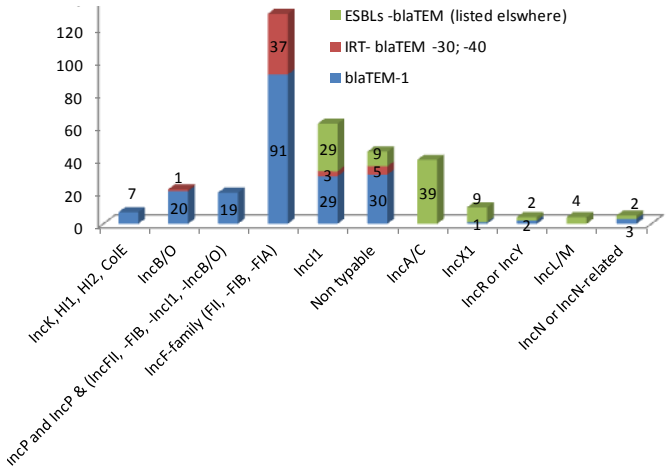


Figure 7. Distribution of replicons on plasmids with different variants of *bla*$_{TEM}$ evaluated based on combined data from this study (Manuscripts I and II) and from Bergenholtz & Jørgensen and Carattoli *et al.* 2009 [12;33]; ESBLs encoding variants were *bla*$_{TEM-3}$, *bla*$_{TEM-10}$, *bla*$_{TEM-135}$, *bla*$_{TEM-15}$, *bla*$_{TEM-19}$, *bla*$_{TEM-20}$, *bla*$_{TEM-21}$, *bla*$_{TEM-24}$, *bla*$_{TEM-52}$ and *bla*$_{TEM-63}$; *bla*$_{TEM-1}$ plasmid with IncI1&IncFIB &IncFIB (study II) was counted as IncI1 replicon; *bla*$_{TEM-1}$ plasmids with IncFII &IncR and with IncFIB & IncY replicons (study II)

were considered as IncR or IncY replicons.

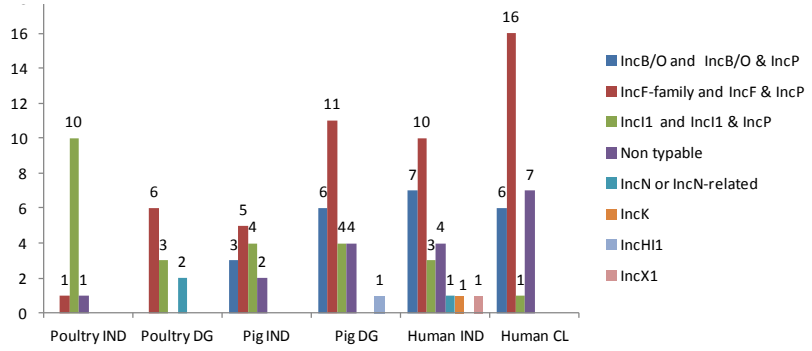


Figure 8. Distrubution of the replicons detected on *bla*$_{TEM-1}$ plasmids from faecal (indicatory) and diagnostic or clinical *E. coli* from humans and food-produciton animals (summary of study II).

The explanation to (and implication of) this distribution is more clear when looking on data reported by Johnson *et al.*[100;102] and data reported in Manuscript II (the latter summarized on Figure 8). In the two cited studies of Johnson, he examined diversity of plasmids not selected based on the resistance. In his studies mainly IncF plasmids, in particular IncFII and IncFIB, were found in pathogenic and commensal *E. coli* in humans and poultry, IncB/O plasmids were the second most abundant in humans (both pathogenic and indicator), while IncI1 were the second most abundant in pathogenic and indicator *E .coli* from poultry; in poultry there were also a notable fractions of IncN and IncP replicons present. In pathogenic avian strains IncB/O were often detected [102]; IncI1 replicons were frequently detected in pathogenic *E. coli* from pigs[100]. According to Manuscript II similar pattern was observed among plasmids with *bla*$_{TEM-1}$ genes from faecal indicator and diagnostic/clinical *E. coli* from humans, cattle, pigs and poultry. Namely, IncFII and IncFIB were frequent in all examined reservoirs (human and animals); IncB/Os were the second most abundant in humans, cattle and pigs but were not detected in poultry; IncI1 were dominating in poultry, to lesser extend were found in pigs and humans and were not detected in cattle; often broad-host range (BHR) IncP[194] replicons were found on the same *bla*$_{TEM-1}$ plasmids with IncB/O, IncFII, -FIA, FIB and IncI1 mainly in *E. coli* from animals (Figure 8).

In summary, the following statements can be made based on the above data:

a) IncF-family plasmids are found in commensal and pathogenic *E. coli* both in humans and animals and hence are well adapted to this bacterium in these reservoirs

b) IncB/O are likely to be adapted specifically for *E. coli* from humans, cattle and pigs (found both in pathogenic and commensal isolates), but were also found among pathogenic *E. coli* in the avian reservoir

c) IncI1 are well adapted for commensal and pathogenic *E. coli* from poultry and are also often found in pathogenic *E. coli* from pigs

d) $bla_{\text{TEM-1}}$ genes usually reside on the same plasmids that are generally predominant in *E. coli* from the given reservoir [57]

e) bla_{TEM} encoding TEM-ESBLs are often found on BHR plasmids like IncA/C and NHR plasmids like IncI1 and IncX1

f) IncP are the most common BHR plasmids with $bla_{\text{TEM-1}}$ and were detected mainly in cattle and often as co-integrates with IncF-family, IncI1 and IncB/O replicons in different examined reservoirs.

Marcade *et al.*[125] previously suggested that the $bla_{\text{TEM-1}}$ genes were able to evolve only to some extend (typically producing IRT variants) on the IncF-family and IncB/O replicons in humans; the bla_{TEM} genes encoding ESBLs might have evolved outside of the human reservoirs, possibly on the IncA/C, IncI1 or IncX1 scaffolds. This implies that the plasmids encoding ESBLs like TEM-52 (Manuscript I) were probably acquired at least by human *E. coli* strains (typically not populated with IncI1, IncX1 or IncA/C plasmids) from external sources; possibly from poultry or pigs where especially high prevalence of IncI1 replicons was detected, or on the IncX1 replicons from other bacteria species as discussed below. Regarding IncA/C, these replicons were not detected in the faecal indicator or in the diagnostic/ clinical *E. coli* of humans and food production animals (study II); as it is explained in the next section these plasmids have generally broad host range and likely this is the reason why such a diversity of bla_{TEM} variants was found on these scaffolds by other authors.

Only one case IncX1 replicon was detected with $bla_{\text{TEM-1}}$ (in human *E. coli*) in study II. Based on that it can be deduced that, contrary to IncF- and IncI-complex replicons, IncX1 replicons are not really prevalent in the *E. coli* from healthy individuals; hence must have been transmitted to *E. coli* of humans and animals (like pigs, cattle or poultry) probably from other bacteria. Among others *S. enterica* could be the source (or at least the important carrier) of IncX1 replicons. This species is found in humans almost exclusively because of consumption of contaminated foods of animal origin (excluding *S.* Typhi and *S.* Paratyphi which are specifically human-associated but these two serovars do not seem to be the problem in the western countries). In European countries the IncX1 and IncI1-encoding TEM-52s were mainly detected (study I); also the detection of TEM-52 in other countries was typically

limited to non-typhoidal *Salmonella* species[43;84;114] thus likely originating from animal sources.

Above findings suggest that IncA/C, IncI1 or IncX1 plasmids with *bla*$_{\text{TEM-52}}$ (Manuscript I) might indeed have been acquired by the human *E. coli* from the animal sources. This also raises the question about the origins of the *bla*$_{\text{TEM-1}}$ genes found in *E. coli* originating both from humans and animals like pigs, cattle and poultry often on the same *E. coli* -host adapted plasmids; an attempt to answer this question is made in further sections.

3.3 Molecular evidences that diverse Enterobacteriaceae from humans and animals may share similar or undistinguishable *bla*$_{\text{TEM}}$ plasmids.

Still little is known regarding the transmission of mobile genetic elements between bacteria from the diverse reservoirs like animals, natural environments and humans. This study delivered several evidences that either undistinguishable or at least closely related plasmids with either *bla*$_{\text{TEM -1}}$ or *bla*$_{\text{TEM-52}}$ circulated in *E. coli* and S. *enterica* from humans, animals and meat (Manuscripts I and –II). Moreover, some of the replicons detected in humans and associated with the *bla*$_{\text{TEM-135}}$ and *bla*$_{\text{TEM-52}}$ encoding genes (IncI1, IncX1 and IncA/C) were deduced not to be typical for human associated *E. coli* (the latter are normally dominated by IncF and IncB/O plasmids) suggesting acquisition of these ESBLs encoding plasmids possibly from food-production animals.

3.3.1 IncX1 plasmids with *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM-52}}$

In study I epidemic *bla*$_{\text{TEM-52b}}$ IncX1 plasmids were found in *E. coli* and various serovars of *S. enterica* from human, poultry, beef and broiler meat. One case of *bla*$_{\text{TEM-1}}$ IncX1 (p1413-CO3) plasmid was detected in a human *E. coli* isolate from Denmark in the IInd study. Additional RFLP profiling (not published in the manuscripts) demonstrated that the two types of IncX1s, namely the epidemic *bla*$_{\text{TEM-52}}$ plasmids and the plasmid with *bla*$_{\text{TEM-1}}$, were not epidemiologically related (data not published). Interestingly, the epidemic IncX1 *bla*$_{\text{TEM-52}}$ plasmids from human and animal/meat isolates from Denmark and The Netherlands (Manuscript I) shared many similarities with pOLA52 isolated from swine manure from *E. coli* [142] as well as with pOG670[23] from *S. enterica* (data not published). A variant of pOLA52 (e.g. pOLA52 *bla*$_{\textit{TEM-1}}$*:ntp*, kan^{R}), was initially used in the incompatibility assay with the pE001 plasmid (Manuscript I). The pOG670 was originally isolated from humans/cattle (exact source not specified) in Scotland in 1917. The epidemic IncX1 *bla*$_{\text{TEM-52}}$ plasmid from study I, pOLA52 and pOG670 may possibly belong to the same similarity cluster compared to the *bla*$_{\text{TEM-1}}$ IncX1 plasmid from study II. It is possible that only a certain

clone or clones of IncX1 plasmids might have been responsible for the spread of the *bla*$_{\text{TEM-52}}$ genes in animals and humans at least in European countries. However, such hypothesis needs to be further investigated.

3.3.2 IncI1 plasmids with *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM-52}}$

By analogy to the IncX1 replicons, two types of epidemic IncI1 plasmids were found in this study harbouring *bla*$_{\text{TEM-1}}$ (pMLST ST36/CC5, paper II) and *bla*$_{\text{TEM-52}}$ (pMLST$^{71;100}$ ST5/CC5, paper I). Interestingly, Leverstein-van Hall *et al.*[116] demonstrated that the ST36/CC5 *bla*$_{\text{TEM-52}}$ plasmids were circulating in animals and humans in also in The Netherlands (described in section 1.2.3).

Despite the differences in their STs the two plasmids belonged to the same CC5; the CC5 is currently comprised of four ST-types (ST5,-10,-21 and -36) which are represented by IncI1 plasmids mainly with *bla*$_{\text{TEM-52}}$ and *bla*$_{\text{TEM-20}}$ [100;158]. These data suggest that these CC5 scaffolds might have been the original platforms for the evolution of the *bla*$_{\text{TEM-1}}$ to *bla*$_{\text{TEM-52}}$ genes.

TEM-52 was initially described in *K .pneumoniae* from stool sample taken from a Greek patient (Athens) in a French hospital (Paris) in 1996[163]. In the reference study it was determined that the *bla*$_{\text{TEM-52}}$ gene resided on a small plasmid (<10 kb; replicon was not identified) harbouring Tn*3*-like element. Other reports of the *bla*$_{\text{TEM-52}}$ informed about rather large plasmids (>40 kb); *bla*$_{\text{TEM-52}}$ were found on plasmids with not determined replicons from *Shigella* spp. isolated betwen 1991-2000 in Korea [182]; on IncA/C, IncN, IncI1, IncL/M, IncR and IncX1 plasmids from *K. pneumoniae, E. coli* and different serovars of *S. enterica* originating from diverse European countries as well as Canada and Korea, isolated from humans, poultry and meat between 1995-2000 (Bergenholts & Jørgensen[11] and Manuscript I); In more recent reports *bla*$_{\text{TEM-52}}$ were located on IncA/C plasmids form clinical *E. coli* from Tunesia[41] (isolation years not specified) and IncN plasmids from clinical isolates of *P. mirabilis* collected in 2008 in Korea[180]. Much of the of data regarding the replicon typing of *bla*$_{\text{TEM}}$ plasmids (as well as plasmids with other *bla* types) come from after 2005 when the PBRT was published and also when already more attention was given to surveying of the resistance and its genetic background in animal reservoirs. In this case it is not possible to state whether this 'first' *bla*$_{\text{TEM-52}}$ in human originated from animal sources or not.

3.3.3 IncB/O and IncK plasmids with *bla*$_{\text{TEM-1}}$

In the IInd study a range of either undistinguishable or at least closely related *bla*$_{\text{TEM-1}}$ IncB/O plasmids designated as RFLP 'p' was detected in *E. coli* from healthy human, human clinical

sample, diseased pig and cattle as well as from healthy cows. Another group of IncB/O plasmids sharing similarities with each other was found among several human isolates of *E. coli* (RFLP 'm' in Manuscript II). The *E. coli* isolates from each reservoir have been compared with each other by *Xba*I PFGE. The strains were shown not to be clonally related which suggests that these similar IncB/O RFLP 'p' and 'm' plasmids were indeed circulating in the diverse *E. coli* isolates. Overall, the $bla_{\text{TEM-1}}$ IncB/O plasmids from the different reservoirs seemed to share many similarities based on their RFLP patterns. Currently no pMLST or similar method exist for IncB/O plasmids; therefore the relationship of these epidemic IncB/O 'p' and 'm' scaffolds with the previously published plasmids like pR3521[150](from clinical *E. coli* in Greece), pO26-vir (FJ386569; from STEC, USA) or pHUSEC41-1[112] (from STEC from Germany) or possibly other not listed plasmids from isolates originating from countries other than Denmark remains to be elucidated in the future studies.

One case of a $bla_{\text{TEM-1}}$ IncK plasmid was detected in human *E. coli* isolate (Manuscript II). Due to the similarity of the IncB/O and IncK replicons it will also be mentioned in this subsection. The IncK replicons have been rather rarely detected in Enterobacteriaceae from humans and from food production animals. Although, this replicon was recently shown to drive the spread of $bla_{\text{CTX-M-14}}$ gene in *E. coli* from humans in Spain [202].

3.3.4 IncFII and/or IncFI plasmids with $bla_{\text{TEM-1}}$

In study II the selected IncF-family plasmids underwent the replicon sequence typing (RST[205]). In three different diagnostic cattle *E. coli* isolates similar multireplicon plasmids IncFII & IncFIB with $bla_{\text{TEM-1c}}$ were found; they shared the same RFLP profiles and according to the FAB formula (each letter representing IncFII, IncFIA and IncFIB, respectively) they were of F2:A-:B1 types. A plasmid from another cattle isolate and harbouring $bla_{\text{TEM-1b}}$ gene was only verified by RFLP in study II but shared similar pattern to these three IncFII & IncFIB, $bla_{\text{TEM-1c}}$ scaffolds. An F2:A-:B1 plasmid called pAPEC-O2-ColV (AY545598) was previously described in the ExPEC strains [187;205]. This implies that the infection in some of the cows from which the amp^R isolates were collected and used in study II might have been caused by the APEC-like strains or at least the F2:A-:B1 plasmids from these cows might have been of avian origin (or *vice versa*). In fact, Skyberg *et al.* demonstrated that this virulence pAPEC-O2-ColV plasmid may contribute to the increased virulence when transmitted to the previously commensal *E. coli* strains[187]. On another hand,

this original plasmid (AY545598) does not encode any *bla* genes and was originally isolated in USA[101]. It is therefore possible that not a horizontal transmission of plasmids is in question here, but the similar plasmid types might be generally hallmarks of the pathogenic *E. coli* strains from the diverse geographical locations [99].

In two diagnostic pig isolates and in one diagnostic cattle isolate similar IncFII $bla_{\text{TEM-1}}$ plasmids sharing the RFLP type 'd' were detected in the study II. Two of these plasmids underwent RST and they were related to ST F35 (both had three nucleotide differences compared to F35). There is no 100% identical match found in GenBank for this F35-related locus. The F35:A-:B- pattern was previously found on $bla_{\text{CTX-M-9}}$ plasmids from *E. coli* isolates from pets in China (pHN0113-2; HQ706665.1)[61] and $bla_{\text{CTX-M-14}}$ (the $bla_{\text{CTX-M-9}}$ family) from en epidemic, human *E. coli* ST131 strain from China (pWCE35; GU462158.1) [219].

Finally, similar IncFII, RFLP 'g', RST type F2:A-:B- plasmids were found in three different clones of human *E. coli* (Manuscript II). This pattern was previously described on the diverse plasmids from human isolates of *E. coli , S. flexneri, S. sonnei* and *K.pneumoniae;* the latter plasmids harboured $bla_{\text{TEM-1,}}$ $bla_{\text{CTX-M-14 ,-15 or -24}}$, or encoded virulence factors like haemmaglutinin, colicin or iron transport systems[205]. Recently, Deng *et al.*[61] also demonstrated an epidemic dissemiation of an F2:A-:B- plasmids with *rmt*B (aminoglycoside resistance), *qep*A (fluoroquinolone efflux pump) and diverse $bla_{\text{CTX-M}}$ variants in different Enterobacteiraceae from humans and animals in China. Madec *et al.*[124] demonstrated dissemination of $bla_{\text{CTX-M-15}}$ F2:A-:B- plasmids among humans and animals in France (also mentioned in section 1.2.3).

3.3.5 IncN plasmids with $bla_{\text{TEM-1}}$ genes

Two IncN plasmids (p7372121-1 and p1308-CO3) detected in the study II were compared with each other by pMLST. IncN $bla_{\text{TEM-1b}}$ p7372121-1 from poultry belonged to ST3; similar ST3 plasmids with $bla_{\text{CTX-M-1}}$ and *qnr*S1 (human isolate) were previously described in *E. coli* and *S. enterica* from Denmark and The Netherlands[73]. The $bla_{\text{TEM-1b}}$ IncN p1308-CO3 from human belonged to ST6 (Manuscript II). The later types of plasmids harbouring $bla_{\text{CTX-M-3}}$, $bla_{\text{KPC-3}}$ and $bla_{\text{OXA-3}}$ were previously reported in *S. enterica* (United Kingdom) and *K. pneumoniae* (USA) [73] .

3.4 NHR, host specific plasmids are recipients of *bla*$_{TEM}$ genes, BHR plasmids are transporters for mobilized genetic traits from the environment or between more distantly related species

In the 1980s Datta and Hughes examined a collection of 400 Enterobacteriaceae from human infections (*E. coli*, *K. pneumoniae*, *Shigella*, *Salmonella* and *Proteus* from diverse geographical locations)[57;103]. The isolates originated from so called pre-antibiotic era (abbreviated PAE) isolates and were collected between 1917 and 1954. They managed to conjugate plasmids from 84 PAE to *E. coli* recipients and none of these plasmids conferred antimicrobial resistance phenotype in the recipient. Moreover, these plasmids represented the same Inc groups as the ones currently found in the key infectious Enterobacteriaceae but harbouring sometimes multiple resistances, namely IncB (= IncB/O), IncN, IncFII, IncI1and IncX[57;103]. This implied that the resistance genes in humans must have been acquired from some external sources rather than being the genes endogenously found in bacteria from humans. In this study (Manuscripts I and II) the *bla*$_{TEM}$ genes in *E. coli* and in other aforementioned enteric bacteria resided mainly on the similar types of plasmids, the IncF-plasmids (IncFII, IncFIIk, IncFIA and –FIB) and IncI1 complex (IncI1, IncB/O, one case of IncK) plasmids. The IncI1 and IncB/O plasmids were both found as epidemic types transmitting either the *bla*$_{TEM-52}$ (IncI1) or *bla*$_{TEM-1}$ (IncI1 and IncB/O).

Considering that the similar IncF or IncI-complex plasmids with the same resistance gene are found both in human and animals it is difficult to point out whether the *bla*$_{TEM-1}$ genes were acquired by humans from animals (or *vice versa*). It can only be speculated that, similarly to other *bla* genes, the *bla*$_{TEM-1}$ genes originated form chromosomes of bacteria other than the well characterised Enterobacteriaceae of which genomes are available in the databases like *E. coli*, *S. enterica* or *K. pneumoniae*. It seems likely that the BHR plasmids like IncP, IncR, IncA/C or IncN detected in this study might have been in the past the links between the yet unrevealed *bla*$_{TEM-1}$ chromosomal progenitors. Also the IncY replicons (being actually forms of bacteriophages) with the *bla*$_{TEM}$ genes were previously sporadically detected[33], one case was detected with *bla*$_{TEM-1}$ in this study (Manuscript II). Probably not all bacteriophages assume the plasmidic forms like the IncY and hence their participation in the transmission of these resistance traits might have been to some extend overlooked. Nevertheless, at the current state of knowledge the participation of bacteriophages in the transmission of resistance is still considered minor in Enterobacteriaceae compared to the plasmid driven transmission[74].

Typically IncP plasmids are known to poses one of the broadest host ranges among the Inc groups of Enterobacteriaceae. They are capable of transfer even to Gram positive bacteria, although they cannot replicate in the latter[189]. Curiously, in study II a rather large fraction of IncFII, IncFIB, IncI1 and IncB/O was detected as co-integrates with the IncP replicons. Particularly in the cattle isolates the IncP plasmids were more prevalent. Actually, Bahl *et al*. previously demonstrated high prevalence of the IncP plasmids in the influents into a Danish wastewater treatment plant, which emphasizes the abundance of these plasmids in the outside environments [5].

The IncR family has not yet been well characterised. First description of this replicon was on pKP245, a plasmid originating from UTI isolate of *K. pneumonia* [41]. IncR were detected on a $bla_{\text{TEM-1}}$ plasmid from *Escherichia fergusonii* (human infection; CU928144), on the $bla_{\text{TEM-52}}$ plasmid pK727 from *E. coli* (Manuscript I) and on a plasmid pLV1403 from *Pantoea agglomerans* (member of Enterobacteriaceae) from lake water sediment[148] (studies I and III). These replicons were actually often detected in *K. pneumoniae* examined in the study III. By analogy to the IncP plasmids from study II it seems that the BHR IncR replicons tend to form co-integrates with the host specific replicons like IncFII_k (Manuscript III).

Two conclusions based on the above can be made in this subsection. Firstly, the 'naive' (resistance free) NHR plasmids in *E. coli* in humans and in animals must have originally depended either on the mutation rate to develop resistance to antimicrobials, or on the occurrence of the external resistance genes carried on the BHR plasmids (or other MGEs) that were passing by[192]. BHR plasmids like IncP, IncA/C, IncR, IncN detected in this study and probably other plasmids (like IncY) species function as transporters for various traits. As these BHR scaffolds plasmids are exposed to variety of potential hosts and conditions, they are likely to be recipients of a large number of genetic elements including transposons or even other plasmids.They may be the key factors needed for mobilization of the *bla* genes from yet undetermined environmental sources.

3.5 Plasmid encoded resistance is an ecological problem and studying plasmids epidemiology calls for specialized tools

Diverse Enterobacteriaceae found in the GI tract of humans and animals without doubts interact with each other and with other bacterial species as well as with viruses (for instance bacteriphages) [192]. The same statement applies to the bacterial species found in the natural environments like soils, plants, surface waters, lake sediments etc. Many bacterial species probably pass through the GI tracts; the transmission of *bla* genes (and possibly other genetic

traits) is driven not only by the defined resistant bacterium, but also by a range of MGEs, some of them being self-transmissible (plasmids, ICEs) or can be mobilized from one bacterium to another[143]. Currently the term plasmid epidemiology is frequently used to underline that not only a given clone of bacterium but a stable 'clone' of plasmid can traverse across different bacterial host organisms. For many years assessment of plasmids incompatibility and since 2005 typing of replicons by PBRT was combined with the RFLP methods and these served as standard means to study epidemiology of plasmids.

3.5.1 PBRT *vs* novel mPCR

Upon the initiation of this study (2009) the published PBRT [35] did not encompass as many replicon variants as it does now. Especially the plasmids from *K .pneumoniae* seemed to escape the detection and hence classification by this PCR-based method. Therefore, an attempt was made in study III to collect the available sequences of plasmids from this species and to design a PCR-based method for rapid detection and classification of plasmids specifically in *K. pneumoniae* (Manuscript III). A novel multiplex PCR was designed (mPCR) and it was targeting a range of *rep* sequences (i. e. encoding the Rep initiator of replication). By the end of 2010 Villa *et al*. presented an updated PBRT scheme[205]. Many of the plasmids from *K. pneumoniae* used as the references for designing of the mPCR turned out to be the $IncFII_k$ variants as defined by Villa *et al.* However, an interesting observation was made combining the data from study III and from the study of Villa *et al.* Namely, multiple plasmids in the same bacterium apparently carried similar incompatibility determinants belonging to the $IncFII_k$ group. The *K. pneumoniae* strain MGH78578 (ATCC 700721; GenBank) harbours three large plasmids; two of these are $IncFII_k$ both with the FAB formulas [K1:A-:B-][205], where 'K' stands for the FII_k locus (pKPN3 and pKPN4; the third plasmid is an IncR plasmid called pKPN5). This challenges the assumption that plasmids from the same Inc group are not suppose to reside in the same bacterium[144]. The mPCR combined with the *in silico* analyses solved this issue indicating that secondary incompatibility factors may be encoded by these co-residing FII_k plasmids in *K. pneumoniae* MGH78578, namely the secondary replicases (which presumably are part of independent replicons). Indeed pKPN3 harbours the *rep*$AFII_k$ and a second *rep* gene belonging to a repIV group (defined in Manuscript 3), while pKPN4 harbours *rep*$AFII_k$ and a *rep* sequence of repVI type. Co-existence of several resistance plasmids belonging to the IncF-family in the same isolate is apparently not unusual; a similar phenomenon was recently described for resistance F-plasmids in the *E. coli* strain by other authors[61].

In conclusion, the above illustrates that the classification of plasmids to the Inc groups requires continuous optimizations of the PCR-based protocols (and species considerations) in order to tract the plasmids relatedness as well as genetic rearrangements in their scaffolds. Designing of the PCR is largely depended on the available sequencing data. Moreover, the designation 'Inc groups' gradually loses its original meaning and it seems to be more proper to consider some of the novel Inc types rather as homology or similarity groups (Manuscript III).

3.5.2 *K. pneumoniae* from humans and from the environment harbours few classical Inc types and a range of potentially novel replicons

Study III revealed that only a limited range of replicons were present in the examined *K. pneumoniae* from humans and from the surface waters[191]. Typically IncFII$_k$, a novel replicon repIV and also IncR were detected. In many cases the plasmids remained non-typable. Possibly inclusion of the secondary replicases collectively termed in Manuscript III as *the remaining rep's* as the targets of the mPCR would solve the problem of some of these non-typable replicons. Furthermore, it is proposed in this thesis that the repIV replicon detected often on plasmids in this study might be a *Klebsiella* specific equivalent of either IncFIA or -FIB replicons usually found in *E. coli* (Manuscript III).

The isolates in study III were not selected based on any resistance markers, hence can be considered as 'normally found' in *K. pneumoniae* in humans or in the environment. Some *K. pneumoniae* can transiently colonize mucosal surfaces in humans and animals, however unlike for *E. coli*, a term commensal seem to be inappropriate in this case as some of the strains were in fact pathogenic causing UTI or bacteraemia (Manuscript III); *K. pneumoniae* is an opportunistic pathogen therefore the strains causing infections in general would be similar to those found normally in the natural environments[159]. In the recent study of Mataseje *et al*. [127] RepFIIA, IncR and also a range of non-typable plasmids were found to be responsible for transmission of *bla*$_{KPC}$ genes in carbapenemase producing *K. pneumoniae* from clinical settings. By analogy to the IInd study it seems that the plasmids typical for *K. pneumoniae* (FII$_k$, possibly repIV or other replicons that were untypable in study III) and also the broad host range IncR, IncN, IncA/C and IncL/M (examples listed in Manuscript III) would also be prone to acquire the diverse *bla* genes.

3.6 Plasmids and transposable elements in enteric bacteria - implications in mobilization and spread of the *bla*$_{TEM}$ genes

Although plasmids are the main focus of this study, the subject of transposable elements should not be overlooked. It was previously shown that the most frequently detected *bla* genes encoding Ambler class A ESBLs, namely *bla*$_{SHV}$, *bla*$_{CTX-M,}$ and *bla*$_{TEM}$, were typically transmitted not only on the similar types of replicons in Enterobacteriaceae (mainly IncF-family, IncI1, IncN, IncA/C[33;34]), but also on the specific transposable platforms. The *bla*$_{SHV}$ were shown to be usually located on DNA segments containing also other resistance genes and flanked by the IS*26* elements thus constituting the composite transposons[131].

The *bla*$_{CTX-M}$ were shown to be typically mobilized by insertion sequences IS*Ecp*1 (*bla*$_{CTX-M-3}$, *bla*$_{CTX-M-14}$, *bla*$_{CTX-M-15}$, *bla*$_{CTX-M-10}$) and ISCR1 (*bla*$_{CTX-M-9}$, *bla*$_{CTX-M-2}$)[4]. The *bla*$_{TEM}$ genes were detected within the Tn*3*-like transposons[6] and this was also observed in study I and II (actually the IncR plasmid pEFER analysed *in silico* in study III also harbours the *bla*$_{TEM-1b}$ on the Tn*2* transposon; data not shown in Manuscript III).

The linking PCR in study I was initially designed based on the sequenced element EF141186 annotated previously as Tn*3* transposon with *bla*$_{TEM-52}$ gene [11]. It was later realized that this element was in fact Tn*2* type [6]. This PCR setup from Manuscript I was re-analyzed *in silico* and the conclusion was made that the primers used in the linking PCR (Manucript I) indeed matched with the Tn*2* type transposons but also with the Tn*1* type elements; Tn*3* transposons would actually not be detected by this set up. In the second study the linking PCR was redesigned, in order to distinguish between the different elements and encompass larger variety of the putative elements upstream of the *bla*$_{TEM-1}$ genes (e.g. Tn*1*, -*2*,-3 and IS*26* in two different orientations with respect to the *bla*$_{TEM}$ gene). The new linking PCR was in fact tested initially on the plasmids from Manuscript I, confirming that the majority of the *bla*$_{TEM-52}$ genes indeed resided on the Tn*2* transposons, although in one case of the pK727 many unspecific bands were produced and it was decided to designate the element upstream of the *bla*$_{TEM-52}$ gene on this plasmid as unknown in order to get more specific information of this sequence in the future studies (erratum to Manscript I).

As indicated previously, PCR based methods are sensitive to the polymorphisms in the primer binding sequences. Moreover, the substitutions between the primers would typically not be detected without further sequencing or application of methods like single-nucleotide polymorphism-specific PCR [134]. This was the reason why in Manuscript II it is particularly emphasized that any result of the linking PCR should be interpreted as 'putative element linked to *bla*$_{TEM}$ gene'.

3.6.1 *Bla*$_{\text{TEM-1b,-1c \& -52}}$ are typically mobilized by Tn*2*; insertions of IS*26* upstream of the *bla*$_{\text{TEM}}$ genes are also frequent

Table 2. Analysis of transposable elements associated with *bla*$_{\text{TEM}}$ genes (study I and II)

	bla TEM allele	*bla* TEM-1b	*bla* TEM-1a	*bla* TEM-1c	*bla* TEM diverse	*bla* TEM-52 b or -c
		N total=112	N total=15	N total=22	N total=10	N total=22
Associated transposable element	size (s) of PCR product[a]	n with the specified element (replicons)	n with the specified element (replicons)	n with the specified element (replicons)	n with the specified element (replicons)	n with the specified element (replicons)
Tn*1*		1 (FII & FIB)			1 (*bla* TEM-1d FII;FIB;FIA)	
Tn*2*		48 (FII; FIB; FIA; I1; P; B/O; N; NT; K; FII&R)		22 (FII;FIB; B/O; NT; IncI1)	4 (*bla* TEM-1g, -1j, 30, -135; FII; FIB; B/O)	20 (X1A; I1; L/M; N-like)
Tn*3*		1 (NT)	15 (B/O; I1, FIB; FII;P; NT; X1A)		2 (*bla* TEM-1c/1d, -30 ; both NT)	
IS26a	0.4 kb	2 (B/O & P)				
IS*26* a	2 kb and 0.4 kb	7 (B/O; P; B /O & IncP; FII & FIB & P; FII & FIB)				
IS*26* a	2 kb	14 (FII; FIB; I1; P; FIB & Y; NT; n-trsf)				
IS*26* a	1.3 kb	17 (FII; FIA; FIB; P)			1 (*bla* TEM-30 ; FII)	
IS*26* a & IS*26* b	2 kb (IS26a)& 0.6 kb (IS26b)	9 (B/O; B/O&P; I1& P)				
IS*26* b	0.6 kb	3 (B/O; IncFII & IncFIB)				
IS*26* b	1 kb				1 (*bla* TEM-40; FIB)	
IS*26* b	1.2 kb	1 (FII &FIB)				
ukn		9 (FII; FIB; NT; n-trsf; HI1)			1 (*bla* TEM-1d ,FII;FIB;FIA)	2 (A/C *bla* TEM-52b; R *bla* TEM-52c)

[a]- refers to linking PCR IV and –V form Manuscript II, relevant only for the ISa and ISb linking PCRs which were expected to generate misslaneous products; n-trsf – the *bla*$_{\text{TEM-1}}$ was non transferable from the wild type and not associated with any of the plasmids detected in the wild type strain; ukn- unknown (non-typable)

The summary of typing of the genetic environment of the *bla*$_{\text{TEM}}$ genes (study I and II) is presented in the Table 2. Overall, the *bla*$_{\text{TEM-1b}}$ located on Tn*2* transposons were most frequently detected on the various plasmids in *E. coli* (Manuscript II); *bla*$_{\text{TEM-52b}}$ and *bla*$_{\text{TEM-52c}}$ were also mainly located on the Tn*2* transposons on various plasmids from *E. coli* and *S. enterica* (Manuscript I). The *bla*$_{\text{TEM-1a}}$ genes were exclusively detected on Tn*3* and *bla*$_{\text{TEM-1c}}$ were only found on Tn*2* transposons (Manuscript II). In many cases in study II the elements detected upstream of the *bla*$_{\text{TEM-1b}}$ were IS*26* (no such case was observed upstream of the *bla*$_{\text{TEM-1a}}$ and the *bla*$_{\text{TEM-1c}}$). Insertions of IS*26* elements must have occurred in different orientations with respect to the *bla*$_{\text{TEM}}$ genes and possibly multiple times (illustrated examples are presented in Manuscript II, S2 supplement). That would explain the presence of multiple or differently sized bands produced in the ISa/b- *bla*$_{\text{TEM}}$ PCR (Table 2).

Tn*1* (suggested previously to be the typical transposon associated with *bla*$_{\text{TEM-2}}$ in *Pseudomonas* spp.) was detected in two cases in the IInd study; a multireplicon IncFII & IncFIA & IncFIB plasmid from human *E. coli* carried Tn*1* -*bla*$_{\text{TEM-1d}}$ and another

IncFII & IncFIB plasmid from poultry carried putative Tn*1* -*bla*$_{\text{TEM-1b}}$ (Manuscript II). The latter is an unusual combination and should preferably be verified in the future studies.

In summary, this study confirmed that the transmission of *bla*$_{\text{TEM}}$ genes is driven by Tn*3*-family elements on the plasmids in Enterobacteriaceae. The two elements, Tn*2* and IS*26* seem to be particularly involved in shaping of the accessory regions of plasmids from Enterobacteriaceae as well as other bacterial families. Partridge *et al*. demonstrated that the Tn*2* and IS*26* also drive the evolution and rearrangements of the regions containing the IS*Ecp1*-*bla*$_{\text{CTX-M-15}}$ on the IncF-family plasmids from *E.coli* [152].

3.6.2 Candidates for chromosomal progenitors of plasmid encoded *bla*$_{\text{TEM-1}}$

There are no records in the literature or in GenBank of a fully sequenced genome of an enteric bacterium that would harbour chromosomally located *bla*$_{\text{TEM-1}}$ gene (or Tn*3*-like-*bla*$_{\text{TEM-1}}$ inserted on the chromosome). *Tnp*R of Tn*2* linked to *bla*$_{\text{TEM-1b}}$ was previously found on a genomic island designated ICE*hinc1056* from *H. influenzae* (AJ627386; the allele was originally annotated as *bla*$_{\text{TEM-1d}}$, however, analysis in this study showed 100% identity of this open reading frame with *bla*$_{\text{TEM-1b}}$). There is no *tnp*A present on ICE*hinc1056*, however further upstream of the *tnp*R-*bla*$_{\text{TEM-1b}}$ there is an integrase gene present [132] (illustrated on the figure S2 in Manuscript II). This genomic island was shown to be typically integrated into the *H. influenzae* chromosome, but could be excised, circularize and also conjugate to other hosts[132]. Related elements with genes either coding for virulence factors or degradative enzymes, but not with *bla*$_{\text{TEM}}$ genes, were detected in other species of *Haemophilus*, *Pseudomonas* spp. and *Salmonella* spp.[132] It is interesting that also other *bla*$_{\text{TEM}}$ variants were described in *Haemophilus* spp. ('full' version of Tn*2*-*bla*$_{\text{TEM-1b}}$ is found on the sequenced chromosome of *H. influenza* CP002277; *bla*$_{\text{TEM-15b}}$ on the small plasmid pSF2[200] from *H. parainfluenzae*, AM849805). The latter are members of the family Pasteurellaceae and these bacteria, similarly to *K. pneumoniae*, can be free living microorganisms. Moreover, *Haemophilus* has increased permeability to penicillin G compared to enteric bacteria like *E. coli* [199;200], hence it seems logical that the β-lactamase driven mechanisms of resitance could have been somehow induced in these (or related) bacteria in the earily history of penicillin usage in humans and animals; or by the presence of this compund naturally produced by fungi in soil (although *H. influenzae* can also modify its outer membrane permeability as a mechanism of resistance to β-lactams). Resistance to aminopenicillins had its outbreak in *H. influenza* almost ten years after the first description of TEM-1 in Enterobacteriaceae [132]. It is therefore a far- reaching hypothesis, but it seems plausible that

the *bla*$_{TEM-1}$ genes could have been in the past derived from these kinds of ICE*hinc1056*-similar elements (possibly originating from other members of environmental or animal associated Pasteurellaceae). Recombination might have led to creation of Tn*3*- like-*bla*$_{TEM-1}$ elements that were later transmitted on BHR plasmids (or the ICE- elements) to Enterobacteriaceae and also *Pseudomonas* species[64] (the latter produce their chromosomal AmpC β-lactamases therefore they seem to be rather recipients of plasmid mediated *bla*$_{TEM}$ genes[121]). It can be further speculated that consequently the [Tn*3*-like -*bla*$_{TEM-1}$] elements transposed onto the endogenous plasmids in species like *K. pneumoniae* (IncFII$_k$, IncR or other scaffolds) as well as *E. coli* plasmids IncF, IncB/O and IncI1 or onto BHR platforms like IncP, IncR, IncN or other replicons.

Naturally, it could also be opposite and the *bla*$_{TEM}$ genes were initially transmitted (likely on plasmids) from Enterobacteriaceae to other families of bacteria. In any case, the question of the *bla*$_{TEM-1}$ origins remains to be answered in the future studies.

4. Conclusions

The following conclusions were deduced from this work:

i) Two types of replicons were mainly detected on the *bla*$_{\text{TEM-1}}$-plasmids from *E. coli* of human and food production animal origin: the IncF-complex (IncFII, IncFIA, IncFIB, IncFIC) and the IncI-complex (IncI1, IncB/O, IncK); in food production animals often IncP plasmids were forming co-integrates of the aforementioned types; overall, in few or single cases IncN, IncHI1, IncR and IncY replicons were detected.

ii) *Bla*$_{\text{TEM-52}}$ plasmids were mainly associated with IncI1 and IncX1 and to lesser extend IncA/C, IncL/M, IncN-related, IncR in *E. coli* and *S. enterica*; IncA/C are frequently found on diverse other *bla*$_{\text{TEM}}$ variants encoding TEM-ESBLs.

iii) Often the same plasmids that are normally prevalent in the given bacterium are also harbouring the resistance genes in this bacterium.

iv) Plasmids from *E. coli* display not only the adaptation to the given host bacterium but also reservoir specificity, namely IncB/O were found rather in *E. coli* of humans, cattle and pigs while IncI1 were typically found in *E. coli* from poultry and to lesser extend also in pigs; further research if required to identify the reason of this phenomenon.

v) IncI1 and IncX1 plasmids with *bla*$_{\text{TEM-52}}$ were likely acquired from food production animals; mainly *E. coli* and *S. enterica* from the poultry are suspected to be reservoirs of these resistance plasmids. It cannot be unequivocally concluded whether the *bla*$_{\text{TEM-1}}$ plasmids found in humans originated from animal sources or not, although the clonally related *bla*$_{\text{TEM-1}}$ IncB/O and IncI1can apparently circulated in *E. coli* from humans and diverse food production animals. This confirms that the plasmids can be horizontally exchanged between bacteria from animals and humans

vi) IncI1 scaffolds belonging to CC5 might have been the main platforms driving the transmission and evolution of the *bla*$_{\text{TEM-1}}$genes to *bla*$_{\text{TEM-52}}$ genes.

vii) BHR scaffolds like IncP, IncA/C, IncR, IncN and also IncL/M probably serve as transporters bringing the new traits (like the *bla*$_{TEM}$ or other resistance genes) from other bacteria species.

viii) *Bla*$_{TEM}$ usually reside on Tn*3*- family transposons but the different gene alleles tend to be associated with the specific members of the Tn*3*-family (*bla*$_{TEM\text{-}1b,\text{-}1c,\ \text{-}52b,\ \text{-}52c}$ typically were located on Tn*2*; *bla*$_{TEM\text{-}1a}$ typically were located on Tn*3*); different plasmids in Enterobacteriaceae can exchange their resistance genes owing to the association of these genes with the transposable elements.

ix) Despite the improvements in the standard PBRT methods for typing of plasmids in *K. pneumoniae* (IncR and IncFII$_k$), this species seem to harbour a variety of secondary replicons detected by the mPCR designed in study III. This further implies that optimizations are still needed in the detection and classification methods for plasmids from the diverse bacteria species in order to tract the epidemiology of these MGEs.

Overall, the studies presented here contributed to understanding that not only enteric bacteria but also mobile genetic elements like plasmids can be transmitted in a zoonotic mode. In some cases it was not possible to unequivocally state what was the direction of the plasmid transmission, i. e. from the animals or natural environments to humans or the other way round. Nevertheless, this thesis delivered evidences that *bla*$_{TEM}$ plasmids can be horizontally transmitted between enteric bacteria of human and animal origin and can also be found in bacteria contaminating meat products. The need for further research in order to determine the genetic background of bacteria from various niches and mobile genetic platforms transmitting resistance genes like the *bla*$_{TEM\text{-}1}$ is emphasized. This might eventually enable the prediction of which bacteria and from which niche/organisms constitute reservoirs of mobilizable resistance traits.

5. Future perspectives

When it comes to Gram negative bacteria, so far the best studied plasmids originated mainly from families like Enterobacteriaceae, in particular plasmids form the model organism (and also the common infectious agent) like *E. coli*. Detailed studies allowed for recognition that plasmids from other enteric species developed certain bacterial host specificities like the FII$_k$, FII$_s$ or FII$_y$ variants of the IncFII replicons. Study III provided data suggesting that plasmids

form *K. pneumoniae* generally may constitute distinct Inc groups than those described for *E. coli*. It is clear, however, that the occurrence of the resistance and virulence genes on plasmids stimulated researchers to focus mainly on these selected types of plasmids. By analogy, mainly resistance (and also degradative) plasmids from *Psudomonas* spp. are also relatively well studied. Recently a replicon typing scheme was published for previously not that well characterized plasmids from *Acinetobacter baumanii* [14]. These plasmids again drew attention mainly because of their association with the resistance genes in *A. baumanii*. Some of the Inc groups (comprised typically of the broad host range plasmids) overlap between the various bacterial families as described in section 2.3.1. It is not difficult to imagine that the resistance genes as well as other genetic traits may be transmitted on the mobile platforms between microorganisms found often in the natural environments. Supposable because of the aforementioned bias in the research foucusing on resistance plasmids, many other plasmids as well as their original hosts have not been yet well characterized and therefore it is difficult to get a full picture of where the hot spots for mobilization of the unwanted resistance genes are located [189]. To address this problem, future studies should preferentially include also plasmids as well as overall genetic diversity in the various environmental niches (soils, water reservoirs, plants, reservoirs of commensal bacteria in humans and animals etc.). This way the presence of potentially novel resistance genes in the environment could be rapidly estimated. Having the knowledge about where the progenitors for resistance genes are born would allow for predicting and in the future preventing of the further spread of these unwanted traits.

Moreover, studies on reservoir-specificity and plasmid- (bacterial) host interactions are needed to fully encompass the ecology of resistance transmission driven by these MGEs.

Finally, tracking down the epidemiology of MGEs requires rapid, cost effective methods with appropriate discriminatory power. PCR methods like PBRT are sensitive to the nucleotide substitutions at the primer binding positions. At the present moment pMLST is probably the most effective way for performing sequence-based comparisons of the diverse scaffolds in the screening studies like this one. However, pMLST is rather a prelude for future methodologies involving the whole genome sequencing. The latter would likely require elaboration of rapid annotation pipelines (or some possibility for instant *in silico* analysis of the obtained sequences, possibly with the use of predefined primers for the specific trait). This would allow for fishing out the traits specific for example for plasmids (like the replicon or genes characteristic for transfer regions); or traits like resistance or virulence.

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SECTION III

Manuscripts I-III

MANUSCRIPT I

Investigation of diversity of plasmids carrying the *bla*$_{\text{TEM-52}}$ gene.

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Investigation of diversity of plasmids carrying the bla_{TEM-52} gene

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Objectives: To investigate the diversity of plasmids that carry bla_{TEM-52} genes among *Escherichia coli* and *Salmonella enterica* originating from animals, meat products and humans.

Methods: A collection of 22 bla_{TEM-52}-encoding plasmids was characterized by restriction fragment length polymorphism (RFLP), replicon typing (by PCR or replicon sequencing), susceptibility testing, assessment of plasmid ability to self-transfer by conjugation and typing of the genetic environment of the bla_{TEM-52} gene. Detected IncI1 plasmids underwent further plasmid multilocus sequence typing.

Results: RFLP profiles demonstrated dissemination of bla_{TEM-52} in Denmark (imported meat from Germany), France, Belgium and the Netherlands from 2000 to 2006 by mainly two different plasmids, one encoding $bla_{TEM-52b}$ (IncX1A, 45 kb) and the other $bla_{TEM-52c}$ (IncI1, 80 kb). In addition, $bla_{TEM-52b}$ was also found to be located on various other plasmids belonging to IncA/C and IncL/M, while $bla_{TEM-52c}$ was found on IncN-like as well as on IncR plasmids. In the majority of cases ($n=21$) the bla_{TEM-52} gene was located on a Tn*3* transposon. Seven out of 10 bla_{TEM-52} plasmids tested in conjugation experiments were shown to be capable of self-transfer to a plasmid-free *E. coli* recipient.

Conclusions: The bla_{TEM-52} gene found in humans could have been transmitted on transferable plasmids originating from animal sources. Some of the bla_{TEM-52} plasmids carry replicons that differ from the classical ones. Two novel replicons were detected, IncX1A and IncN-like. Unlike its predecessor bla_{TEM-1}, the bla_{TEM-52} gene was not detected on F-type replicons suggesting that this gene evolved on other types of plasmid scaffolds.

Keywords: antibiotic resistance, ESBLs, human and non-human isolates

Introduction

Plasmids are usually circular, double-stranded DNA entities that can self-replicate.[1] They often encode functions giving extra advantages to their hosts in the presence of selective pressure.[1] The ability of some plasmids to transfer antibiotic resistance genes from one bacterial host to another constitutes a real threat to human health.[1,2] Extended-spectrum β-lactam antibiotics are commonly used for treatment of severe infections caused by Gram-negative bacteria, in particular those caused by Enterobacteriaceae. Resistance to these β-lactams is often plasmid encoded and the frequency of resistance is alarmingly increasing.[1]

Plasmid-encoded Bla_{TEM}-type enzymes capable of degrading β-lactam antibiotics were first described in 1965 and have since then disseminated worldwide.[1] The bla_{TEM-52} gene, encoding an extended-spectrum β-lactamase (ESBL), was first described in 1998 in France.[3] Since then it has been detected in clinical isolates and/or production animals from Canada, Portugal, France, Greece, the Netherlands, Germany, Belgium, Great Britain, Croatia and Japan, and has become the most prevalent ESBL in Korea, where it seemed to spread both clonally and horizontally.[4–17] Currently Bla_{TEM-52} β-lactamases constitute one of the most common types of ESBLs along with the Bla_{SHV} and Bla_{CTX-M} enzymes.[1] Many of the reports on the occurrence of bla_{TEM-52} genes come from ESBL prevalence studies, which did not focus on detailed characterization of plasmid species associated with the bla_{TEM-52} genes.[4–6,8–12,15–17] Thus little is known about the possible relationship between plasmids encoding the Bla_{TEM-52}-type β-lactamases. Knowledge about the mechanisms of dissemination of β-lactam resistance traits by mobile elements like plasmids can facilitate development of methods for predicting and further controlling that dissemination.

Currently, the largest amount of data on plasmids harbouring ESBL genes exists for plasmids carrying bla_{CTX-M} genes. Replicons belonging to IncI1, IncN, IncFIB, IncFIA, IncFII, IncA/C, IncL/M and IncHI2 families were, in the majority of cases, associated with diverse subtypes of bla_{CTX-M}.[1] Overall, other β-lactam

resistance genes like bla_{CMY}, bla_{SHV}, bla_{VIM} and diverse bla_{TEM} subtypes were also most often localized on plasmids carrying the aforementioned replicons.[1] Replicons belonging to other Inc families were detected sporadically on the ESBLs encoding plasmids.[1]

Different bla_{TEM} genes, including bla_{TEM-52}, evolved from bla_{TEM-1} and bla_{TEM-2}.[2] Bla_{TEM-1} and Bla_{TEM-2} β-lactamases are not considered ESBLs due to their narrow substrate spectrum. Subsequent mutations in the bla_{TEM} genes led to amino acid substitutions that expanded the substrate spectrum of the encoded enzyme due to an enlargement of the active site.[2] Bla_{TEM-52} differs from the Bla_{TEM-1} β-lactamase by three amino acid substitutions; Glu(104)→Lys, Met(182)→Thr and Gly(238)→Ser.[18] Also the silent point mutations are useful in tracing the evolutionary origin of the resistance genes.[18] Thus far two variants of the bla_{TEM-52} gene ($bla_{TEM-52b}$ and $bla_{TEM-52c}$) have been described.[4–7,9–11,19] Detection of similar plasmids harbouring different alleles of the bla_{TEM} genes would indicate that the plasmids might have acquired these genes possibly on transposable elements from different sources.[6]

The sparse knowledge about the possible relationship between plasmids harbouring the bla_{TEM-52} resistance genes prompted us to conduct a study on these plasmids in order to obtain further insight into their dissemination among the Enterobacteriaceae. Plasmids from both human and animal (or meat) isolates were analysed to investigate a potential plasmid-associated transfer of bla_{TEM-52} from animal to human reservoirs.

Materials and methods

Selection of strains

Twenty-two strains including *Escherichia coli* ($n=13$) and various serovars of *Salmonella enterica* ($n=9$) and carrying a version of the bla_{TEM-52} gene were collected from different sources and further characterized in this study (see Table 1). Isolates were collected during the period from 1995 to 2006 in different countries [Denmark (German meat), France, the Netherlands, Belgium, Spain, Korea and Canada]. They were kindly provided by different researchers and institutes and originated from poultry, poultry meat, beef meat or clinical samples from humans.[4–7,9–11,19]

Isolation of individual plasmids carrying the bla_{TEM-52} gene

Plasmidic DNA was purified from wild-type isolates and later from transformants using a Qiagen Plasmid Mini or Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

Plasmids were introduced to electrocompetent plasmid-free *E. coli* GeneHogs® (Invitrogen) cells by means of transformation by electroporation (Bio-Rad, Micropulser™). The transformation set-up was as follows: 2.5 kV; resistance 200 Ω; and capacitance 25 μF. Electroporants (transformants) were resuspended in 1.2 mL of brain hearth infusion (BHI) broth (Becton, Dickinson & Co., Bacto™), incubated for a minimum of 1 h at 37°C and plated onto selective BHI (Becton, Dickinson & Co., Difco™) agar plates.

Verification of transformants harbouring individual plasmids carrying the bla_{TEM-52} gene

Plasmids from the 22 donor strains were used for transformation by electroporation as described above. Selection of transformants (further designated with the suffix TF) was done on agar plates containing 2 mg/L cefotaxime. If necessary, plasmids from transformants were purified as described previously and the procedure was repeated until transformants with single bla_{TEM-52} plasmids were isolated for all 22 corresponding primary strains.

The presence of plasmids in the transformants and their sizes were determined using S1-PFGE;[20] 5 U of S1 nuclease (Fermentas) was used per plug slice. Plug slices with XbaI-digested *Salmonella* Branderup were used as size ladders.[21] Samples were run on a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA) and the conditions used were a 1% agarose gel (SeaKem Gold Agarose/Lonza) in 0.5× Tris/borate/EDTA, a voltage gradient of 6 V/cm, with phase from 6.8 to 38.4 s, and a run time of 19 h. Staining and image capture were performed as stated in Ribot *et al.*[21]

Verification of bla_{TEM-52} genes

The presence of ESBL genes was confirmed for both donor strains and transformants by PCR targeting conserved regions flanking the bla_{TEM} gene, as described by Hasman *et al.*[9] Unless the bla_{TEM-52} gene was already sequenced, PCR products were purified using GFX columns (Amersham Biosciences) and fully sequenced (Macrogen Inc., Korea).

Plasmid characterization by restriction fragment length polymorphism (RFLP)

RFLP was performed on bla_{TEM-52} plasmids from the 22 transformants. Plasmids from all transformants were purified as described and digested with EcoRI. The resulting fragments were separated and visualized on a 0.8% agarose gel (SeaKem®LA Agarose/Lonza) after 5 h at 4.0 V/cm or 21 h at 1.2 V/cm.

Replicon typing

Replicons of plasmids from the transformants were typed as described by Carattoli and co-workers.[22,23] In cases when it was impossible to determine the replicon by this method, cloning of the replicon or full plasmid sequencing was performed using a GS FLX pyrosequencer (Roche).

From plasmid preparations of pE001 (located in *E. coli* 2161TF) and pGOC049 (located in *E. coli* GOC049TF), a standard FLX sequencing library was built using 5 μg of DNA according to the manufacturer's guidelines (Roche). Test emulsion PCRs were performed to obtain the best copies/bead ratio. DNA containing beads with each plasmid library were sequenced in two regions using the GS FLX standard sequencing kit on a 4-region 25×75 pico titre plate. A total of 11000 and 20000 reads from each of plasmids pE001 and pGOC049 were aligned and assembled using the Newbler assembler software version 2.0.01.14 provided with the GS FLX instrument.

In the case of the plasmid from 727TF, a fragment carrying the putative replicon was generated by digestion of the plasmid with BglI and BamHI (Fermentas) and subsequent purification on the GFX column (Amersham Biosciences). The fragment was ligated to the chloramphenicol resistance gene that was PCR amplified from the vector pLOW1 (Amersham Life Science). The construct was transformed by electroporation into electrocompetent GeneHogs®. Transformant was selected on BHI agar plates supplied with 25 mg/L chloramphenicol. Plasmid was purified from this transformant and used as the template for sequencing. Sequencing was performed by the standard Sanger sequencing method at Macrogen Inc. (Korea).

Results were further processed using Vector NTI Suite 11 (Invitrogen, Inc.) and then BLASTN and BLASTX searches against known replicon sequences from the GenBank database were performed to identify putative replication proteins located on the plasmids.

Multilocus sequence typing of IncI1 plasmids

All plasmids from transformants positive for the IncI1 replicon in the multiplex PCR[22] underwent further plasmid multilocus sequence typing (pMLST) as described by Garcia-Fernandez *et al.*[24]

Incompatibility assay

Incompatibility testing was performed for bla_{TEM-52} plasmids located in *E. coli* GOC049TF (designated as pGOC049) and *E. coli* 2161TF (designated as pE001). In separate transformations, *E. coli* GeneHogs® carrying plasmid R46 (IncN plasmid, kindly provided by Alessandra Carrattoli, Istituto Superiore di Sanità, Rome, Italy; accession number AY046276) and the IncX1 plasmid pOLA52 *bla::npt* (Kan^r),[25] respectively, were obtained. Plasmids were purified from these by the described method. Each of the four transformants mentioned in this section was made electrocompetent using a standard protocol for preparation of electrocompetent *E. coli* cells.[26] The protocol employed to perform the incompatibility assay was as described by Norman *et al.*[25] with modification so that the tested plasmid was introduced into the electrocompetent cells harbouring the second plasmid by means of transformation by electroporation. pGOC049 (CTX^r) was tested against IncN representative plasmid R46 (Tet^r). Selection of transformants with both pGOC049 and R46 was made on an agar plate supplied with cefotaxime together with tetracycline (2 mg/L and 16 mg/L, respectively). pE001 (CTX^r) was tested against IncX1 representative pOLA52 *bla::npt* (Kan^r). An agar plate supplied with cefotaxime together with kanamycin (2 mg/L and 50 mg/L, respectively) was used to select transformants harbouring both pOLA52 *bla::npt* and pE001.

Each of the two tested plasmids was used in the incompatibility assay both as the incoming and as the residual agent. To assess the plasmid losses in the incompatibility assay for pGOC049 and R46, selective plates with 16 mg/L tetracycline and 2 mg/L cefotaxime were used, respectively. To assess plasmid losses in the incompatibility assay for pE001 against pOLA52 *bla::npt*, selective plates supplied with 2 mg/L cefotaxime and 50 mg/L kanamycin were used, respectively. As controls, each of the four transformants originally harbouring only one plasmid was inoculated into the broth without selection, and plasmid stability was further assessed following the method described by Norman *et al.*[25] The presence/absence of specific replicon types in the obtained transformants was confirmed by PCR targeting replicons of R46, pOLA52 *bla::npt*, pE001 and pGOC49, respectively.

PCR conditions for detecting IncN replicon of R46 were as described by Carattoli *et al.*[27] Primers for detecting the remaining replicons were designed based on the obtained sequencing data. For the sequences of the primers and for PCR details please see Table S1 (available as Supplementary data at *JAC* Online).

Examination of clonal relationship of wild-type isolates harbouring similar plasmids

If plasmids carrying the same replicons and displaying similar RFLP profiles were detected in more than one of the transformants, the corresponding *E. coli* wild-type strains harbouring similar plasmids were tested by a PCR phylotyping method as described by Clermont *et al.*[28] to pre-determine the potential clonality of these strains.

Genetic environment upstream of the bla_{TEM-52} gene

PCR linking for the presence of the *tnpA* gene of Tn*3* upstream of the bla_{TEM-52} gene was performed on the transformants (n=22). The primers used and PCR details are given in Table S1. The PCR product obtained from strain 54.12TF was purified using GFX columns (Amersham Biosciences) and sequenced. The sequence was aligned with the sequence of Tn*3*-bla_{TEM-52} (EF141186).

Plasmid transmissibility

bla_{TEM-52}-carrying plasmids from selected strains (representing each of the different RFLP groups (76-33094TF, 44.02TF, ESBL 140TF, ESBL 424TF, YMC 95/4/4199TF, YMC 96/7/4035TF, 549TF, 641TF, GOC043TF and 727TF) were tested for the ability to self-transfer to the plasmid-free recipient *E. coli* MT101 (Nal^r, Rif^r). Conjugation was set-up as follows; sterile paper filter (pore diameter 0.2 μm, Advantec®) was placed in the centre of a blood agar plate, 2 mL of donor and recipient cultures in exponential phases of growth were mixed together and 500 μL of the mixture was placed on the paper filter, allowing the liquid to soak into the medium. After overnight incubation, filters were washed with 4 mL of 0.9% salt water and 100 μL of the suspension was inoculated onto BHI (Becton, Dickinson & Co., Difco™) agar plates with 2 mg/L cefotaxime, 32 mg/L nalidixic acid and 25 mg/L rifampicin. After overnight incubation at 37°C the presence of transconjugants was assessed.

Susceptibility testing

Unless stated in the references, the primary strains were tested for their susceptibility to a range of antimicrobial agents by means of a commercially available panel for Enterobacteriaceae (Sensititre®). The antimicrobial agents included were amoxicillin/clavulanic acid, ampicillin, apramycin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamicin, nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim. Testing was performed according to the recommendations of the CLSI (formerly the NCCLS). CLSI breakpoints (2003) were used for interpretation of the results except for cefotaxime. For cefotaxime, and if no CLSI breakpoints were available for a tested compound, the criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) were used for interpretation of the results.

Based on the resistance profiles obtained for donor strains, transformants were tested for susceptibility to the selected antimicrobials. Susceptibility testing of transformants was performed using the disc diffusion method according to the guidelines of the CLSI. The recipient strain of *E. coli* GeneHogs® was included as a control. CLSI zone diameter interpretative standards (2003) were used for interpretation of the results. Results for ceftiofur were interpreted as recommended by Aarestrup *et al.*[29]

Results

Verification of bla_{TEM-52} genes

Twenty-two strains carrying a bla_{TEM-52} plasmid were obtained from different sources as listed in Table 1 and transferred to a plasmid-free *E. coli* recipient. The obtained transformants were given the same names as the corresponding donor isolates, but with a TF suffix. Both the primary strains and the obtained transformants were positive in PCR targeting the bla_{TEM} gene. Among these isolates, 13 were found to carry a plasmid with the $bla_{TEM-52b}$ version of the gene and 9 carried the $bla_{TEM-52c}$ version.

Plasmid characterization by RFLP

Purified plasmids from all 22 transformant strains were digested with the EcoRI enzyme. RFLP patterns indicated that strains 2161TF, 7633094-7TF, 36.52TF, 44.02TF, 46.20TF, 48.78TF,

Table 1. List of wild-type strains harbouring the bla_{TEM-52} plasmids characterized in the study and the results of plasmid characterization

Wild-type strain	Phylotype	Isolation source (country of origin)	Year of isolation	bla_{TEM-52} allele	Plasmid RFLP type	Replicon type	Self-transmissibility	Plasmid size (kb)	Element upstream of bla_{TEM-52}	Resistances associated with the bla_{TEM-52} plasmid	Reference
E. coli 2161	B1	broiler meat (DE[a])	2006	TEM-52b	a	IncX1A	ND	45	Tn*3*	AMP, CEF, CPD, XNL	4
E. coli 7633094-7	B1	beef (DE[a])	2004	TEM-52b[b]	a	IncX1A	yes	45	Tn*3*	AMP, CEF, XNL	10
Salmonella Blockley 36.52	NA	poultry meat (NL)	2001	TEM-52b[b]	a	IncX1A	ND	45	Tn*3*	AMP, CEF, XNL	9
Salmonella Paratyphi 44.02	NA	poultry (NL)	2001	TEM-52b[b]	a	IncX1A	yes	45	Tn*3*	AMP, CEF, XNL	9
Salmonella Blockley 46.20	NA	poultry meat (NL)	2001	TEM-52b[b]	a	IncX1A	ND	45	Tn*3*	AMP, CEF, XNL	9
Salmonella Typhimurium 48.78	NA	poultry (NL)	2002	TEM-52b	a	IncX1A	ND	45	Tn*3*	AMP, CEF, XNL	9
Salmonella Blockley 51.09	NA	poultry (NL)	2002	TEM-52b[b]	a	IncX1A	ND	45	Tn*3*	AMP, CEF, XNL	9
Salmonella Virchow 54.12	NA	poultry (NL)	2002	TEM-52b[b]	a	IncX1A	ND	45	Tn*3*	AMP, CEF, XNL	9
E. coli 660	A	human (F)	2001	TEM-52b[b]	a	IncX1A	ND	45	Tn*3*	AMC, AMP, XNL, CTX	6
Salmonella Typhimurium 44.78	NA	poultry (NL)	2001	TEM-52c[b]	b	IncI1	ND	80	Tn*3*	AMP, CEF, SPT, XNL	9
E. coli 549	A	human (F)	2000	TEM-52c[b]	b	IncI1	no	80	Tn*3*	AMP, XNL, CTX	6
E. coli 641	B1	human (F)	2002	TEM-52c[b]	b	IncI1	yes	80	Tn*3*	AMC, AMP, XNL, CTX	6
E. coli 692	A	human (F)	2001	TEM-52c[b]	b	IncI1	ND	80	Tn*3*	AMC, AMP, XNL, CTX	6
E. coli 710	D	human (F)	2002	TEM-52c[b]	b	IncI1	ND	80	Tn*3*	AMP, XNL, [CTX]	6
E. coli Cloeckaert TK[c]	NA	human/poultry (B, F)	2001–05	TEM-52c[b]	b	IncI1	ND	80	Tn*3*	AMP, [CAZ], CEF, [CRO], [XNL]	7
E. coli GOC043	B2	poultry (ES)	unknown	TEM-52c	c	IncN-like	yes	40	Tn*3*	AMP, XNL, CTX	5
E. coli GOC049	B1	poultry (ES)	unknown	TEM-52c	c	IncN-like	ND	40	Tn*3*	AMP, XNL, CTX, SPT	5
E. coli 727	ND	human (F)	2003	TEM-52c[b]	d	IncR	no	40	Tn*3*	AMP, [XNL], [CTX], GEN, NEO	6
E. coli ESBL 140	ND	human (CA)	1999–2000	TEM-52b	e	IncA/C	no	81	unknown	AMP, XNL, [CTX], GEN	17
E. coli ESBL 424	ND	human (CA)	1999–2000	TEM-52b	f	IncI1	yes	115	Tn*3*	AMP, XNL, CTX, GEN, SPT, TET	17
Salmonella Saintpaul YMC 95/4/4199	NA	human (KR)	1995–97	TEM-52b[b]	g	IncL/M	yes	65	Tn*3*	AMC, AMP, XNL, CTX, SPT, SMX, TMP	11
Salmonella Stanley YMC 96/7/4035	NA	human (KR)	1995–97	TEM-52b	h	IncL/M	yes	80	Tn*3*	AMC, AMP, XNL, CTX, GEN	11

DE, Denmark; NL, the Netherlands; F, France; B, Belgium; ES, Spain; CA, Canada; KR, Korea; ND, not determined; NA, not applicable; AMP, ampicillin; AMC, amoxicillin/clavulanate (2:1); CAZ, ceftazidime; CEF, cefalotin; CPD, cefpodoxime; CRO, ceftriaxone; CTX, cefotaxime; GEN, gentamicin; NEO, neomycin; SMX, sulfamethoxazole; TET, tetracycline; TMP, trimethoprim; XNL, ceftiofur; SPT, spectinomycin; [], intermediate resistance.

[a]Gene was sequenced by the researcher/institute providing the strain.

[b]Isolated in Denmark.

[c]Plasmid originally isolated from *S. enterica*; transconjugant provided by Cloeckaert *et al.*[7]

51.09TF, 54.12TF and 660TF shared a very similar $bla_{TEM-52b}$-type plasmid with an approximate size of 45 kb. The RFLP profile of these plasmids was designated type a. Strains 44.78TF, 549TF, 641TF, 692TF, 710TF and Cloeckaert TF were shown to share a similar ~80 kb bla_{TEM-52}-type plasmid, which differed from the already mentioned p$bla_{TEM-52b}$ RFLP type a. This RFLP type was designated type b. pGOC043 and pGOC049 shared an almost identical pattern, as they differed only by the presence of one extra band in pGOC049, and were thus designated as type c. RFLP profiles of plasmids from the remaining transformants were significantly different from each other (difference of more than six bands) and from the three described types. These plasmids ranged in size from ~40 kb to ~146 kb and were designated with RFLP profile letters d to h.

Replicon typing

All transformants carrying plasmids with RFLP profile b and ESBL 424TF (RFLP type f) were positive for the IncI1 replicon in the multiplex PCR. YMC 95/4/4199TF (RFLP type g) and YMC 96/7/4035TF (RFLP type h) were positive for the IncL/M replicon. In one case for ESBL 140TF (RFLP type e) the replicon IncA/C was detected and 727TF (type d) gave a positive signal for the IncR replicon.

Replicons of plasmids from 2161TF (representing type a) and GOC49TF (representing type c) did not produce positive results by the standard multiplex PCR and were therefore detected by partial or full plasmid sequencing. As the IncR replicon is not so commonly detected in *E. coli*, the replicon of plasmid from 727TF (plasmid designated as p727) was also cloned and sequenced to perform further analysis. The sequences were compared with the GenBank database to identify similar plasmids with known replicons. The sequence encoding the putative replicase of plasmid p727 showed 100% similarity with *repB* sequences of two different IncR plasmids, namely pEFER (GenBank accession number CU928144) and pK245 (DQ449578), originating from *Escherichia fergusonii* and *Klebsiella pneumoniae*, respectively.

The putative *rep* sequence of the plasmid pE001 from strain 2161TF shared 100% similarity with the *pir* sequence of pMAS2027 classified as IncX1 (FJ666132). No significant similarity was observed between the putative *rep* of pE001 and *pir* of another IncX1 plasmid, pOLA52 (EU370913) (40% similarity at the amino acid level). What is more, a fragment of 590 bp upstream of *repB* of pE001 shared 96% identity at the nucleotide level with a fragment of the same size determining the incompatibility properties of a classical IncX1 plasmid R485 (M11688). Also the *stbE* and *stbD* genes determining the stability properties of R485 (AF072126) were shown to be present on pE001. The remaining components of the replicon and the entire transfer region of pE001 shared a large number of similarities with pOLA52 (IncX1), pMAS2027 and other plasmids considered as IncX1, namely pSE34 (EU219533), pOU1115 (DQ115388) and pOU1114 (DQ115387). These included fragments or full sequences of replication origins, genes encoding diverse accessory proteins found on the replicon (among others *bis*, *taxD*, *parA*) and components encoding the conjugation machinery (*taxA*, *taxB*, *taxC*, *taxD*, *pilx1-6*, *pilx8-11*). The comparison of the pE001 replicon with other IncX1 replicons mentioned is presented in Figure 1. Based on the sequence analysis, we propose to classify the replicon of pE001 as an IncX1 variant, namely IncX1A. All strains sharing the RFLP profile 'a' in this study appeared positive in *rep*-pE001 PCR, thus they were also assigned to the IncX1A subgroup.

The *rep* sequence of pGOC049 showed 72% identity at the DNA level (and 78% at the amino acid level) with the *repA* sequence of the IncN plasmid R46 and also *repA* of plasmid pKOX105 (HM126016). Due to the similarity to the IncN replicon of R46, pGOC049 was assigned to be a type of IncN plasmid. PCR targeting the replicon of pGOC049 was also performed on the plasmid from GOC043TF due to the similarity of their RFLP patterns. A positive product for pGOC043 was observed in this PCR.

The sequences of pE001, the *rep* fragment of pGOC049 and the *rep* fragment of p727 were deposited in GenBank with accession numbers JF776874, JF708955 and JF708954, respectively.

Multilocus sequence typing of IncI1 plasmids

Plasmids positive for the IncI1 replicon in the multiplex PCR, namely from ESBL 424TF, 44.78TF, 549TF, 641TF, 692TF, 710TF and Cloeckhaert TF, underwent further pMLST.[22,24] Based on the sequencing results, the plasmid from ESBL 424TF (RFLP type f) was assigned to be of sequence type (ST) 2. The remaining tested plasmids (sharing RFLP type b) were assigned by pMLST to ST5. Various insertions or deletions were observed in the sequences obtained for the six IncI1 plasmids of ST5 compared with the allele variants described in the reference. However, these mutations were not located on the sites corresponding to the relevant nucleotides, e.g. those determining the STs on the reference sequences.

Incompatibility testing

Incompatibility testing was performed for bla_{TEM-52} plasmids originating from *E. coli* GOC049TF (pGOC049) and *E. coli* 2161TF (pE001) to investigate their incompatibility affiliation. In transformants harbouring only one of the respective plasmids, e.g. pGOC049, R46, pOLA52 *bla::npt* or pE001, and grown for 50 generations without selective pressure, no loss of original resistance was observed. The presence of respective replicons was additionally confirmed in selected colonies from the transformants by the PCRs targeting these replicons. This demonstrated the stability of the plasmids in the recipient. The pE001 plasmid turned out to be compatible with pOLA52 *bla::npt* (an IncX1 plasmid). Regardless of which of the plasmids was the incoming or residing agent in the assay, in both cases 99% of transformants retained the initial resistance to both cefotaxime and kanamycin, indicating that the two plasmids could co-exist in the same cell. Two selected transformants with resistance to both antibiotics underwent S1-PFGE, and they were shown to harbour two plasmids at the same time, thus demonstrating that no co-integration had occurred. This led to the conclusion that the two plasmids are compatible. However, based on the large homology of the overall pE001 sequence with pOLA52 and 100% identity of the putative *rep* of pE001 with *pir* of pMAS2027 (classified as IncX1[30]), we suggest assigning this plasmid to the IncX family, and further to the IncX1A subgroup.

The *rep* sequence of pGOC049 showed 72% identity with the *repA* sequence of the classical IncN plasmid R46. Attempts to introduce pGOC049 into electrocompetent cells already

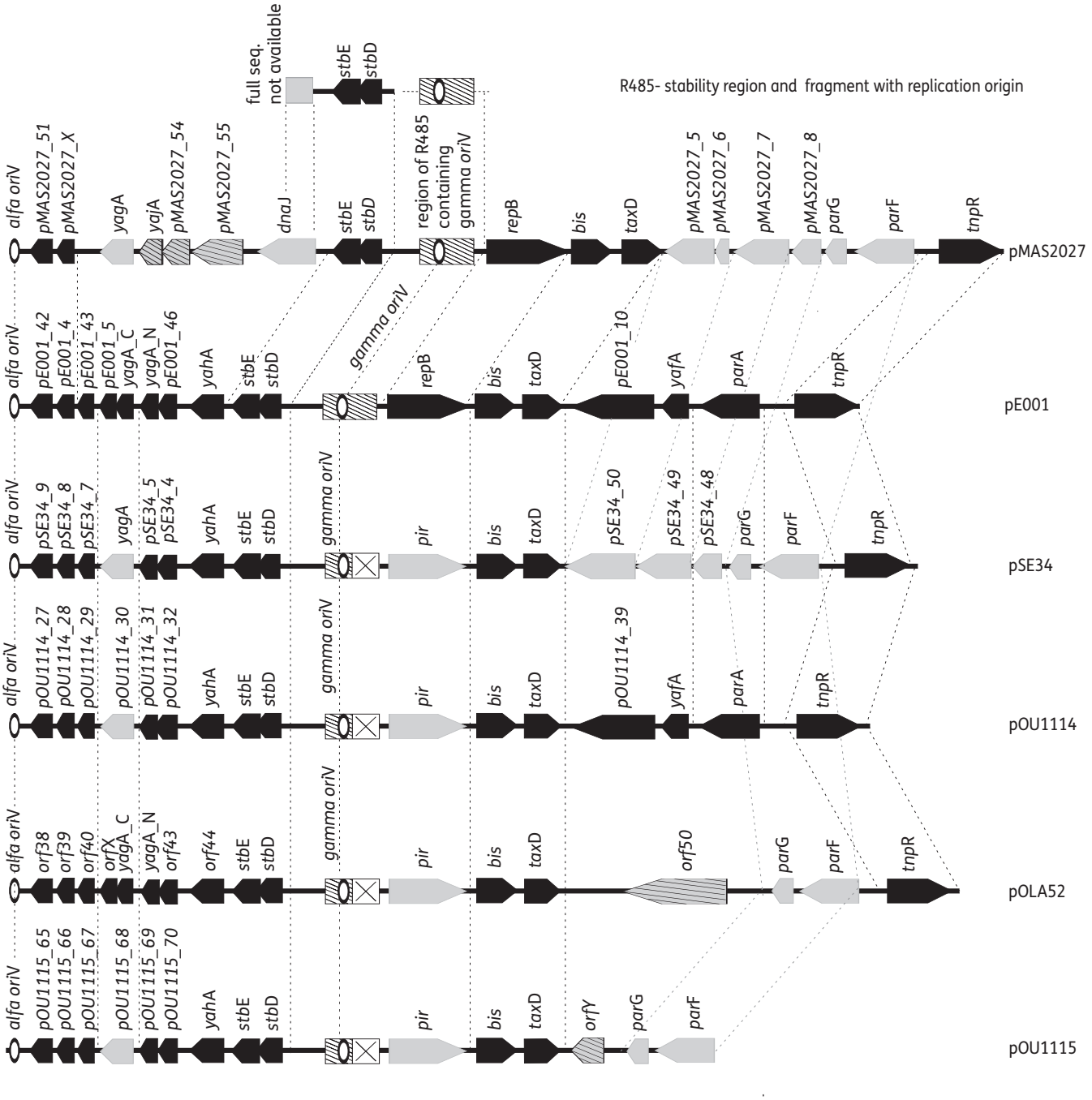

Figure 1. Schematic comparison of the pE001 replicon with the replicons of IncX1 and IncX1-related plasmids. Black arrows indicate the genes and open reading frames (ORFs) found on pE001 and similar genes and ORFs found on other plasmids included in the scheme, grey arrows indicate genes and ORFs that were not found on pE001 but are shared by at least two or more other plasmids described, grey arrows filled with slanting lines represent genes and ORFs found only on the individual plasmids indicated, white rectangles with black slanting lines correspond to the fragment of the sequence of R485 (M11688), black crosses indicate the position at which the sequence of the given plasmid differs significantly from the described R485 fragment (M11688) and black ovals indicate origins of replication. The sequences were obtained from GenBank. ORFs were predicted for all the plasmids examined in this study with Vector NTI Suite 11 software (Invitrogen, Inc.). *orfX* on pOLA52 and *orfY* on pOU1115 were predicted in this study, but were not found in the original annotations of the plasmids.

harbouring R46 did not produce transformants on the plate with selection for both plasmids at the same time. However, transformants were observed on this selective plate when R46 was used as the incoming agent and pGOC049 as the residing one. What is more, no loss of resistance either to cefotaxime or to tetracycline was observed after cultivating that transformant for 50 generations without selection. At the end of the incompatibility assay a selected transformant colony that was resistant to

both cefotaxime and tetracycline was tested using S1-PFGE and was shown to harbour the two plasmids at the same time. The results obtained from the incompatibility assay are difficult to interpret since transformants harbouring two plasmids simultaneously were obtained only when R46 was the incoming plasmid, but not when the pGOC049 was the incoming agent. Due to the similarity of the two replicons we propose to term the replicon of pGOC049 as IncN-like.

Examination of clonal relationship of wild-type isolates harbouring similar plasmids

Three *E. coli* strains 2161, 7633094-7 and 660 harboured similar IncX1A plasmids. *E. coli* 2161 and 7633094-7 were shown to belong to the same phylotype B1, and thus could possibly be clonally related. *E. coli* 660 was assigned to phylotype A and would not be expected to be clonally related to the two phylotype B1 isolates with similar plasmids. *E. coli* 549, 641, 692 and 710 strains harboured very similar IncI1 plasmids. Two of these isolates, *E. coli* 549 and 692, shared the same phylotype A and could be clonally related. *E. coli* 641 and 710 were shown to belong to B1 and D phylotypes, respectively. *E. coli* GOC043 and GOC049 harboured similar IncN-like plasmids. These two strains gave a positive signal in the PCR to B2 and B1 phylotypes, respectively.

The *Salmonella* Blokley wild-type isolates 36.52, 46.20 and 51.09 harbouring similar IncX1A plasmids have been previously examined by XbaI-digested PFGE by Hasman and Aerstrup[31] and were shown to be undistinguishable.

Genetic environment upstream of the bla_{TEM-52} gene

In the plasmid originating from the Cloeckaert TK isolate the $bla_{TEM-52c}$ gene had previously been reported to reside on a Tn*3* element.[7] Thus the upstream regions of bla_{TEM-52} genes in plasmids from the remaining transformants of this study were also investigated. For the plasmid originating from ESBL 140TF (RFLP type e), no signal was observed in the PCR linking the presence of *tnpA* with the bla_{TEM-52} gene. The remaining plasmids were positive in the described PCR, indicating that the bla_{TEM-52} genes were located within the Tn*3* transposon and downstream of the transposase.

Plasmid transmissibility

bla_{TEM-52} plasmids belonging to different RFLP groups (indicated in parentheses) and originating from selected transformants that were shown not to carry any other plasmids—76-33094TF (RFLP type a), 44.02TF (RFLP type a), ESBL 140TF (RFLP type e), ESBL 424TF (RFLP type f), YMC 95/4/4199TF (RFLP type g), YMC 96/7/4035TF (RFLP type h), 549TF (RFLP type b), 641TF (RFLP type b), GOC043TF (RFLP type c) and 727TF (RFLP type d)—were tested for the ability to self-transfer to the plasmid-free recipient *E. coli* MT101. Transconjugants were observed for all the above listed strains except three; 549TF (RFLP type b/IncI1), ESBL 140TF (RFLP type e/IncA/C) and 727TF (RFLP type d/IncR).

Susceptibility testing

Not surprisingly, all primary strains were resistant to ampicillin, ceftiofur and cefotaxime. All 22 transformants that carried only a variant of a bla_{TEM-52} plasmid were likewise resistant to the tested β-lactam antimicrobials.

Thirteen of the primary strains were resistant to sulphonamides and trimethoprim. In one case these resistances were apparently associated with the $bla_{TEM-52b}$/IncL/M plasmid from YMC 95/4/4199TF. Ten primary strains were resistant to tetracycline. Tetracycline resistance associated with the $bla_{TEM-52b}$ IncI1 plasmid was observed in one case in the ESBL 424TF strain. Five of the primary strains were resistant to neomycin. This resistance was observed in one of the corresponding transformants, namely 727TF, indicating that it was residing on the IncR $bla_{TEM-52c}$ plasmid. Five of the primary isolates were resistant to gentamicin and in four cases this resistance was associated with $bla_{TEM-52b}$ and $bla_{TEM-52b}$ plasmids from ESBL 140TF (IncA/C plasmid), ESBL 424TF (IncI1), 727TF (IncR) and YMC 96/7/4035TF (IncL/M). Five of the primary strains were resistant to one or both of the tested amphenicol compounds (chloramphenicol and florfenicol). Resistance to the tested amphenicols was not observed in the corresponding transformants.

Discussion

Little is known about the possible relationship between plasmids harbouring bla_{TEM-52}. Therefore we characterized plasmids from a collection of 22 bla_{TEM-52}-positive isolates from animals, humans and food products originating from several different European countries as well as Canada and Korea.

Thirteen plasmids in our study carried the $bla_{TEM-52b}$ allele, while nine carried the $bla_{TEM-52c}$ allele. Both alleles were disseminated among plasmids from human and non-human isolates and they were generally associated with different plasmid incompatibility groups. Clearly the IncI1 ($n=7$) and IncX1A ($n=9$) replicons dominated among the bla_{TEM-52} plasmids characterized in the study. Six of the seven IncI1 plasmids carried the $bla_{TEM-52c}$ allele. These belonged to RFLP type b and all represented ST5. One IncI1 plasmid carried the $bla_{TEM-52b}$ allele, represented RFLP type f and was found to be ST2. Curiously the six ST5 IncI1 $bla_{TEM-52c}$ plasmids originated from strains isolated in European countries, while the ST2 $bla_{TEM-52b}$ plasmid originated from Canada (human isolate). One of the ST5 IncI1 plasmids found in *E. coli* Cloeckaert TK and described in this study was originally isolated by Cloeckaert *et al.*[7] from *S. enterica* species. Apparently epidemic $bla_{TEM-52c}$ ST5 IncI1 plasmids circulated in European countries during the time between diverse strains of *E. coli* (phylotypes A, B1 and D; this study) and serovars of *S. enterica*.[7] The strains were isolates from humans and poultry, indicating possible transmission of the ST5 IncI1 $bla_{TEM-52c}$ plasmid between these two reservoirs.

Nine of the 13 $bla_{TEM-52b}$ plasmids appeared identical in RFLP profiles (RFLP type a) and they shared the same IncX1A replicon. *E. coli* (phylotypes A and B1) and various *S. enterica* serovars harbouring the nine plasmids originated from poultry, poultry meat, broiler meat and beef, as well as one from a human infection. These originated from Germany, France and the Netherlands between 2001 and 2006, which demonstrates a relatively wide spread of the similar $bla_{TEM-52b}$ IncX1A plasmids among the mentioned reservoirs.

Both the IncI1 and the IncX1A plasmids described above originated generally from diverse serovars of *S. enterica* and diverse phylotypes of *E. coli*. Although in some of the cases it is possible that the wild-type strains harbouring the similar plasmids were clonally related, it is clear that these very similar plasmids were capable of residing in diverse strains.

Other replicons associated with bla_{TEM-52} genes detected in this study belonged to IncL/M ($n=2$; both carried $bla_{TEM-52b}$), IncA/C ($n=1$; $bla_{TEM-52b}$), IncR ($n=1$; $bla_{TEM-52c}$) and IncN-like ($n=2$; both carried $bla_{TEM-52c}$) incompatibility families. The two IncL/M plasmids originated from different *S. enterica* serovars and they did not seem to be closely related, as their RFLP patterns were very different. The two IncN-like plasmids originated from different *E. coli* strains (phylotypes B1 and B2) isolated from Spanish poultry. Their RFLP profiles were very similar, suggesting an interspecies transmission of these similar IncN-like plasmids.

Overall, the findings described above indicate that both the $bla_{TEM-52b}$ and $bla_{TEM-52c}$ genes may be distributed on the diverse plasmid replicons, most probably due to the association with Tn*3* elements. Once integrated onto the plasmid backbone, the Tn*3*-bla_{TEM-52} element may possibly have been transferred on that plasmid both horizontally and clonally.

Interestingly, several of the bla_{TEM-52} plasmids in this study were negative in the standard multiplex PCR for replicon typing.[27] These replicons were sequenced and the corresponding plasmids were tested in incompatibility assays with known representatives of classical Inc families. The putative Rep protein of pE001 from *E. coli* 2161 shared 100% identity at the amino acid level with the π replicase of plasmid pMAS2027 and 40% identity at the amino acid level with the π replicase of pOLA52. pE001 turned out to be compatible with the latter, and this could have been due to the differences between pE001 and pOLA52 replicases. All of the remaining components of the replicon and also the transfer regions of the pE001 shared from 74% to 100% amino acid identities with pMAS2027, pOLA52 and several other plasmids classified as IncX1-like (Figure 1). pMAS2027 was assigned by Ong *et al*.[30] to the IncX1 family based solely on its sequence analysis; therefore we can only speculate that pMAS2027 could display similar incompatibility properties to the IncX1A pE001. The two plasmids share high sequence similarity with the incompatibility fragment of the classical IncX1 plasmid R485 (M11688), while the remaining IncX1 plasmids share only a partial similarity with the R485 fragment (Figure 1). The full sequence of R485 is not yet publicly available. It is highly possible that pE001 and pMAS2027 represent a separate branch of the IncX1 family termed in this study as IncX1A.

In the case of pGOC049, the results of the incompatibility assay with R46 (IncN) were difficult to interpret. Due to the similarity of pGOC049 replicase and RepB of the IncN plasmids and the lack of data on the remaining part of the pGOC049 sequence, we decided to term this plasmid as IncN-like. Another $bla_{TEM-52c}$ plasmid from *E. coli* GOC043 was found to be very similar to pGOC049. It is likely that this type of IncN-like replicon represents a separate branch of the IncN family.

The *rep* sequence of p727 shared 100% identity with *repB*s of IncR plasmids originating from *K. pneumoniae* (pKP245) and *E. fergusonii* (pEFER). IncR replicons apparently have a broad host range. Surprisingly, the sequences of pEFER and pKP245 do not seem to contain the functional and typical conjugative transfer elements.[32] This could explain why the $bla_{TEM-52c}$ IncR plasmid p727 examined in the study was incapable of self-transfer in conjugation. This further indicates that IncR replicons could be mobilizable.

An important observation drawn from our study is that bla_{TEM-52} seemed to be primarily associated with a limited number of aforementioned classical replicons or replicons closely related to the classical IncX1 and IncN, but not with the IncF family. This is contrary to the bla_{TEM-52} predecessor, namely bla_{TEM-1}, which is most often associated with IncFII/FIB/FIA families.[1] The question is raised as to why bla_{TEM-52} is not observed on the same IncF scaffolds as bla_{TEM-1} if bla_{TEM-52} evolved from bla_{TEM-1}. The reason could be that the bla_{TEM-52} gene did not evolve on IncF plasmids, but evolved from bla_{TEM-1} that transposed initially to IncI1, IncA/C, IncL/M, IncR or other plasmid backbones. Supporting this theory is the fact that bla_{TEM-1} as well as its evolutionary followers bla_{TEM-3}, bla_{TEM-21} and bla_{TEM-24} were detected on other than IncF scaffolds.[1] The bla_{TEM-52} gene residing on Tn*3* was apparently not able to re-associate with IncF scaffolds that already harboured the Tn*3* elements due to transposon immunity, thus the occurrence of this type of ESBL is limited to other mentioned replicons.[33] Occurrence of bla_{TEM-19} and bla_{TEM-15}, which are the most probable intermediates of evolution from bla_{TEM-1} to bla_{TEM-52}, has been reported.[34,35] However, there are no sufficient data available on replicons of plasmids carrying these. This makes the model explaining where and when the upgrade occurred from Bla_{TEM-1} to the extended-spectrum Bla_{TEM-52} incomplete.

Our study underlined that primarily the conjugative and relatively broad host range plasmids belonging to IncI1, IncX1A, IncA/C, IncL/M and IncN-related types are the transporters for bla_{TEM-52} genes. Also, not so commonly detected and possibly mobilizable IncR plasmids play a role in bla_{TEM-52} transmission. A conclusion drawn from our study is that IncX and IncN families of replicons might be more diverse than previously thought. In particular, the occurrence of the IncX plasmids could be generally underestimated due to the lack of suitable detection methods currently available, as the classical multiplex PCR targets only the IncX2 replicons.[22]

Moreover, the bla_{TEM-52} plasmids were found in enteric bacteria from food-production animals, meat products and humans and in many cases they were capable of self-transfer in conjugation. Some of them conferred other than ESBL resistances to the host bacteria, namely to aminoglycosides, tetracycline and sulphonamides. This underscores the potential risk of selection for co-resistances when bla_{TEM-52} plasmids are present in enteric bacteria. The discovery of new plasmid types like IncX1A and the N-related replicons that were not detectable by means of currently available screening methods underscores the importance of further research within the area of plasmid biology, with a focus on plasmid-associated antibiotic resistance.

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Transparency declarations

None to declare.

Supplementary data

Table S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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Erratum

Investigation of diversity of plasmids carrying the *bla*$_{TEM-52}$ gene

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There were errors in Table 1. Except for plasmid p727, in all cases where the element upstream of *bla*$_{TEM-52}$ is currently indicated to be Tn*3*, this should be changed to Tn*2*. In the case of p727, the element upstream of *bla*$_{TEM-52}$ should be labelled 'unknown'. The cause of the errors was the design of the Tn-*bla*$_{TEM}$ linking PCR (Materials and methods section, Genetic environment upstream of the *bla*$_{TEM-52}$ gene) based on the sequence EF141186, which was previously classified as Tn*3*-*bla*$_{TEM-52}$. This element should be correctly classified as Tn*2*-*bla*$_{TEM-52}$.[1] The authors apologize for the errors.

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Supplementary data

Table S1. Primers and PCR conditions used in the study to detect the selected targets

Primer sequence	Target sequence	Amplicon size (bp)	Annealing temperature used in PCR (°C)	Reference or accession number
Pfw 5'-GCAGATTGATTCACGTGAAG-3'	*pir* gene of pOLA52 *bla::npt*	720	58	25
Prv 5'-CCTCTGAAACCGTATGGTATTC-3'				
Pfw 5'-AGGCTTCAGGTGAAAACTGT-3'	*repB* of pE001	838	58	JF776874[a,b]
Prv 5'-TATCAAACTCTTCCAAGAATTTAGCT-3'				
Pfw 5'-GAATAAAAAGGTTAATGTAAAACAGG-3'	*rep* of pGOC049	703	58	JF708955[b]
Prv 5'-CAGCTAATGGCTTGTTGATG-3'.				
Pfw 5'-TGAAGCTCATCCGGTTCAC-3'	Tn*3*-*bla*TEM-52	1268	60	EF141186
Prv 5'-CTGAGAATAGTGTATGCGGCGAC-3'				19

Standard PCR conditions were applied in all cases.

[a]Sequence obtained in collaboration with L. Hansen, Copenhagen University (lh@10-12.dk).

[b]This study.

MANUSCRIPT II

Characterization of plasmids carrying *bla*$_{\text{TEM-1}}$ gene from humans, poultry, cattle and pigs.

Bielak, E., Knudsen, B. E., Haugaard, E., Andersen, L., Hammerum A. M., Schønheyder, H., C., Porsbo, L. and Hasman H.

Characterization of plasmids carrying *bla*$_{\text{TEM-1}}$ gene from humans, poultry, cattle and pigs.

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Running title: Diversity of *bla*$_{\text{TEM-1}}$ plasmids from human and food production animals

Key words: *bla*$_{\text{TEM-1}}$, replicon typing, transferable resistance

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Abstract

Objectives: To examine the relationship between *bla*$_{\text{TEM-1}}$ plasmids from *Escherichia coli* from humans and food-production animals.

Methods: Plasmids with *bla*$_{\text{TEM-1}}$ genes were isolated from pathogenic, indicatory and faecal commensal *E. coli* isolates pigs, poultry, cattle and humans. These plasmids were characterized by replicon typing PCR, restriction fragment length polymorphism (RFLP), susceptibility testing and typing of the genetic environment of the *bla*$_{\text{TEM-1}}$ gene by PCR.

Results: Overall, respectively [68.4 %, 86.2 %, 50.0 % and 30.4 %] of *bla*$_{\text{TEM-1}}$ plasmids from humans, cattle, pigs and poultry harboured IncF-family and/or IncB/O replicons; IncFII and IncFIB replicons were detected on *bla*$_{\text{TEM-1}}$ plasmids from all reservoirs, IncB/O were not detected in poultry, IncI1 were not detected in cattle. 56.5 % and 20.0 % of *bla*$_{\text{TEM-1}}$ plasmids from poultry and pigs, respectively, carried IncI1 replicons. IncF-family, IncB/O and IncI1 were often present on the same scaffolds with IncP replicons. Single cases of IncX1, IncK, IncHI1, IncN, IncY and IncR were detected. Typically, *bla*$_{\text{TEM-1b, -1c}}$ alleles resided on Tn*2* while *bla*$_{\text{TEM-1a}}$ on Tn*3* transposons. The majority of the *bla*$_{\text{TEM-1}}$ plasmids conferred co-resistances to sulfamethoxazole, trimethoprim or tetracycline, or combination of these.

Conclusions: Similar distribution of replicons was observed among pathogenic and faecal commensal *E. coli*. However, IncI1 replicons were predominant for poultry and pigs; IncB/Os were predominant in humans, cattle and pigs. Related IncB/O, IncI1 and IncFII *bla*$_{\text{TEM-1}}$ plasmids were found in different *E. coli* clones across human and animal reservoirs. This indicates transmission of *bla*$_{\text{TEM-1}}$ plasmids between bacteria from humans and food- production animals.

Introduction

In 1960s and -70s respectively ampicillin and amoxicillin were introduced to chemotherapy and these aminopenicillins were able to efficiently penetrate the outer membranes of the Gram-negative bacteria.([1;2]) Aminopenicillins cause relatively few side effects to humans and animals and have broad spectrum of activity, among others against *Escherichia coli*, *Salmonella enterica*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*.([3]) However, bacteria soon developed resistance to these drugs. Today the TEM-1 β-lactamase encoded by the $bla_{\text{TEM-1}}$ gene is the most common cause of resistance to aminopenicillins and to some of the first generation cephalosporins (cephalotin and cephaloridine) in Enterobacteriaceae.([3]) TEM-1s are currently found also in *Pseudomonas aeruginosa*, *H. influenzae* and *N. gonorrhoeae*.([3;4]) High frequency of occurrence of TEM-1 β-lactamases not only hampers the treatment of infections caused by the resistant isolates; it is also most likely that the selective pressure induced by usage of other classes of β-lactam antimicrobials might induce mutations in the $bla_{\text{TEM-1}}$ genes leading to production of diverse TEM enzyme variants with a broader substrate spectrum.([3;4]) The majority of such variants detected so far in clinical and in experimental laboratory settings are either Extended Spectrum β-Lactamases (ESBLs) or they confer resistance to β-lactam inhibitors (IRTs).([3;4]) An alarming fact is that mutations in the $bla_{\text{TEM-1}}$ gene leading to substitution of only one of the key amino acids in the active centre of the TEM-1 enzyme can be enough to encode variants with ESBLs properties. This is exemplified by TEM-17 or TEM-19 that differ by single amino acid substitutions compared to their TEM-1 predecessor (www.lahey.org/Studies/temtable.asp).[3]

The bla_{TEM} genes in Enterobacteriaceae are almost exclusively located on plasmids.([5;6]) Plasmids are usually double stranded, circular and often self-transmissible DNA molecules that replicate autonomously from the bacterial chromosomes.([7]) The minimal region required for a plasmid to replicate and maintain itself is termed replicon. Based on the replicon similarities plasmids can be classified to incompatibility groups (Inc groups).([8]) Previous studies reported the occurrence of $bla_{\text{TEM-1}}$ genes primarily on the IncF-related scaffolds.([5;6]) Diverse bla_{TEM} variants encoding ESBLs (TEM-3, -10, 21, -24 and -52) have been typically reported on broad host range IncA/C replicons and it was suggested that these TEM-ESBLs evolved outside of the human reservoir of *E. coli*.([6]) Recent studies indicated that the $bla_{\text{TEM-52}}$ genes were generally located on IncX1, IncI1,

IncA/C, IncL/M and IncN-related plasmid scaffolds found in *E. coli* and *S. enterica* mainly from poultry and from humans. ([9]) Contrary to $bla_{\text{TEM-1}}$, there is no report in the literature on $bla_{\text{TEM-52}}$ alleles associated with IncF- family or IncB/O replicons. Moreover, previous studies showed also that the related or undistinguishable IncI1 and IncX1 plasmids with $bla_{\text{TEM-52}}$ circulated among different *E. coli* and *S. enterica* isolates from humans, poultry and meat products.[9]

Previously it has been shown that various bla_{TEM} genes usually resided on Tn*3*- related transposons found on variety of plasmid scaffolds.([9;10]) Tn*3*- like family of transposons was subdivided into Tn*1, 2* and *-3* groups.([10]) Question should be therefore raised not only about the diversity of $bla_{\text{TEM-1}}$ associated replicons, but also whether there are possible differences in distribution of these distinct Tn- types transmitting $bla_{\text{TEM-1}}$ genes in humans and in animals.

The aim of this study was to examine the diversity of $bla_{\text{TEM-1}}$ plasmids from *E. coli* isolated from humans and food production animals like cattle, poultry and pigs. Both the diagnostic (from diseased humans or animals; the diagnostic human isolates were termed as clinical) and the faecal indicatory (from healthy individuals) isolates were analyzed in this study. The focus was stated on the plasmids originating from *E. coli* as this bacterium is a common inhabitant of human and animal gastro-intestinal tracts that may also cause infections in the aforementioned hosts. ([11]) Here we present data on the possible differences in distribution of plasmid replicons in the healthy and diseased individuals and we investigate whether the $bla_{\text{TEM-1}}$ genes found in humans could have arrived to this reservoir on the plasmids originating from food-production animals (poultry, cattle, and pigs). An attempt is made to point out on which mobile platforms (plasmids or specific variants of transposons) the transmission and evolution of the $bla_{\text{TEM-1}}$ genes occurred in humans and animals. This knowledge is essential to generate models illustrating mobilization, transmission and evolution routes of bla_{TEM} resistance genes. Such models could be applied in the future as tools to prevent further spread of the resistance in bacteria.

Materials and Methods

Selection of wild type *E. coli* strains harbouring $bla_{\text{TEM-1}}$ genes

Ampicillin resistant *E. coli* wild type (WT) isolates from cattle, poultry and pigs have been selected from the previously published collection of Olesen et al.([12]) The selected WTs included diagnostic samples as well as indicatory isolates collected from animals routinely sampled at slaughtering .([12]) Ampicillin resistant faecal commensal *E. coli* WTs from humans originated from a collection published previously as part of the NorMat/ Danmap studies. ([13]) Ampicillin resistant clinical *E. coli* WTs have been collected from bacteraemia patients with different initial sites of infection and originated from Aalborg Hospital, Denmark.([14]) All of the WT isolates were collected in Denmark in the period 2000-2004.

Verification of the presence of the bla_{TEM} by PCR

PCR targeting the bla_{TEM} genes was initially performed on all of the ampicillin resistant WTs. PCR set-up was as described by Hasman et al.([15]) Unless the bla_{TEM} genes found in the selected WTs were sequenced by the corresponding reference studies, the PCR products have been sequenced in this study by standard capillary sequencing method (Macrogen Inc., Korea). Obtained sequences were blasted against the bla_{TEM} reference database available at Lahey Clinic (http://www.lahey.org/Studies/temtable.asp). WT isolates harbouring the $bla_{\text{TEM-1}}$ genes were selected for further analysis. In individual cases WTs harbouring other bla_{TEM} variants were also included in further analysis to obtain broader overview of the plasmids associated with bla_{TEM} genes in the examined *E. coli* collections.

Phylotyping and Pulse Field Gel Electrophoresis (PFGE)

The *E. coli* WT isolates harbouring $bla_{\text{TEM-1}}$ genes or other included bla_{TEM} variants underwent phylotyping by a triplex PCR method described by Clermont et al. ([16])Then Xba I- PFGE ([17]) was performed on the WT strains; Plugs were run on a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA). PFGE running conditions, staining and image capture procedures applied were as described by Cao et al.([18]) *Salmonella* Branderup was used as a size ladder.([17]) The analysis of the obtained images was performed with BioNumerics version 4.6.1 software (Applied Maths, Belgium). Program parameters were set up on Dice and unweighted -pair group method using average

linkages (UPGMA) with 1% band tolerance. Cut off value assumed for designation of the strains as possibly related was 80%.([19])

Isolation of plasmids

WT strains with *bla*$_{\text{TEM-1}}$ genes from cattle and poultry were initially used in conjugation to verify the transmissibility of the ampicillin resistance; conjugation assay was performed as described previously by Bielak et al.([9]) Plasmid free recipients used in conjugation were either *E. coli* MT102 (naldixic acid (NAL) and rifampicin (RIF) resistant)([9]) or *E. coli* K-12 HEHA4 (kanamycin (KAN) resistant).([12]) Transconjugants (TCs) were isolated on the plates with the selection adjusted to the recipient type (described below). TCs in which no other than the individual *bla*$_{\text{TEM-1}}$ plasmids were detected (see below) were selected for further analysis.

For WT isolates, where conjugation did not work as well as all WTs from pigs and humans, total plasmid content was purified Plasmids were introduced to electrocompetent *E. coli* GeneHogs ® or *E. coli* DH10B^{TM} (Invitrogen, Inc.) by electroporation method; conditions were as described by Bielak et al.([9]) Transformants (TFs) in which no other than the individual *bla*$_{\text{TEM-1}}$ plasmids were detected were selected for further analysis.

Plasmid purifications from the WTs and later from TFs and TCs were done using Qiagen Plasmid Mini or Midi Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions.

Media, antibiotics and growth conditions

Brain-Heart Infusion (BHI; Becton, Dickinson & Co., Bacto™) agar plates were used for cultivation of the strains. Ampicillin (100 mg/L) was used as selection for the WTs, isolation of TFs in GeneHogs ® and DH10B^{TM} recipients (Invitrogen, Inc), and later for sub-cultivation of the obtained TFs and TCs with individual *bla*$_{\text{TEM}}$ plasmids. Ampicillin (100 mg/L) together with nalidixic acid (32 mg/L) and rifampicin (25 mg/L) were used to select for TCs when *E. coli* MT102 (NAL^{R}, RIF^{R}) was used as the recipient strain; ampicillin (100 mg/L) together with kanamycin (50 mg/L) were used to select for TCs when *E. coli* K-12 HEHA4([12]) was used as the recipient. All strains were typically incubated overnight at 37°C.

Verification of plasmid transfer to TFs and TCs

The TFs and TCs obtained underwent the PCR targeting the *bla*$_{TEM}$ genes.([15]) TFs and TCs in which the presence of *bla*$_{TEM}$ genes was confirmed were then subjected to S1-PFGE (S1, supplementary material).([20]) Plugs were run on a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA). Concentration of S1 nuclease (Fermentas) per plug slice, running conditions for the S1-PFGE, conditions of staining and image capture applied were as described by Bielak et al.([9]) This procedure allowed for verification of presence of plasmids and estimation of their sizes in the obtained TFs and TCs. In the further text plasmids isolated from the verified TFs or TCs are designated as p[with specified strain number].

Verification of WTs harbouring non-transferable *bla*$_{TEM}$ genes

Selected WTs from which neither TFs nor TCs with *bla*$_{TEM}$ genes could be generated underwent S1-PFGE as described above. Individual plasmids, if detected, were extracted from the agarose gels and purified at GFX columns (Amersham Life Sciences) according to manufacturer instructions. These extractions underwent *bla*$_{TEM}$ PCR as described above; samples yielding positive signals were further used as templates for replicon typing PCRs (described below). This allowed for estimation of which replicons might be possibly present on these plasmids. Additionally, a standard electrophoresis of the plasmid purifications from the selected WTs was performed (18 h at 50% in 0.8% agarose, Lonza) to visualise plasmid content in these WTs.

Replicon typing of *bla*$_{TEM}$ plasmids

PCR-based replicon typing (PBRT) was performed on the obtained and verified TFs and TCs. Primers and the PCR conditions used were as described by Carattoli et al. ([8]). If no positive PCR signal was observed in the latter, other replicon typing PCRs targeting variants of the following replicons: IncFII, IncX1 and IncN-like have been applied as described by Villa et al. ([21]) and Bielak et al. ([9])

Analysis of *bla*$_{TEM-1}$ plasmids by restriction fragment length polymorphism (RFLP)

Selected plasmids with *bla*$_{TEM}$ genes originating from poultry, cattle, pigs and humans were purified from the corresponding TFs and TCs as described above and underwent RFLP profiling. Plasmids harbouring IncB/O replicons were digested with *Bam*HI. The

remaining plasmids were digested either *Hin*cII or with *Eco*RV to access best possible resolution of bands on the produced fingerprints. The fragments were separated by 4 h electrophoresis in 0.8 % agarose gel (Lonza) at 120 V and visualised by standard staining in ethidium bromide. Whenever possible the fingerprints were analysed in BioNumerics software (version 4.6.1, Applied Maths, Belgium) with parameters set up as described above for PFGE analysis.

Examination of the genetic environment upstream of the *bla*$_{\text{TEM-1}}$ genes

Five simplex PCRs were designed for the purpose of this study linking the presence of either Tn*1* (PCR I), Tn*2* (PCR II), Tn*3* (PCRIII) or IS*26* in two different configurations ([10]) (PCR IV and V) with the *bla*$_{\text{TEM}}$ genes. Three newly designed forward primers were used in PCR I, -II and –III targeting respectively *tnp*A sequences common for all three Tn -types, *tnp*R fragment specific for Tn*2* and *tnp*A fragment specific for Tn*3* (Figures S1-3 in Table S2). The forward primers used in PCR IV and V were as described by Bailey et al. ([10]) The same reverse primer targeting the *bla*$_{\text{TEM}}$ gene was used in each of the five aforementioned simplexes.([9]) PCR conditions, the primers used and interpretation of the results are specified in the Table S2.

Susceptibility testing

Unless resistance phenotypes were specified in the reference studies for corresponding WT strains, the susceptibility tests were performed on WT isolates by means of the commercial microtitre panels for Enterobacteriaceae (Sensititre®). Antimicrobials included on the panels were amoxicillin/ clavulanic acid, ampicillin, apramycin, cefotaxime, ceftiofur, chloramphenicol, ciprofloxacin, colistin, florfenicol, gentamycin, nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim. Testing and interpretation of the results were performed according to the CLSI standards. For cefotaxime and other antimicrobials for which no CLSI breakpoints were available, the results were interpreted using criteria of the European Committee on Antimicrobial Susceptibility Testing (EUCAST).

Based on the resistance profiles observed for the WT strains the corresponding transformants and transconjugants were further tested to the selected antimicrobials by means of the disc diffusion method. Testing was performed according to the CLSI standards (2003). Each of the recipient strains used for production of transformants and

transconjugants, namely GeneHogs ® , DH10B^{TM}, MT102 and HEHA4 were included as controls. The results were interpreted using CLSI zone diameter interpretation standards (2003).

Plasmid Multilocus SequenceTyping (pMLST)

Selected plasmids with IncF –family, IncI1 and IncN replicons underwent the corresponding pMLST with conditions as described respectively by Villa et al. ([21])(here the term replicon sequence typing-RST will be further used), Garcia- Fernandez et al. 2008 ([22]) and Garcia-Fernandez et al. 2010 ([23]).

Results

The list of selected *E. coli* WT strains and the detailed results of plasmids characterizations described in the following sections are specified in the Tables S3a-e.

Selection of wild type *E. coli* strains harbouring *bla*$_{\text{TEM-1}}$ genes

A collection of ampicillin resistant *E. coli* strains from diverse reservoirs (poultry, cattle, pigs and humans) underwent PCR targeting *bla*$_{\text{TEM}}$ genes. The positive PCR products were sequenced and annotated. 24 strains from poultry (12 indicator strains and 12 from diagnostic samples), 33 strains from cattle (2 indicator strains and 31 from diagnostic samples), 40 strains from pigs (14 indicator and 26 from diagnostic strains) and 62 from humans (31 indicator and 31 clinical isolates) were selected for further studies. 2 of the selected WT diagnostic strains from cattle harboured *bla*$_{\text{TEM-30}}$ genes (*E. coli* 74123511 and 74129511) and 3 WT indicator strains from humans harboured respectively *bla*$_{\text{TEM-40}}$ (*E. coli* 257-CO2), *bla*$_{\text{TEM-30}}$ (*E. coli* 875-CO3) and *bla*$_{\text{TEM-135}}$ (*E. coli* 1112-CO3). The remaining selected WTs harboured the *bla*$_{\text{TEM-1}}$ genes.

Isolation of TFs and TCs with individual *bla*$_{\text{TEM}}$ plasmids

Conjugation of WT strains harbouring *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM-30}}$ genes from cattle and poultry reservoirs was initially performed to isolate the individual *bla*$_{\text{TEM}}$ plasmids. In the remaining cases an attempt was made to transfer the plasmids with *bla*$_{\text{TEM-1}}$ or other included *bla*$_{\text{TEM}}$ variants into the plasmid free recipients by means of electroporation. The presence of the *bla*$_{\text{TEM}}$ genes was confirmed in the obtained TFs and TCs by PCR. Transfer of single plasmids from WTs to the recipients was verified by S1-PFGE.

From the 33 WT cattle *E. coli* isolates 7 TCs and 22 TFs with individual $bla_{\text{TEM-1}}$ plasmids were obtained. For 2 of the cattle WT isolates neither TC nor TF were produced (WT 74-13295-1 and WT 74-11793-1). In both cases plasmids of variable sizes were detected in WTs; these plasmids were negative in bla_{TEM} PCR after gel extraction suggesting that the $bla_{\text{TEM-1}}$ genes were most likely integrated into the chromosomes in these two strains. 1TC and 1TF with individual $bla_{\text{TEM-30}}$ plasmids were obtained (74 -12351-1TF and 74-12951-1 TC), however in the 74-12351-1TF it was suspected that two plasmids might have transferred to the recipient.

From the 24 poultry WT isolates 7 TCs and 14 TFs with individual $bla_{\text{TEM-1}}$ plasmids were obtained; in 3 cases (strains 7430605-1, 7277386-1 and 7278662-1) neither TFs nor TCs were produced from the corresponding WT isolates. The WT isolates 7430605-1 and 7277386 -1 were shown in S1-PFGE to harbour two plasmids each. In the strain 7430605-1 plasmids sized to 85 kb produced the positive signal to the bla_{TEM} gene; in WT 7277386-1 plasmids sized to 138 kb produced the positive signal in the bla_{TEM} PCR. No plasmid was detected by S1-PFGE in WT 7278662-1.

TFs with individual $bla_{\text{TEM-1}}$ plasmids were obtained from all except one (WT 74-04083-1) of the 40 WT isolates from pigs. Here it was suspected that two plasmids with estimated sizes of 180 kb and 30 kb had transferred to 74-04083-1 TF based on the S1 PFGE.

From the 31 human faecal indicator isolates 27 TFs with individual $bla_{\text{TEM-1}}$ plasmids were obtained and 3 TFs were obtained with the $bla_{\text{TEM-135}}$, $bla_{\text{TEM-40}}$ and $bla_{\text{TEM-30}}$ plasmids, respectively. In one case (WT 1437-CO3) TF was not produced. No signal corresponding to a plasmid was observed in S1-PFGE for this WT *E. coli* indicating that the $bla_{\text{TEM-1}}$ gene could be located on the chromosome. From the 31 clinical human isolates 24 TFs with individual $bla_{\text{TEM-1}}$ plasmids were obtained. In one case of WT 2002-27242 no TF with $bla_{\text{TEM-1}}$ plasmid was obtained. In 6 cases the TFs obtained from human WTs were suspected to harbour more than one plasmid (2000-14183TF, 2000-27668TF, 2000-49043TF, 2000-78651 TF, 2001-13567TF and 2001-71294TF). These TFs shared a common pattern, namely they all contained one larger plasmid of approximately 20kb/30 kb and one or two smaller plasmids (<10 kb). Co-transmission of cryptic plasmids during transformation has previously been reported in the literature.([24]) Therefore, 74-12351-1TF from cattle, 74-04083-1TF from pig and the six TFs from humans suspected to harbour two plasmids underwent further characterization with a

remark that these results should be interpreted as features potentially associated with the *bla*$_{TEM}$ plasmids in these TFs.

Replicon typing of *bla*$_{TEM\ -1}$ plasmids

The results of the replicon typing are summarized in Table 1 and Table 2. Symbol '&' is used for indication of multiple replicons on the same plasmid. Overall, 57 TFs with *bla*$_{TEM-1}$ plasmids from humans and 92 TFs and TCs with *bla*$_{TEM-1}$ plasmids from animals underwent PCR-based replicon typing (PBRT). Both in human and animal reservoirs the most commonly detected were IncFII replicons and also multireplicons composed of diverse combinations of IncFII, IncFIB, IncFIA and/or IncP groups; 46 % and 48 % of the *bla*$_{TEM-1}$ plasmids from humans and from animals, respectively, carried one or more of the IncF- type replicons listed above. Individual cases of multireplicons IncFII & IncR (73-30615-7TF; pig faecal indicator isolate) and IncFIB & IncY (2002-48269 TF; human, clinical isolate) were also detected. In addition, 23 % and 14 % of *bla*$_{TEM-1}$ plasmids from human and animal sources, respectively, belonged to IncB/O family or carried double replicons IncB/O & IncP; 7 % of *bla*$_{TEM-1}$ plasmids from humans harboured IncI1 or IncI1 in combination with other replicons. IncI1 replicons were almost three times more frequently detected on *bla*$_{TEM-1}$ plasmids from animals (23 %) than humans. Rather large fraction of *bla*$_{TEM-1}$ plasmids turned out to be non-typable by the applied PBRT methods (19 % from human and 10 % from animal isolates). This group of non-typable plasmids contained among others all of the 8 TFs suspected to harbour more than one plasmid (74-12351-1TF from cattle, 74-04083-1TF from pig and 2000-14183TF, 2001-71294TF, 2001-13567 TF, 2000-27668TF, 2000-49043TF and 2000-78651 TF from humans).

Other replicon types were detected sporadically. Single cases of IncX1 (1413-CO3 TF) and IncK (1084-CO3 TF) replicons were detected on *bla*$_{TEM-1}$ plasmids from human faecal indicator *E. coli*. An IncHI1 replicon was detected on the *bla*$_{TEM-1}$ plasmid from 74-12901-2 TF (from diagnostic pig isolate); 2 *bla*$_{TEM-1}$ plasmids with no other than IncP replicons were detected in cattle diagnostic *E. coli* isolates 74-12891-1and 74-30138-2; in 3 cases IncN replicons were present on the examined plasmids; one of these plasmids originated from human faecal indicator sample (1308-CO3TF) and two from poultry diagnostic samples (7372121-1 TF and 7373304-1 TF; here, the corresponding WT strains were later shown to be clonally related by PFGE); 3 *bla*$_{TEM-30}$ plasmids examined in the study harboured the following replicons: IncB/O (p875-CO3 from human faecal

indicator strain), IncFII (p74-12951-1 from cattle diagnostic strain) and a non typable replicon was present on p74-12351-1T (from cattle diagnostic isolate). The *bla*$_{\text{TEM-40}}$ and *bla*$_{\text{TEM-135}}$ plasmids from human faecal indicator isolates harboured respectively IncFIB (p257-CO2) and IncI1 (p1112-CO3) replicons.

Restriction fragment length polymorphism (RFLP)

Plasmids with the same or similar combinations of replicons were grouped together and underwent RFLP. The characterizations of IncFII, IncB/O and IncI1 plasmids across the diverse reservoirs are summarized in Tables 3-5, respectively. The remaining results for the tested plasmids are listed in the Table S3. RFLP fingerprints are available in supplementary materials (S4). Plasmids differing by up to 3 bands (corresponding to approximately 85 % identity) were designated with the same symbols.

EcoRV digestion of the IncFII plasmids

IncFII plasmids from humans, cattle and pigs were initially digested with *Hin*cII and subsequently with *Eco*RV to access which enzyme generates sufficient number of bands for analysis. The *Eco*RV digestions generated fingerprints most suitable to compare the IncFII plasmids across the reservoirs. 3 *bla*$_{\text{TEM-1b}}$ plasmids from diagnostic isolates of cattle (p73-14678-1) and pigs (p74-13348-1 and p74-13297-1) shared similar RFLP fingerprints differing from each other by 1 to 2 bands; they were designated as RFLP type d; 3 *bla*$_{\text{TEM-1b}}$ plasmids from human clinical isolates produced similar RFLP fingerprints designated as type g (plasmids p2001-49440 and p2002-37995 had undistinguishable band patterns; plasmid p2002-134976 differed by 2 bands). Otherwise, the plasmids with no other than IncFII replicons and originating from human and animal isolates produced diverse RFLP types (Table 3).

HincII digestions of selected plasmids with multiple IncF-type replicons

Selected plasmids from cattle and poultry, which were positive in PBRT to two or more of the F- type replicons (IncFII, IncFIB and/or IncFIA) were digested with *Hin*cII (12 plasmids from cattle and 7 from poultry);4 IncFII & IncFIB plasmids detected in cattle shared similar RFLP profiles designated hinc2 (Table S3a); 3 of these plasmids harboured *bla*$_{\text{TEM-1c}}$ (p 74-12867-1; p 74-12969-1 and p 74-13379-1; these RFLP profiles were undistinguishable) and one plasmid harboured *bla*$_{\text{TEM-1b}}$ (p 74-12866-1; this fingerprint

differed by 3 bands from hinc2 profile). The remaining F-family plasmids originating from the different cattle isolates produced diverse RFLP profiles (Table S3a). The IncFII & IncFIB as well as IncFII & IncFIB & IncP plasmids from the different isolates from poultry yielded diverse RFLP profiles (Table S3c). Due to the common presence of the IncF-family multireplicon *bla*$_{\text{TEM-1}}$ plasmids, the latter were not further compared by RFLP across the reservoirs. Selected plasmids underwent RST as described below to complement the RFLP analysis.

BamHI digestion of the IncB/O, IncP and IncK plasmids

27 plasmids with individual IncB/O replicons as well as with double IncB/O & IncP (14 plasmids from humans, 9 from pigs and 4 from cattle) underwent RFLP profiling. Fingerprints clustered into two groups of similarities. In the first group 3 *bla*$_{\text{TEM-1b}}$ plasmids from humans (p457-CO2, p2001-131351 and p2001-37255), 3 *bla*$_{\text{TEM-1b}}$ plasmids from pigs (p74-12934-1, p74-12933-1, p74-30367-2) and 2 *bla*$_{\text{TEM-1b}}$ plasmids from cattle (p74-12865-1 and p74-12894-1) produced undistinguishable RFLP profiles designated as p (Table 4); 3 other plasmids produced similar profiles to p with differences ranging from 1 to 3 bands (*bla*$_{\text{TEM-1a}}$ p433-CO2 from human, *bla*$_{\text{TEM-1b}}$ p74-13266-1 from pig and *bla*$_{\text{TEM-1b}}$ p74-30014-3 from cattle).

The second similarity cluster was comprised of 9 plasmids from humans which shared RFLP profiles designated as m; 5 of these plasmids produced undistinguishable RFLP fingerprints (*bla*$_{\text{TEM-1c}}$ plasmids: p438-CO2, p2002-50 and p1082-CO3; *bla*$_{\text{TEM-1g}}$ plasmid p1297-CO3 and *bla*$_{\text{TEM-30}}$ p875-CO3). In 4 cases 1 to 3 three bands differences were observed compared to the RFLP m patterns (*bla*$_{\text{TEM-1c}}$ plasmids: p2002- 14946, p2001-25022 and p2002-110474, and *bla*$_{\text{TEM-1b}}$ p 97-CO2).

2 *bla*$_{\text{TEM-1a}}$ plasmids from pigs, p74-12927-1 and p74-11919-1, produced identical RFLP patterns o; 2 *bla*$_{\text{TEM-1b}}$ plasmids from pigs, p73-30819-1 and p74-30165-7, produced similar (3 bands difference) RFLP patterns q and q_1, respectively. The remaining plasmids with incB/O replicons yielded distinct RFLP types (Table 4). Also, 2 IncP plasmids from cattle and the IncK plasmid from human underwent the RFLP and generated distinct RFLP types (Table S3a and S3d).

EcoRV digestions of the IncI1 plasmids

Digestion with *Eco*RV generated most suitable fingerprint for analysis of IncI1 plasmids.7 *bla*$_{\text{TEM-1}}$ IncI1 plasmids from poultry isolates (p7430186-1, p7430287-1,

p7430521-1, p7370817-2, p7430125-1, p7430557-1, and p7430237-1) and 1 IncI1 *bla*$_{\text{TEM-1}}$ plasmid from human (p1341-CO3) produced similar RFLP fingerprints designated with t (Table 5). In two of these plasmids the same difference of 2 bands compared to the 't' profile was observed (p7430521-1 and p7370817-2); 4 IncI1 plasmids originating from different reservoirs and carrying different *bla*$_{\text{TEM}}$ alleles produced similar RFLP profiles designated with 'u' (*bla*$_{\text{TEM-1b}}$ p1033-CO3 and *bla*$_{\text{TEM-135}}$ p1112-CO3 from human; *bla*$_{\text{TEM-1b}}$ p7430284-1 from poultry and *bla*$_{\text{TEM-1a}}$ p74-30164-4 from pig). These plasmids shared overall from 80% to 100% similarities hence they were designated as 'u' variants.

2 plasmid from humans, IncI1& IncP *bla*$_{\text{TEM-1b}}$ p2002-70903 and IncI1 *bla*$_{\text{TEM-1c}}$ p545-CO2 shared over 80% similarity and were designated as RFLP 'w' and 'w_1' types. Otherwise the RFLP profiles of the detected IncI1 plasmids differed from each other (Table 5).

Relatedness of the WTs harbouring similar plasmids

E. coli WTs that were shown to harbour plasmids with similar replicons and RFLP profiles were initially examined by phylotyping and also by Xba-I PFGE (if they belonged to the same phylotypes); 6 of the InFII *bla*$_{\text{TEM-1}}$ plasmids from cattle shared an identical RFLP profile 'a' and their corresponding WTs were shown to be the same or closely related clones (WT 7413236-1, WT 7313865-1, WT 7412972-1 and WT 7413296-1, WT 7412973-1 and WT 7413208 shared from 85 % to 100% identity in XbaI PFGE); 2 WT strains from pigs, WT 74-12934-1 and WT 74-12933-1, harbouring similar RFLP types p IncB/O plasmids also produced indistinguishable band patterns in the PFGE. 2 WT 74-13361-2 and WT 74-12892-1 shared 93% band identity with each other; these two strains harboured similar IncFII&IncFIA &InFIB plasmids. 2 poultry WT isolates 7276769-1 and 7277386 harbouring the similar IncFII &IncFIB plasmids produced indistinguishable band patterns ; also two poultry isolates, WT 7372121-1 and WT 7373304-1 harbouring the IncN plasmids turned out to be clonally related (Table S3). In the remaining cases the WTs harbouring the similar plasmids were not epidemiologically related with each other. Notably, no clonal relatedness was seen among isolates originating from different animal species or humans. Since clonally related WTs originated from different individuals [probably sampled at different dates over a four-year period], their resident *bla*$_{\text{TEM}}$ plasmids were characterised and included in the analyses as

independent units (Tables1-7). This enabled estimation if these similar plasmids from the related clones possibly acquired diverse features.

Examination of the genetic environment upstream of the *bla*$_{\text{TEM-1}}$ genes

It has been previously suggested that *bla*$_{\text{TEM-52}}$ genes often resided on the Tn*3*- family transposons.([9;10]) The linking PCR in the previous study of Bielak et al. targeted primarily Tn*2* elements.([9]) A set of new linking PCR protocols was designed in this study to detect the three subtypes of the Tn*3* –like transposons and IS*26* elements possibly present upstream of the *bla*$_{\text{TEM}}$ genes (Table S2). Plasmids with the *bla*$_{\text{TEM-1}}$ genes and the selected variants were tested with the use of these new PCR schemes. Overall, similar pattern was observed in humans and in animals (Table 6). Namely, on the majority of plasmids *bla*$_{\text{TEM-1b}}$ and *bla*$_{\text{TEM-1c}}$ genes were located on Tn*2* transposons. However, insertions of IS*26* elements were also frequently detected upstream of the *bla*$_{\text{TEM-1b}}$ alleles. *Bla*$_{\text{TEM-1a}}$ genes resided exclusively on Tn*3* elements (Table 6).

A range of other *bla*$_{\text{TEM-1}}$ alleles was detected mainly in human faecal indicator isolates, namely *bla*$_{\text{TEM -1d}}$, *bla*$_{\text{TEM -1g}}$ and a novel allele *bla*$_{\text{TEM -1j}}$ (JQ423955) . The *bla*$_{\text{TEM -1g}}$ (IncB/O p1297-CO3) and *bla*$_{\text{TEM -1j}}$ (IncFII & FIB; p904-CO3) resided on Tn*2* elements; *bla*$_{\text{TEM-1d}}$ was found on p862-CO3 (IncFII & IncFIB) and on p2000-103495 (this plasmid had a non-typable replicon and harboured apparently two copies of the *bla*$_{\text{TEM}}$ gene, each being a different allele, namely *bla*$_{\text{TEM -1c}}$ and *bla*$_{\text{TEM -1d}}$); in p862-CO3 *bla*$_{\text{TEM -1d}}$ gene resided on Tn*1*; 2000-103495 TF was positive to Tn*3*, however it cannot be concluded which of the alleles was linked to this transposon. Unusual combinations were also detected, namely Tn*1* -*bla*$_{\text{TEM-1b}}$ was found on p7370940-1 (IncFII & IncFIB replicon from poultry, diagnostic isolate), Tn*3* -*bla*$_{\text{TEM-1b}}$ on p 74-30181-3 (non-typable replicon from pig) and *bla*$_{\text{TEM-30}}$ was detected either on Tn*3* in cattle (p74-12351-1; non-typable replicon) or on Tn*2* in a human isolate (p 875-CO3; IncB/O; RFLP type ‘m’).

Tn*2*-*bla*$_{\text{TEM-135}}$ was found on p 1112-CO3 (IncI1, human) and IS*26* was found upstream of *bla*$_{\text{TEM-40}}$ on p 257-CO2 (IncFIB, human).

In 2 WTs from cattle, 1 WT from poultry and 2 WTs from humans either no plasmid was detected or the detected plasmids were negative to the *bla*$_{\text{TEM}}$ PCR. These WTs were also subjected to the linking PCR. WT 7278662-1 from poultry produced positive signal to Tn*2*-*bla*$_{\text{TEM}}$; in WT 2002-27242 and WT 1437-CO3 from humans and in WT 74-11793-1

from cattle, IS*26* were detected upstream of the *bla*$_{\text{TEM-1}}$ genes; in WT 74-13295-1 the element upstream of the *bla*$_{\text{TEM-1}}$ gene was non-typable.

Susceptibility testing

In general, no significant differences were observed in the distributions of resistances associated with *bla*$_{\text{TEM-1}}$ plasmids between the faecal indicator and diagnostic/clinical isolates from animals and humans (Table 7). Overall, many of the *bla*$_{\text{TEM-1}}$ plasmids from all reservoirs conferred accessory resistances to sulphonamides, tetracycline and trimethoprim. Co-transfer of sulphonamide resistance was seen among 42% to 56% of the *bla*$_{\text{TEM-1}}$ plasmids from humans and animals, respectively. Overall, 35% and 25 % of *bla*$_{\text{TEM-1}}$ plasmids from humans and 49 % and 32% of *bla*$_{\text{TEM-1}}$ plasmids from animals co-transmitted resistances to tetracycline and to trimethoprim, respectively (Table 7). To a lesser extend the resistances to aminoglycosides (neomycin, spectinomycin or gentamycin) and surprisingly to amoxicillin/clavulanate were found to be associated with the *bla*$_{\text{TEM-1}}$ plasmids. In total, resistance to at least one of the aforementioned aminoglycosides was associated with approximately 12 % of plasmids from human reservoirs and with 24 % of plasmids from animals. In particular higher occurrence of *bla*$_{\text{TEM-1}}$ plasmids associated with neomycin resistance was found in animals (19 % of examined plasmids) than in humans (3.5 %). Conversely, a larger fraction of the *bla*$_{\text{TEM-1}}$ plasmids from humans than from animals conferred the reduced susceptibility to the amoxy-clavulanate (respectively 25 % and 5 % of *bla*$_{\text{TEM-1}}$ plasmids from humans and animals).

pMLST of selected IncF-family, IncN and IncI1 *bla*$_{\text{TEM-1}}$ plasmids

7 IncI1 plasmids underwent IncI1 pMLST([22]). 3 of these, namely p74-13303-1 (pig, IncI1 & IncFIB & IncFIC, RFLP s), p7430125-1 and p1341-CO3 (RFLP types t from poultry and human, respectively) shared the same ST36 pattern (clonal complex CC5); the FIB and FIC loci on p74-13303-1 were B16-related:C2 according to RST (1 nucleotide difference was seen in the FIB locus compared to the C16- reference allele);

3 IncI1 & IncP plasmids were compared and they produced different patterns, namely p7365811-1 (poultry, RFLP θ) was pMLST ST3/CC3, p74-12848-1 (pig, IncP& IncI1, not readable in RFLP) was typed as a new ST and p2002-70903 (human, IncI1 & IncP,

RFLP w) was ST97-related (at least 3 nucleotide differences), whereas p74-30166-3 (pig RFLP α) belonged to ST27/CC26 type.

Two *bla*$_{\text{TEM-1b}}$ IncN plasmids detected in the study (not compared with each other by RFLP) were compared by pMLST and they turned out not to be related; p7372121-1 from poultry belonged to ST 3 and p1308-CO3 from human belonged to ST6.

2 IncFII, RFLP types d plasmids were compared by RST (p73-14678-1 from cattle and p74-13297-1 from pig); they produced F-35 related:A-:B- patterns. In both cases the F-35 allele was the closest match and the same mutation with respect to this reference allele was detected on both plasmids (3 nucleotide difference). 2 of the IncFII, RFLP types g plasmids from humans were compared and they represented F2: A-:B- pattern (Table 3).

Multireplicon p73-14130-2 plasmid from cattle was verified by RST (RFLP hinc3, IncFII & IncFIA & IncFIB) and it produced RST pattern F35-related: A2:B24; the same mutations as in the two RFLP d IncFII plasmids described above were observed in the FII loci of this multireplicon plasmid compared to the reference allele F35. 3 plasmids from cattle with IncFII & IncFIB double replicons were examined (p74-12867-1, p74-12969-1 and p74-13379-1; RFLP hinc2); they belonged to RST types F2:A-:B1 (Table S3a).

Discussion and conclusions

This study provided evidences that similar, possibly epidemic *bla*$_{\text{TEM-1}}$ plasmids circulated in commensal and pathogenic *E. coli* from humans and food-production animals (cattle, pigs and poultry) in Denmark. Related IncFII RFLP types d (related to RST types F35:A-:B-) plasmids with *bla*$_{\text{TEM-1b}}$ genes were found in diagnostic *E. coli* isolates from pigs and cattle. Similar IncI1 *bla*$_{\text{TEM-1b}}$ RFLP types t (pMLST ST36/CC5) plasmids were found in indicator *E. coli* from human and diverse poultry isolates. Possibly related IncI1 plasmids (RFLP type u and u_variants) harbouring *bla*$_{\text{TEM-1a}}$, *bla*$_{\text{TEM-1b}}$ *bla*$_{\text{TEM-135}}$ were detected in indicator *E. coli* strains of pig, poultry and human origin. Similar IncB/O plasmids with different *bla*$_{\text{TEM-1}}$ alleles belonging to two major RFLP types m and p were detected both in humans (here only RFLP m) and in animals in this study. IncI1, IncB/O and IncK plasmids are members of the same, I-complex family.([25]) It should also be considered whether the high degree of similarity observed in

RFLPs on the *bla*$_{TEM-1}$ IncB/O plasmids could be due to the general conservation of these I-complex backbones in the different host.([26])

Previously, similar backbones to the *bla*$_{TEM-1}$ IncI1 ST3/CC3 (poultry, RFLP θ) and ST36/CC5 plasmids(RFLP t, poultry and human) were described on plasmids encoding CTX-M-1 (ST3/CC3) and TEM-52 (ST36/CC5) from humans and poultry isolates in the Netherlands.([27]) Based on the RST profiles of the IncFII &IncFIB RFLP hinc2 plasmids from cattle (F2:A-:B1) they may be related to the previously described virulence plasmid from avian pathogenic *E. coli* pAPEC-02-ColV.([28]) The ST3 and ST6 backbones of the two *bla*$_{TEM-1}$ IncN plasmids detected in *E. coli* from poultry and human, respectively, were previously detected on *bla*$_{CTX-M-1}$, *qnr*S1 (ST3)and *bla*$_{CTX-M-3}$, *bla*$_{KPC-3}$ and *bla*$_{OXA-3}$ (ST6) plasmids from *E. coli, K. pneumoniae* and *S. enterica* of various origins.([23])

Overall, based on the results it can be concluded that IncF-family and IncB/O replicons were predominant in *bla*$_{TEM-1}$ plasmids on *E. coli* from humans, cattle and pigs. IncI1 were also found in pigs and sporadically in humans. IncI1 replicons dominated on *bla*$_{TEM-1}$ plasmids from *E. coli* in poultry. A similar distribution of replicons to the one described above was previously observed in commensal and pathogenic *E. coli* from poultry and human reservoirs by Johnson et al. ([11;26]) In these studies the plasmids were not selected based on the resistance, hence they represented the replicons normally found in these reservoirs. In summary, the collected data suggest that *bla*$_{TEM-1}$ genes might have been acquired by endogenous, host or reservoir (human, pigs, poultry and cattle) specific IncI1, IncB/O and IncF-family plasmids.

Other replicon types like IncX1, IncK, IncHI1, IncN, IncY and IncR were detected on *bla*$_{TEM-1}$ plasmids in single isolates from the different reservoirs. Several of the plasmids turned out to be non-typable by the PBRT method. This could suggest a presence of either novel plasmid types or variants with polymorphisms within the primer binding areas for the PBRT primers. Further investigation will be needed to verify this.

Interestingly, in the study relatively large fraction of *bla*$_{TEM-1}$ plasmids from humans and in rare cases the *bla*$_{TEM-1}$ plasmids from food-production animals conferred reduced susceptibility to amoxy-clavulanate in the previously susceptible recipients (25 % and 5% of plasmids form humans and animals, respectively). This was surprising as the TEM-1 does not confer the resistance to clavulanate.([3])Although, a similar phenomenon was previously observed.([29;30])Two hypotheses were suggested, one that the reduced susceptibility to the inhibitor was due to the hyper -production of TEM-1 β-lactamases in

bacteria; or due to decreased permeabillity especially in the clinical isolates of *E. coli*.([29]) The overproduction of the TEM-1 may be related to the strength of the promoters of the $bla_{\text{TEM-1}}$ genes.([31]) Lartigue et al. showed previously that one nucleotide difference in the proposed -35 signal in the promoter *P3* of $bla_{\text{TEM-1b}}$ gene resulted in the production of promoter *P5* leading to increase in the MIC for AMC from 32 µg/mL to 1024µg/mL.([31]) In our study the primer used for sequencing of the bla_{TEM} genes was located to close to the putative -35 signal upstream of the $bla_{\text{TEM-1}}$ genes to determine the promoter types in the isolated with the reduced susceptibility to clavulanate. Finally, another possibility could be that other than bla_{TEM} –type genes are present on the same plasmid scaffolds contributing to the decreased susceptibility to the β-lactamase inhibitor in these isolates. Further examination of this phenomenon will be persuaded.

Conversely to the above, a larger fraction of $bla_{\text{TEM-1}}$ of plasmids from animals conferred co-resistance to aminoglycosides (neomycin, spectinomycin or gentamycin) compared to plasmids from humans. This discrepancy seems to be a natural consequence of increased usage of these aminoglycosides in veterinary (in particular neomycin and spectinomycin) rather than in human chemotherapy, which probably led to higher occurrence of aminoglycoside resistance genes in animal reservoirs.([32]) In fact, the data obtained in this study illustrate that the occurrence of resistance in the given reservoir corresponded to the increased usage of the specific antimicrobials in this reservoir. According to DANMAP report in the period 2001-2004 the annual consumption of active compounds aminoglycosides in animals was over 10^3 kg and approximately 30 kg in humans.([33]) Particularly in pigs aminoglycosides were used more frequently.([33]) Accordingly, we noticed that 33 % of $bla_{\text{TEM-1}}$ plasmids from pigs conferred co-resistance to neomycin which is at minimum 3 times more compared to other reservoirs (cattle 11%, poultry 5% and humans 4%; Table 7).

The study supported previous observations that Tn*2* were the most common transposons linked to bla_{TEM} genes in *E. coli*.([9;10]) Further correlation was deduced based on the results of the linking PCR, namely $bla_{\text{TEM -1b}}$ and $bla_{\text{TEM -1c}}$ gene alleles typically resided on Tn*2* transposons, while $bla_{\text{TEM -1a}}$ alleles was found exclusively on Tn*3*. Other bla_{TEM} variants were generally located on or linked to different elements (Tn*1*, *-2*, *-3*, IS*26* or untypable elements). Interestingly, on one of the IncFII &IncFIB plasmid from human a Tn*1* -$bla_{\text{TEM-1d}}$ was detected and similar combination to this one, namely Tn*1* -$bla_{\text{TEM-2}}$ was previously found on IncP-1plasmids originating from *P. aeruginosa* (these are IncP

plasmids in *E. coli*) ([10]; [33]); *bla*$_{\text{TEM-1d}}$ and *bla*$_{\text{TEM-2}}$ differ by one nucleotide substitution at position 109 from the first nucleotide of the *bla*$_{\text{TEM}}$ start codon (GenBank AF188200 and BN000925, respectively). Moreover, the following unusual combinations were detected: Tn*1* -*bla*$_{\text{TEM-1b}}$ on IncFII & IncFIB plasmid from poultry, Tn*3* -*bla*$_{\text{TEM-1b}}$ located on non-typable replicon from pig; *bla*$_{\text{TEM-30}}$ was detected in cattle on Tn*3* (non-typable replicon) and on Tn*2* in human isolate (IncB/O plasmid; RFLP type m).These examples implied that possible recombination between the transposons and similar *bla*$_{\text{TEM}}$ alleles probably occurred on the examined plasmids and generated new Tn-*bla*$_{\text{TEM}}$ variants. ([34])

Curiously, in some of the examined WTs it was not possible to transfer the *bla*$_{\text{TEM-1}}$ genes to plasmid free recipient. In some of these WTs the *bla*$_{\text{TEM -1}}$ genes were shown be linked to the Tn*2* or IS*26* elements. It was suspected that the *bla*$_{\text{TEM}}$ genes might be residing on the chromosomes in these strains. In the view of the above findings the question should be raised about the very origins of the plasmid encoded *bla*$_{\text{TEM-1}}$ in Enterobacteriaceae in humans and animals. It is likely that the Tn*2* transposons first mobilized the *bla*$_{\text{TEM-1}}$ genes from their original chromosomal locations and then these transposable elements were further spread on plasmids in different bacteria species.

In conclusion, both indicator and pathogenic *E. coli* from humans, cattle, pigs and poultry apparently shared similar plasmids with *bla*$_{\text{TEM-1}}$ genes; these *bla*$_{\text{TEM-1}}$ plasmids might have been acquired by humans via direct contact or food chain from food-production animals.([27]) The progenitors of *bla*$_{\text{TEM-1}}$ genes might have been mobilized in the past by Tn*3*- like elements from yet undetermined chromosomal locations. Further research is required to investigate that hypothesis. IS*26* were often found inserted upstream of the *bla*$_{\text{TEM-1}}$ genes probably contributing to further acquisitions of diverse resistance genes by these plasmids.([10]) Co-resistance to sulfamethoxazole, trimethoprim and/or tetracycline was conferred by the relatively large number of examined *bla*$_{\text{TEM-1}}$ scaffolds. This constitutes a threat for selection for such multi-resistant bacteria in the infected individuals treated with these classes of antimicrobials. Moreover, more discriminative, preferably sequencing based tools like pMLST or full genome sequencing needs to be designed to obtain better perspective of the apparently conserved backbones of IncB/O plasmids.

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Transparency declarations

None to declare.

List and description of Tables and Figures

Table 1. Summary of replicon typing results of $bla_{\text{TEM-1}}$ and bla_{TEM} variants plasmids from human and animal reservoirs

^{x}- TFs harbouring possibly two plasmids were also included in the general analyses as in all cases the replicons turned out to be non- typable in these TFs, these cases are indicated with the 'x' superscript throughout the Table 1; $^{\text{RFLP [type]}}$ – subgroups of plasmids sharing similar RFLP profiles were indicated with this superscript, for example six of the nine IncFII plasmids from cattle harboured similar RFLP 'a' profiles etc.; detailed results of RFLP analyses of plasmids across the reservoirs are listed Tables 3-5 and the same letters were used here to designate the RFLP types of the specified plasmids; NT- non -typable

Table 2. Distrubution of replicons among the *bla*$_{\text{TEM-1}}$ plasmids from humans, cattle, poultry and pigs

[a]The TFs from pigs and humans that possibly harboured two plasmids were negative in PBRT, therefore these were counted as single cases of NT plasmids with *bla*$_{\text{TEM-1}}$ genes

Table 3. Characterization of selected IncFII *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM-30}}$ plasmids from humans, pigs and cattle

IND- faecal indicator; CL- clinical; DG-diagnostic; NR- results not readible; NT- non typable ; NAP- not applicable, plasmid DNA was isolated from agarose gel; [a]- numbers next to the same RFLP submbols indicate that there were some differences observed between these marked fingerprints, otherwise the profiles were closely related (max. 3 bands difference was the criterium to designate plasmids with the same symbol); [b]- should be interpreted as a putative element upstream of the *bla*$_{\text{TEM}}$ gene; $^{\text{Xba\_[number]}}$ WT strains marked with the same Xba_[numbers] were shown to be clonally related in Xba I - PFGE; [c] -phylotype A from the original study of Clermont et al. is sub-divided here into *yja*A positive A1 type and *yja*A negative A2 type; AMP- amplicillin; AMC- amoxy-clavulanate 2:1; APR- apramycin; CHL- chloramphenicol; GEN- gentamycin; NEO- neomycin, SMX- sulfamethoxazole; SPT- spectinomycin; SXT- co-trimoxazole (trimethoprim/ sulfamethoxazole 1:5); TET- tetracyclin; TMP- trimethoprim; []- intermediate resistance; the different RFLP profiles are indicated with letters from a to z ,if necessary additional symbols of Greek alphabet were used; RST- Replicon Sequence Typing; pMLST- plasmid Multilocus Sequence Typing; Highlighted background is applied to cases when similar plasmids were found across the different reservoirs (e.g. humans, pigs, cattle or poultry)

Table 4. Characterization of IncB/O *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM-30}}$ plasmids from humans, pigs and cattle.

The legend is as described for Table 3.

Table 5. Characterization of IncI1 *bla*$_{TEM-1}$ and *bla*$_{TEM-135}$ plasmids from humans, pigs and poultry

The legend is as described for Table 3.

Table 6. Diversity of elements detected upstream of the *bla*$_{TEM}$ genes in AMP^{R} *E. coli* from humans and from food-production animals

[a]- Wild type strains with non- transferable *bla*$_{TEM}$ genes were transferred to the corresponding were also tested in the PCR I-V in order to obtain a general overview of the diversity of the elements linked to the *bla*$_{TEM}$ genes in the examined strains collection; [b]- two copies of the *bla*$_{TEM-.1}$ gene were detected on the same plasmid in 2000-103495 TF, the two copies were -1c and -1d alleles , respectively; ukn- unknown

Table 7. Summary of resistances typically co-transferred to the recipients on the *bla*$_{TEM-1}$ plasmids from humans and food-production animals

[a]- some of the plasmids co-transferred simultaneously resistances to a combinations of the listed antimicrobials; however, in the Table 7 each plasmid was counted independently each time for the analysed antimicrobial; aabbreviations for antimicrobials are explained in the legend to Tables 3; [b]TFs suspected to harbour more than one plasmid were not included in this analysis

Table S1. Explanatory material 1

Table S2. Primers used in five simplex PCRs for typing of the regions upstream of the *bla*$_{TEM}$ genes.

Table S3a. Summary of results for *E. coli* from cattle
Table S3b. Summary of results for *E. coli* from pigs
Table S3c. Summary of results for *E. coli* from poultry

Table S3d. Summary of results for *E. coli* from humans- faecal indicator isolates

Table S3e. Summary of results for *E. coli* from humans- clinical isolates

S4. Supplementary RFLP data

Figure S1. Targets of the PCR I, II and III on the typical TnpA-*bla*$_{TEM}$ elements

Figure S2. Fragment of ICE*hin1056* from *Haemophilus influenzae* (AJ627386); *in silico* analysis indicated primers for PCR I would not match to this sequence as *tnp*A gene of Tn*2* is not present on this element; however the sequence matches with PCR II primers targeting the *tnp*R of Tn*2* and *bla*$_{TEM}$ gene

Figure S3. The *bla*$_{TEM-1}$ region on IncB/O plasmid p3521 (GU256641). Based on the *in silico* analysis, in PCR IV two products of lengths 551 kb and 2122 kb would be produced for this sequence (indicated with lines below the open reading frames)

Figure S4. The structure of transposon Tn6039B previously described for pHCM1 (AL513383) plasmid by Bailey et al. PCR IV and V would be expected to generate products of 1393 bp and 1888 bp lengths, respectively, for this sequence.

Figure S5. Putative arrangement of IS*26* inserted upstream of *bla*$_{TEM-1}$ genes on plasmids producing in this study an approximately 2 kb signal in IS*26*a-*bla*$_{TEM}$ PCR and approximately 0.6 kb signal in IS*26*b-*bla*$_{TEM}$ PCR.

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Table 1. Summary of replicon typing results of *bla*$_{\text{TEM-1}}$ and *bla*$_{\text{TEM}}$ variants plasmids from human and animal reservoirs

Isolation source	CATTLE		POULTRY		PIGS		HUMANS	
Total of *E. coli* amp^{r} WT isolates examined	33		24		40		62	
Isolation source	faecal com.	diagnostic	faecal com.	diagnostic	faecal com.	diagnostic	faecal com.	clinical
Total of WTs from the specified source	2	31	12	12	14	26	31	31
TFs or TCs with plasmids harboring other than *bla*$_{\text{TEM-1}}$ variants		2 *bla*$_{\text{TEM-30}}$ (1 IncFII and 1 NT^{A})					3 (1 *bla*$_{\text{TEM-30}}$ IncB/O; 1 *bla*$_{\text{TEM-135}}$ IncI1 $^{\text{RFLP u}}$; 1 *bla*$_{\text{TEM-40}}$ IncFIB)	
Total *E.coli* WT with *bla*$_{\text{TEM-1}}$	2	29	12	12	14	26	28	31
E. coli WT with *bla*$_{\text{TEM-1}}$ on a plasmid or other transferable element	2	27	12	11	14	26	27	30
Cases when *bla*$_{\text{TEM-1}}$ possibly located on the chromosome		2		1			1	1
Replicons on the *bla*$_{\text{TEM-1}}$ plasmids								
IncB/O		1			3 (2 $^{\text{RFLP q}}$)	6 (4 $^{\text{RFLP p}}$, 2 $^{\text{RFLP o}}$)	6 (4$^{\text{RFLP m}}$; and 1$^{\text{RFLP p}}$)	4$^{\text{RFLP m}}$
IncB/O & IncP	1	2					1 $^{\text{RFLP p}}$	2 $^{\text{RFLP p}}$
IncFIB		1				1		
IncFII		8 (6 $^{\text{RFLP a}}$)			1	4 (2 $^{\text{RFLP d}}$)	2	5(3$^{\text{RFLP g}}$, 2$^{\text{RFLP j}}$)
IncFII & IncFIB		4$^{\text{RFLP hinc2}}$	1	3 (2 $^{\text{RFLP poul2}}$)	2	5	6	8
IncFIB & FIA		1						
IncFII & IncFIB & IncFIA		6 (2 $^{\text{RFLP hinc5}}$)					1	1
IncFII & IncFIB & IncP		1		3 (2 $^{\text{RFLP poul4}}$)	1		1	1
IncFII & IncP						1		
IncFII & IncR					1			
IncFIB & IncY								1
IncI1			10 (6$^{\text{RFLP t}}$)	2 (1 $^{\text{RFLP t}}$)	4	2	4 (1$^{\text{RFLP t}}$, and 2$^{\text{RFLP u}}$)	
IncI1 & IncP				1		1		1
IncI1 & IncFIC & IncP						1		
IncHI1						1		
IncK							1	
IncN				2 $^{\text{RFLP poul3}}$			1	
IncP	1	1						
IncX1							1	
NT		2	1		2	4 (1^{A})	3	7 (6^{A})

^{A}- TFs harbouring possibly two plasmids were also included in the general analyses as in all cases the replicons turned out to be non- typable in these TFs; $^{\text{RFLP [type]}}$ – subgroups of plasmids sharing similar RFLP profiles were indicated with this superscript, for example six of the nine IncFII plasmids from cattle harboured similar RFLP ‘a’ profiles etc.; detailed results of RFLP analyses of plasmids across the reservoirs are listed Tables 3-5 and the same letters were used here to designate the RFLP types of the specified plasmids; NT- non – typable.

Table 2. Distrubution of replicons among the *bla*$_{TEM-1}$ plasmids from *E. coli* isolates from humans, cattle, poultry and pigs

Reservoir	Human[a]	Animals[a]	Cattle	Poultry[a]	Pigs[a]
Total of *bla*$_{TEM-1}$ plasmids typed from different WT	57 (100 %)	92 (100 %)	29 (100 %)	23 (100 %)	40 (100 %)
Replicons distrubution					
IncFI; IncFII and IncF –family combined with other replicons	26 (45.6 %)	44 (47.8 %)	21 (72.4 %)	7 (30.4 %)	16 (40.0 %)
IncB/O and IncB/O combined with IncP	13 (22.8 %)	13 (14.1 %)	4 (13.8 %)	-	9 (22.5 %)
IncI1 and IncI1 combined with other replicons	4 (7.0 %)	21 (22.8 %)	-	13 (56.5 %)	8 (20.0 %)
Non typable	11 (19.3 %)	9 (9.8.0 %)	2 (6.2 %)	1 (4.3 %)	6 (15.0 %)
IncK, IncN, IncX, IncP, IncHI1	3 $^{IncK, IncN, IncX1}$ (5.3 %)	5 (5.4 %)	2^{IncP} (6.2 %)	2^{IncN} (4.3 %)	1 ^{IncHI1} (2.5 %)

[a]The TFs from pigs and humans that possibly harboured two plasmids were negative in PBRT, therefore these were counted as single cases of NT plasmids with *bla*$_{TEM-1}$ gen

Table 3. Characterization of selected IncFII *bla*$_{TEM-1}$ and *bla*$_{TEM-30}$ plasmids from humans, pigs and cattle

E. coli WT number	Isolation source	WT phylotype [c]	*bla*$_{TEM}$ allele	Replicon(s) (RST if performed)	Plasmid RFLP[a]	Plasmid size [kb]	Element upstream of *bla*$_{TEM-1}$ [b]	Plasmid associated resistances
74-12973-1	Cattle DG	A2 $^{Xba\_1}$	*bla*$_{TEM-1b}$	IncFII	a	51	IS*26*	AMP, SMX, TMP
74-13208-1	Cattle DG	A2 $^{Xba\_1}$	*bla*$_{TEM-1b}$	IncFII	a	51	unknown	AMP, SMX, TMP
74-13236-1	Cattle DG	A2 $^{Xba\_1}$	*bla*$_{TEM-1b}$	IncFII	a	51	unknown	AMP, SMX, SXT, TMP
73-13865-1	Cattle DG	A2 $^{Xba\_1}$	*bla*$_{TEM-1b}$	IncFII	a	51	IS*26*	AMP, SMX, SXT, TMP
74-12972-1	Cattle DG	A2 $^{Xba\_1}$	*bla*$_{TEM-1b}$	IncFII	a	51	IS*26*	AMP, SMX, SXT, TMP
74-13296-1	Cattle DG	A2 $^{Xba\_1}$	*bla*$_{TEM-1b}$	IncFII	a	51	IS*26*	AMP, SMX, TMP
74-12951-1	Cattle DG	A2 $^{Xba\_1}$	*bla*$_{TEM-30}$	IncFII	b	151	IS*26*	AMP, AMC, AMX, SMX, TMP
74-13287-1	Cattle DG	B1	*bla*$_{TEM-1b}$	IncFII	c	148	unknown	AMP, NEO, SMX, TMP
73-14678-1	Cattle DG	A2	*bla*$_{TEM-1b}$	IncFII (F35-rel.:A-:B-)	d_1	77,2	unknown	AMP, SMX, SXT, TMP
74-13348-1	Pig DG	A1	*bla*$_{TEM-1b}$	IncFII	d	70	IS*26*	AMP, GEN, TMP
74-13297-1	Pig DG	A1	*bla*$_{TEM-1b}$	IncFII (F35-rel.:A-:B-)	d_2	70	IS*26*	AMP, SMX
74-13205-1	Pig DG	B1	*bla*$_{TEM-1b}$	IncFII	e	105[c]	Tn*2*	AMP, SMX, TET
73-30620-6	Pig IND	D2	*bla*$_{TEM-1b}$	IncFII	f	70	IS*26*	AMP, SMX, TET, TMP
2001-49440	Human CL	D	*bla*$_{TEM-1b}$	IncFII (F2:A-:B-)	g	64	IS*26*	AMP, SMX, TMP
2002-37995	Human CL	D	*bla*$_{TEM-1b}$	IncFII	g	65	IS*26*	AMP,SMX, TMP
2002-134976	Human CL	B2	*bla*$_{TEM-1b}$	IncFII (F2:A-:B-)	g_1	60	IS*26*	AMP, SMX
1307-CO3	Human IND	B2	*bla*$_{TEM-1b}$	IncFII	h	70	IS*26*	AMP, SMX, TMP
2001-59632	Human CL	B2	*bla*$_{TEM-1b}$	IncFII	i	70	IS*26*	AMP, SMX, [GEN]
2001-106784	Human CL	B2	*bla*$_{TEM-1a}$	IncFII	j	70	Tn*3*	AMP
855-CO3	Human IND	B2	*bla*$_{TEM-1b}$	IncFII	k	60	Tn*2*	AMP, AMC
74-13224-1	Pig DG	A2	*bla*$_{TEM-1b}$	IncFII	l	80	Tn*2*	AMP

IND- faecal indicator; CL- clinical; DG-diagnostic; NR- results not readible; NT- non typable ; NAP- not applicable, plasmid DNA was isolated from agarose gel; [a]- numbers next to the same RFLP types indicate that there were some differences observed between these marked fingerprints, otherwise the profiles were closely related (max. 3 bands difference was the criterium to designate plasmids with the same symbol); [b]- should be interpreted as a putative element upstream of the *bla*$_{TEM}$ gene; $^{Xba\_[number]}$ WT strains marked with the same Xba_[numbers] were shown to be clonally related in Xba I -PFGE; [c] -phylotype A from the original study of Clermont et al. is sub-divided here into *yja*A positive A1 type and *yja*A negative A2 type; AMP- amplicillin; AMC- amoxy-clavulanate 2:1; APR- apramycin; CHL- chloramphenicol; GEN- gentamycin; NEO- neomycin, SMX- sulfamethoxazole; SPT- spectinomycin; SXT- co-trimoxazole (trimethoprim/ sulfamethoxazole 1:5); TET- tetracyclin; TMP- trimethoprim; []-intermediate resistance; the different RFLP profiles are indicated with letters from a to z ,if necessary additional symbols of Greek alphabet were used; RST- Replicon Sequence Typing; pMLST- plasmid Multilocus Sequence Typing.

Table 4. Characterization of IncB/O *bla*$_{TEM-1}$ and *bla*$_{TEM-30}$ plasmids from humans, pigs and cattle.

E. coli WT number	Isolation source	WT phylotype	*bla*$_{TEM}$ allele	Replicon(s)	Plasmid RFLP[a]	Plasmid size [kb]	Element upstream of *bla*$_{TEM-1}$[b]	Plasmid associated resistances
1297-CO3	Human IND	NT	*bla*$_{TEM-1g}$	IncB/O	m	70	Tn*2*	AMP, SMX
875-CO3	Human IND	D	*bla*$_{TEM-30}$	IncB/O	m	80	Tn2	AMP, AMC, SMX
438-CO2	Human IND	B2	*bla*$_{TEM-1c}$	IncB/O	m	88	Tn*2*	AMP, SMX
2002-110474	Human CL	B2	*bla*$_{TEM-1c}$	IncB/O	m_1	70	Tn*2*	AMP, SMX
2002-50	Human CL	D	*bla*$_{TEM-1c}$	IncB/O	m	80	Tn*2*	AMP, SMX, SPE
1082-CO3	Human IND	B2	*bla*$_{TEM-1c}$	IncB/O	m	92	Tn*2*	AMP, SMX
97-CO2	Human IND	B2	*bla*$_{TEM-1b}$	IncB/O	m_2	95	Tn*2*	AMP, SMX
2002-14946	Human CL	B2	*bla*$_{TEM-1c}$	IncB/O	m_3	80	Tn*2*	AMP, SMX
2001-25022	Human CL	D	*bla*$_{TEM-1c}$	IncB/O	m_4	90	Tn*2*	AMP, SMX
1231-CO3	Human IND	B2	*bla*$_{TEM-1b}$	IncB/O	n	85	Tn*2*	AMP
74-11919-1	Pig DG	A1	*bla*$_{TEM-1a}$	IncB/O	o	105	Tn*3*	AMP, SMX, TET, TMP
74-12927-1	Pig DG	A2	*bla*$_{TEM-1a}$	IncB/O	o	100	Tn*3*	AMP, SPT, SMX, TET, TMP
74-13266-1	Pig DG	A2	*bla*$_{TEM-1b}$	IncB/O	p_1	105	IS*26*	AMP, NEO,SMX, TET
433-CO2	Human IND	A1	*bla*$_{TEM-1a}$	IncB/O	p_2	95	Tn*3*	AMP
457-CO2	Human IND	B2	*bla*$_{TEM-1b}$	IncB/O & IncP	p	110	IS*26*	AMP, NEO, SMX, TET
2001-131351	Human CL	A1	*bla*$_{TEM-1b}$	IncB/O & IncP	p	110	IS*26*	AMP, SMX, TET
2001-37255	Human CL	A1	*bla*$_{TEM-1b}$	IncB/O & IncP	p	125	IS*26*	AMP, SMX, TET, TMP
74-12934-1	Pig DG^{XbaI_2}	A1	*bla*$_{TEM-1b}$	IncB/O	p	105	IS*26*	AMP, NEO,SMX, TET
74-12933-1	Pig DG^{XbaI_2}	A1	*bla*$_{TEM-1b}$	IncB/O	p	105	IS*26*	AMP, NEO,SMX, TET
74-30367-2	Pig DG	A1	*bla*$_{TEM-1b}$	IncB/O	p	105	IS*26*	AMP, NEO,SMX, TET
74-12894-1	Cattle DG	B1	*bla*$_{TEM-1b}$	IncB/O & IncP	p	115	IS*26*	AMP, SMX, TET
74-12865-1	Cattle DG	A2	*bla*$_{TEM-1b}$	IncB/O & IncP	p	115	IS*26*	AMP, SMX, TET
74-30014-3	Cattle IND	B1	*bla*$_{TEM-1b}$	IncB/O & IncP	p_3	115	IS*26*	AMP, NEO,SMX, TET
73-30819-1	Pig IND	A2	*bla*$_{TEM-1b}$	IncB/O	q	105	IS*26*	AMP, SMX, TMP
74-30165-7	Pig IND	D2	*bla*$_{TEM-1b}$	IncB/O	q_1	140	IS*26*	AMP, NEO,SMX, TET
74-13196-1	Cattle DG	D2	*bla*$_{TEM-1b}$	IncB/O	r	106	IS*26*	AMP, SMX
73-30622-3	Pig IND	B1	*bla*$_{TEM-1b}$	IncB/O	NR	105	IS*26*	AMP, NEO,SMX, TET

The legend is as described for Table 3.

Table 5. Characterization of IncI1 *bla*$_{TEM-1}$ and *bla*$_{TEM-135}$ plasmids from humans, pigs and poultry

E. coli WT number	Isolation source	WT phylotype	*bla*$_{TEM}$ allele	Replicon(s) (pMLST type, if tested)	Plasmid RFLP[a]	Plasmid size [kb]	Element upstream of *bla*$_{TEM-1}$[b]	Plasmid associated resistances
74-13303-1	Pig DG	A1	*bla*$_{TEM-1b}$	IncI1, IncFIB, IncFIC (ST36/CC5:FIB16:FIC2)	s	80	Tn*2*	AMP
1341-CO3	Human IND	NT	*bla*$_{TEM-1b}$	IncI1 (ST36/CC5)	t	85	Tn*2*	AMP
7430186-1	Poultry IND	A1	*bla*$_{TEM-1b}$	IncI1	t	82	Tn*2*	AMP
7430287-1	Poultry IND	A2	*bla*$_{TEM-1b}$	IncI1	t	90	Tn*2*	AMP
7430521-1	Poultry IND	B1	*bla*$_{TEM-1b}$	IncI1	t_1	85	Tn*2*	AMP
7370817-2	Poultry DG	B1	*bla*$_{TEM-1b}$	IncI1	t_1	85	Tn*2*	AMP
7430125-1	Poultry IND	A1	*bla*$_{TEM-1b}$	IncI1 (ST36/CC5)	t	82	Tn*2*	AMP
7430557-1	Poultry IND	A1	*bla*$_{TEM-1b}$	IncI1	t	85	Tn*2*	AMP
7430237-1	Poultry IND	A2	*bla*$_{TEM-1b}$	IncI1	t	85	Tn*2*	AMP
1033-CO3	Human IND	NT	*bla*$_{TEM-1b}$	IncI1	u	100	Tn*2*	AMP, SMX
7430284-1	Poultry IND	A2	*bla*$_{TEM-1b}$	IncI1	u_1	104	Tn*2*	AMP, SMX
1112-CO3	Human IND	A1	*bla*$_{TEM-135}$	IncI1	u_2	104	Tn*2*	AMP, SMX, TMP
74-30164-4	Pig IND	A2	*bla*$_{TEM-1a}$	IncI1	u_3	80	Tn*3*	AMP
73-30816-2	Pig IND	A1	*bla*$_{TEM-1b}$	IncI1	v	80	Tn*2*	AMP
2002-70903	Human CL	NT	*bla*$_{TEM-1b}$	IncI1 (ST97-rel.) & IncP	w	120	IS*26*	AMP, SMX, TET, TMP
545-CO2	Human IND	D	*bla*$_{TEM-1c}$	IncI1	w_1	105	Tn*2*	AMP, SMX, TET, TMP
74-13304-2	Pig DG	D	*bla*$_{TEM-1b}$	IncI1	x	150	IS*26*	AMP, SMX, TET, TMP
74-13309-1	Pig DG	A1	*bla*$_{TEM-1b}$	IncI1	y	80	IS*26*	AMP
74-30167-1	Pig IND	B1	*bla*$_{TEM-1b}$	IncI1	z	105	IS*26*	AMP, NEO, SMX, TMP
74-30166-3	Pig IND	A1	*bla*$_{TEM-1b}$	IncI1	α	105	IS*26*	AMP
7430621-1	Poultry IND	D	*bla*$_{TEM-1b}$	IncI1	β	82	Tn*2*	AMP
7365811-1	Poultry DG	A2	*bla*$_{TEM-1b}$	IncI1 (ST3/CC3) & IncP	θ	125	Tn*2*	AMP, SMX, SXT, TET, TMP
7430605-1	Poultry IND	A1	*bla*$_{TEM-1b}$	IncI1	NAP	85	Tn*2*	NAP
7430641-1	Poultry IND	B1	*bla*$_{TEM-1b}$	IncI1	NR	85	Tn*2*	AMP
7275882-1	Poultry DG	A2	*bla*$_{TEM-1b}$	IncI1	NR	85	Tn*2*	AMP, AMC
74-12848-1	Pig DG	A1	*bla*$_{TEM-1b}$	IncI1 (ST-new) & IncP	NR	100	IS*26*	AMP, TET

The legend is as described for Table 3.

Table 6. Diveristy of elements detected upstream of the *bla*$_{TEM}$ genes in amp^r *E. coli* from humans and from food-production animals

Total of amp^r isolates examined				159 (100 %)[a]					
Allele occurence among the total number of amp^r isolates examined									
bla$_{TEM-1a}$		*bla*$_{TEM-1b}$		*bla*$_{TEM-1c}$	Other *bla*$_{TEM-1}$ alleles (*bla*$_{TEM-1c/d, -1d, -1g, -1j}$)	Other examined variants (*bla*$_{TEM-30, -40,- 135}$)			
14 (8.8 %)		113 (71.1 %)		22 (13.8 %)	5 (3.1 %)	5 (3.1 %)			
Distribution of *bla*$_{TEM}$ alleles									
Element detected upstream	Human isolates (n= 62)[a] *bla*$_{TEM}$ alleles					Animal isolates (n=97)[a] *bla*$_{TEM}$ alleles			
	-1a n=6	-1b n=33	-1c n=15	-1c/d, -1d, -1g, -1j	-30, -40,-135	-1a n=8	-1b n=80	-1c n=7	-30 n=2
Tn*1*				1 *bla*$_{TEM-1d}$			1		
Tn*2*		15	15	1 *bla*$_{TEM-1g}$ 1 *bla*$_{TEM-1j}$	1 *bla*$_{TEM-30,}$ 1 *bla*$_{TEM-135}$		33	7	1
Tn*3*	6			1 [b] *bla*$_{TEM-1c/d}$		8	1		1
IS*26*		18			1 *bla*$_{TEM-40}$		35		
Ukn				1 *bla*$_{TEM-1d}$			10		

[a]- Wild type strains with non- transferable *bla*$_{TEM}$ genes were transferred to the corresponding were also tested in the PCR I-V in order to obtain a general overview of the diversity of the elements linked to the *bla*$_{TEM}$ genes in the examined strains collection; [b]- two copies of the *bla*$_{TEM-.1}$ gene were detected on the same plasmid in 2000-103495 TF, the two copies were -1c and -1d alleles , respectively; ukn- unknown

Table 7. Summary of resistances typically co-transferred to the recipients on the *bla*$_{TEM-1}$ plasmids from humans and food-production animals

Reservoir	Humans		Animals		Cattle		Pigs		Poultry	
TFs/ TCs with *bla*$_{TEM-1}$ plasmids [b]	total[c]	(%)	total	(%)	total	(%)	total	(%)	total	(%)
	57	(100 %)	88	(100 %)	27	(100 %)	40	(100 %)	21	(100 %)
Plasmid co-transferred resistance to: [a]										
SMX	34	(42.1 %)	49	(55.7 %)	19	(70.4 %)	22	(55.0 %)	8	(38.1 %)
TET	20	(35.1 %)	43	(48.9 %)	17	(63.0 %)	17	(42.5 %)	9	(42.9 %)
TMP	14	(24.6 %)	28	(31.8 %)	11	(40.7 %)	11	(27.5 %)	6	(28.6 %)
NEO	2	(3.5 %)	17	(19.3 %)	3	(11.1 %)	13	(32.5 %)	1	(4.8 %)
SPT	3	(5.3 %)	3	(3.4 %)	1	(3.7 %)	2	(5.0 %)	-	
AMC	14	(24.6 %)	4	(4.5 %)	1	(3.7 %)	2	(5.0 %)	1	(4.8 %)
CHL	2	(3.5 %)	3	(3.4 %)		-	1	(2.5 %)	2	(1.5 %)
GEN	2	(3.5 %)	1	(1.1 %)		-	1	(2.5 %)	-	

[a]- some of the plasmids co-transferred simultaneously resistances to a combinations of the listed antimicrobials; however, in the Table 7 each plasmid was counted independently each time for the analysed antimicrobial; abbreviations for antimicrobials are explained in the legend to Table 3; [b]TFs suspected to harbour more than one plasmid were not included in this analysis; [c]- [%] of the specified total number

Table S1. Explanatory material 1.

In two of the obtained TFs (74-13205-1TF and 74-13344-1 TF; pigs) the sizes of the individual *bla*$_{\text{TEM-1}}$ plasmids observed in S1-PFGE did not correspond to sizes of any of the plasmids detected in the respective WTs. Size of the plasmid in the 74-13205 TF was estimated to105 kb; in the corresponding WT the only plasmid detected in S1-PFGE was approximately 140 kb. The *bla*$_{\text{TEM-1}}$ plasmid in the 74-13344-1TF was sized to 75 kb while two larger plasmids (sized to 85 kb and 145 kb) were observed in the respective WT isolate. Possibly in WT 74-13344-1the largest plasmid was a co-integrate of two *bla*$_{\text{TEM-1}}$ plasmids that was resolved in the TF. The 140 kb plasmid from the WT 74-13205 could also be a co-integrate of two plasmids; in this case one would expect to see a second plasmid in the corresponding TF sized to approximately 30-35 kb. Plasmids of these sizes might be hardly visible in S1-PFGE. In 2001-37255TF (human reservoir) the size of the individual *bla*$_{\text{TEM-1}}$ plasmid (125 kb) observed in the S1-PFGE did not correspond to the size of the single plasmid detected in the WT (60 kb). The plasmid from this TF could be a co-integrate of two plasmids of approximate 60 kb sizes.

Co-integrate formation between plasmids in conjugation and upon transformation have been previously reported [1,2]. Since only individual plasmids were observed in the above TFs, these TFs were treated as harbouring individual *bla*$_{\text{TEM-1}}$ plasmids and were included in further characterizations.

References

1. Grishina EV, Pekhov AP: Cointegration between F-like conjugative plasmids. Biull Eksp Biol Med 1991, 112:1312-1314.

2. Aleksenko AY: Cointegration of transforming DNAs in Aspergillus nidulans: a model using autonomously-replicating plasmids. Curr Genet 1994, 26:352-358.

Table S2. Primers used in five simplex, linking PCRs for typing of the regions upstream of the *bla*$_{TEM}$ genes.

PCR type	Primer sequence	Target	Control	Amplicon size [bp]	T $_{annealing}$ °C	Time $_{annealing}$ [min.]	Reference (accession number)
PCR I	P_{fw} 5'-CGTATCAGCGCTGCATGCTCA-3'	*tnp*A of Tn*1, -2, -3*	JQ423956	1521	60°C	1.5	Bailey *et al.*
PCR II	P_{fw} 5'-GGTGTGAAAGCAAACCGTATA-3'	*tnp*R of Tn*2*	JF776874.1	953	55°C	1	Bielak *et al.*
PCR III	P_{fw} 5'-CGGCTTTTTTAACACAACTG-3'	*tnp*A of Tn*3*	R6K*	1174	55°C	1	R6K *
PCR IV	P_{fw} 5'-ACCTTTGATGGTGGCGTAAG-3'	IS*26*a	For details see explanation below this table	variable	60°C	1.5	RH1270 in Bailey *et al.*
PCR V	P_{fw} 5'-GATGCGTGCACTACGCAAAG-3'	IS*26*b		variable	60°C	2	RH882 in Bailey *et al.*
PCR I, II, III, IV and V	P_{rv} 5'-CTGAGAATAGTGTATGCGGCGAC-3'	*bla*$_{TEM}$					
PCR conditions	Reaction volume 25 µL, primers and dNTPs concentrations: 0.25 µM per reaction, 0.5 U of VWR (Promega®) polymerase was used per reaction. PCR set-up: initiation step at 94°C; 5 min. followed by 30 cycles of i) 94°C; 1 min, ii) T $_{annealing}$; Time $_{annealing}$ iii) 72°C; 90 sec. ; and final elongation step at 72°C; 5 min.						

Interpretation of the results of the linking PCRs I-V

	PCR I	PCR II	PCR III	PCR IV	PCR V
Putative *Tn*1-*bla*$_{TEM}$	+	-	-	PCR IV and V were performed only if no signal was produced in PCR I, II and III	
Tn*2*-*bla*$_{TEM}$	+	+	-		
Tn*3*-*bla*$_{TEM}$	+	-	+		
IS26a - *bla*$_{TEM}$	-	-	-	+	+/-
IS26b - *bla*$_{TEM}$	-	-	-	+/-	+
IS*26* 'a' and 'b' refer to the orientation of the inverted repeats (IRs) of the IS*26* as described by Bailey *et al*.					

* In Bailey *et al*. the R6K was designated as harbouring Tn*1*; however *in silico* analysis of the sequnce of R6K available at Wellcome Trust Sanger Institute, www.sanger.ac.uk predicted the presence of Tn*3*; the latter was confirmed by obtaining a positive signal in PCR III and I, but no signal in PCR II for the R6K; therefore the plasmids was used as positive control for PCR III.

Description to Table S2

Plasmid purifications or boiled lysates from TFs or TCs harbouring individual plasmids of interests were used as templates. Controls used were as follows: plasmid R6K harbouring Tn*3*-bla_{TEM-1} sequence (sequence available at Wellcome Trust Sanger Institute, www.sanger.ac.uk) was positive control in PCR III, plasmid pE001 carrying Tn*2*-bla_{TEM-52} (JF776874.1) was positive control for PCR II. The Tn*1*-bla_{TEM-1} was initially found on p862-CO3 (bla_{TEM-1d} plasmid from human, this study). The PCR I product from this plasmid was sequenced, and deposited in GenBank (JQ423956). The p862-CO3 served later as the control in PCR I. Caution should be taken in interpretation of the results of the PCR I. Only if a given plasmid produced positive signal in PCR I, while no signal was produced in PCR II (Tn*2* specific) and III (Tn*3* specific), the element linked to bla_{TEM} was interpreted in this study as putative Tn*1*. PCRs IV and V were performed only when no signal was observed in PCRI-III. No control was prepared for these two simplexes as the obtained products were expected to be of different sizes as observed in Bailey *et al*. 2011. Any positive product observed in PCR IV or V was interpreted as putative IS*26*a or IS*26*b linked to the corresponding bla_{TEM} gene, respectively.

It needs to be underlined that the study aimed at giving only an overview of the diversity of Tn*1-3* and IS*26* types associated with the bla_{TEM} genes in the examined collection of strains. Therefore no sequencing was performed for further confirmation of the observed PCR results. Tn*1-3* and the IS*26* elements detected upstream the bla_{TEM}-genes should be considered as putative in order to leave the labelling opened for discussion in the future when sequencing would be performed and more details obtained.

Predicted outputs of the linking PCR for the different sequences available in GenBank are indicated on the figures below. The schemes were generated in Vector NTI suit 11 software (Invitrogen, Inc.)

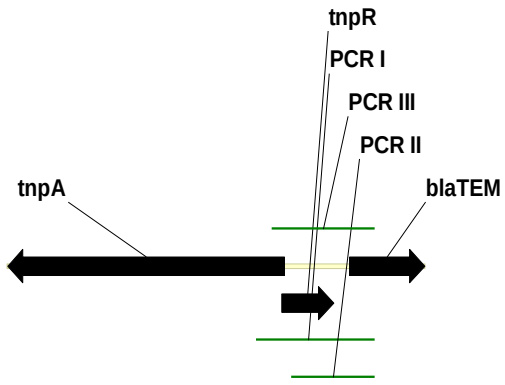


Figure S1. Targets of the PCR I, II and III on the typical *tnpA*-bla_{TEM} elements.

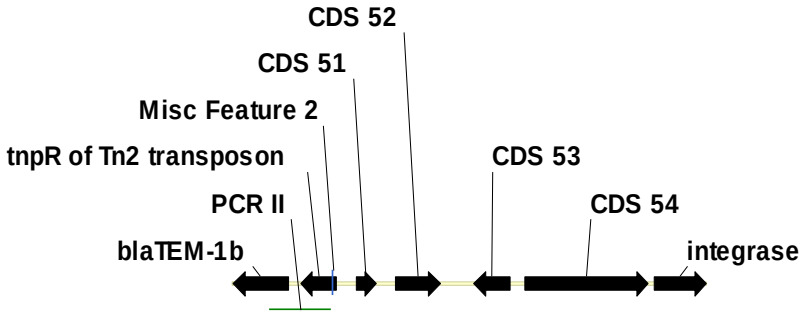


Figure S2. Fragment of ICE*hin1056* from *Haemophilus influenzae* (AJ627386); *in silico* analysis indicated primers for PCR I would not match to this sequence as *tnp*A gene of Tn*2* is not present on this element; however the sequence matches with PCR II primers targeting the *tnp*R of Tn*2* and bla_{TEM} gene.

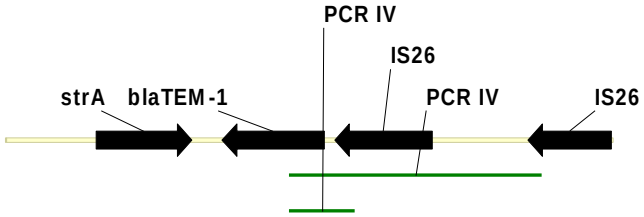


Figure S3. The $bla_{\text{TEM-1}}$ region on IncB/O plasmid p3521 (GU256641). Based on the *in silico* analysis in PCR IV two products of lenghts 551 kb and 2122 kb would be produced for this sequence (indicated with lines below the open reading frames)

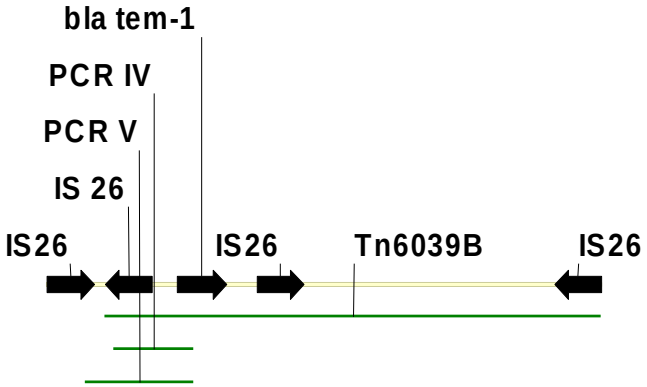


Figure S4. The structure of transposon Tn6039B previously described for pHCM1 (AL513383) plasmid by Bailey *et al*. PCR IV and V would be expected to generate products of 1393 bp and 1888 bp lengths, respectively, for this sequence.

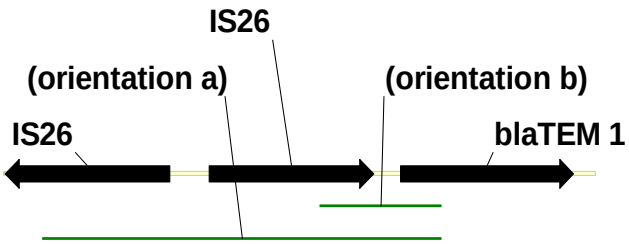


Figure S5. Putative arrangement of IS*26* inserted upstream of *bla*$_{\text{TEM-1}}$ genes on plasmids producing in this study an approximately 2 kb signal in IS*26*a-*bla*$_{\text{TEM}}$ PCR and approximately 0.6 kb signal in IS*26*b-*bla*$_{\text{TEM}}$ PCR.

Table S3 a. Summary of results for *E. coli* from cattle (Manuscript II).

Reservoir	CATTLE
Species	*E. coli*

wild type (WT)	Strain number	WT phylotype	PFGE relatedness of WT strains	W ild type (WT) associated resistances	Type of recipient in which the plasmid was characterized	*bla* TEM allele	Resistances associated with blaTEM plasmids	Replicon (s) detected	RFLP (pMLST or RST types if available)	Plasmid size [kb]	Putative element upstrem the blaTEM gene	Selftrasmissibility * only applicable to TCs obtained from corresponding WTs which harboured no more than 1 plasmid
diagnostic	74-13196-1	D2	not rel.	AMP, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX	IncB/O	r	106	IS*26*a (2 kb and 0.4 kb)	
faecal commensal	74-30014-3	B1	not rel.	AMP, NEO, SMX, STR, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, NEO, SMX, TET	IncB/O + IncP	p_3	115	IS*26*a (2 kb and 0.4 kb)	
diagnostic	74-12894-1	B1	not rel.	AMP, SMX, STR, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET	IncB/O + IncP	p	115	IS*26* a (0.4 kb)	
diagnostic	74-12865-1	A2	not rel.	AMP, SMX, STR, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET	IncB/O + IncP	p	115	IS*26*a (2 kb and 0.4 kb)	
diagnostic	74-12891-1	D1	not rel.	AMP,CHL, SMX, STR, TET	TF (GeneHogs)	*bla* TEM-1b	AMP,CHL, SMX, TET	IncP	Ω (omega)	100	IS*26*a (2 kb and 0.4 kb)	
faecal commensal	74-30138-2	D1	not rel.	AMP, AMC, CEF, SPT, SPR, SMX, TET	TC (MT101)	*bla* TEM-1b	AMP, AMC, SMX, TET	IncP	Δ (delta)	100	IS*26*a (2 kb and 0.4 kb)	yes
diagnostic	74-13300-1	A1	not rel.	AMP, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET, TMP	IncFII, IncFIB, IncP	hinc1	153	IS*26*a (2 kb and 0.4 kb)	
diagnostic	74-12867-1	A1	not rel.	AMP, SMX, STR, TET, TMP	TC (MT101)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	hinc2 (FII2 :FIB1)	130	Tn*2*	
diagnostic	74-12969-1	A1	not rel.	AMP, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	hinc2 (FII2: FIB1)	130	Tn*2*	
diagnostic	74-13379-1	A2	not rel.	AMP, SMX, STR, TET, TMP	TC (MT101)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	hinc2 (FII2: FIB1)	139	Tn*2*	
diagnostic	74-12866-1	B1	not rel.	AMP, SMX, STR, TET, TMP	TC (MT101)	*bla* TEM-1b	AMP, SMX, TET, TMP	IncFII, IncFIB	hinc2_1 (3 bands diff. compared to hinc2)	139	IS*26*a (2 kb and 0.4 kb)	
diagnostic	73-14130-2	B1	Xba_5	AMP, SMX, SXT, STR, TET, TMP	TC (MT101)	*bla* TEM-1b	AMP, SMX, SXT, TMP	IncFII, IncFIB, IncFIA	hinc3 (FII35-related: [FIA2 or 3][A]: FIB24)	135	Tn*2*	yes
diagnostic	74-13361-2	A2	Xba_3	AMP, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, TET	IncFII, IncFIB, IncFIA	hinc5	90	Tn*2*	
diagnostic	74-12892-1	A2	Xba_3	AMP, NAL, SPT, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, TET	IncFII, IncFIB, IncFIA	hinc5 (FII closest match to FII 33; FIA,-B not tested)	90	Tn*2*	no
diagnostic	74-13289-1	A1	(80 % Xba_3)	AMP, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET	IncFII, IncFIB, IncFIA	hinc6	90	IS*26*a (1.3 kb)	
diagnostic	73-13939-1	A2	not rel.	AMP, SMX, SXT, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, TET	IncFII, IncFIB, IncFIA	hinc7	93	Tn*2*	
diagnostic	74-13274-1	A2	not rel.	AMP, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, TET	IncFIB, IncFIA	hinc8	81	Tn*2*	
diagnostic	74-12084-1	A2	not rel.	AMP, CEF, NAL, SPT, STR, SMX, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, SPT, TET	IncFII, IncFIB, IncFIA	not readible	160	Tn*2*	
diagnostic	73-14678-1	A2	not rel.	AMP, GEN, NEO, STR, SMX, SXT, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, SXT, TMP	FII	d_1 FII35-related, the same mutation as in the pig isolate	77,2	unknown	
diagnostic	74-12973-1	A2	(85%)	AMP, NAL, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TMP; (SXT- not tested)	FII	a	51	IS*26*a (1.3 kb)	
diagnostic	74-13208-1	A2	(86%)	AMP, NAL, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TMP; (SXT- not tested)	FII	a	51	unknown	
diagnostic	74-13236-1	A2	(100%)	AMP, NAL, STR, SMX, SXT, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, SXT, TMP	FII	a	51	unknown	
diagnostic	73-13865-1	A2	(100%)	AMP, NAL, STR, SMX, SXT, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, SXT, TMP	FII	a	51	IS*26*a (1.3 kb)	
diagnostic	74-12972-1	A2	(100%)	AMP, NAL, STR, SMX, SXT, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, SXT, TMP	FII	a	51	IS*26*a (1.3 kb)	
diagnostic	74-13296-1	A2	(98%)	AMP, NAL, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TMP; (SXT- not tested)	FII	a	51	IS*26*a (1.3 kb)	
diagnostic	74-12951-1	A2	Xba 1 1_2 (93%	AMP, NAL, STR, SMX, TET, TMP	TC (MT101)	*bla* TEM-30	AMP, AMC, SMX, TMP; (SXT- not tested)	FII	b	151	IS*26*a (1.3 kb)	yes
diagnostic	74-13287-1	B1	Xba_5	AMP, NAL, STR, SMX, TET, TMP	TC (MT101)	*bla* TEM-1b	AMP, NEO, SMX, TMP	FII	c	148	unknown	yes
diagnostic	74-13209-1	A1	not rel.	AMP, NEO, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET	IncFIB	hinc9	112	unknown	
diagnostic	74-13318-1	B1	not rel.	AMP, CEF, GEN, NEO, STR, SMX, TET	TC (MT101)	*bla* TEM-1b	AMP, TET	NT	not readible	240	unknown	yes
diagnostic	74-12351-1	A2	not rel.	AMP, AMC, NAL, NEO, SPT, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-30	AMP, AMC	NT	hinc10	138 & 30 kb	Tn*3*	no
diagnostic	74-13301-1	A2	not rel.	AMP, CIP, NAL, NEO, SPT, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1a	transformant not preserved [B]	NT	not tested	<10 kb	Tn*3*	
diagnostic	74-13295-1	B2	not rel.	AMP, CHL, STR, SMX,TET	*bla* TEM non transferable	*bla* TEM-1b	*bla* TEM non transferable	{2 *bla* TEM -negative plasmids in WT }	not applicable	not applicable	unknown	
diagnostic	74-11793-1	A2	not rel.	AMP, SMX, TET, TMP	*bla* TEM non transferable	*bla* TEM-1b	*bla* TEM non transferable	{1 *bla* TEM -negative plasmid in WT }	not applicable	not applicable	{WT positive to IS*26*a (0.9 kb kb)}	

[A]- sequencing result for this locus and strain were of low quality or only partial sequence was blasted agains reference sequence

[B]- purified DNA from TF used for analyses

* selfrasmissibility - only applicable to TCs obtained from corresponding WTs which harboured no more than one plasmid

	Recipient resistances
Recipeint strains	Based on disc diffusion method
E. coli K12HEHA4	STR, CEF,NEO, [CHL]
E. coli GeneHogs ®	STR, CEF
E. coli MT101 (rif, nal) variant	STR, RIF, NAL (this variant was used in the majority of cases)
E. coli MT101	STR, CEF
[] intermediate resistance	
* recipient intrinsically resistant	

Table S3 b. Summary of results for *E. coli* from pigs (Manuscript II).

Reservoir	PIGS											
Species	*E. coli*											
		WT	PFGE	WT	Type of recipientin which the plasmid was characterized	*bla* TEM allele	Resistances associated with	Replicon (s)	RFLP (pMLST types if available)	Plasmid	Putative element upstrem	Selftransmissiblity
wild type (WT)	Strain number	phylotype	relatedness of selected WT strains	Resistances			*bla* TEM plasmid	(NT- non typable)	Profiles designations correspond to the symbols used in Tables 3-5 in the manuscript	size [kb]	the *bla* TEM gene	NOT TESTED in this reservoir
faecal commensal	74-30165-7	D2	not rel.	AMP, NEO, STR, SMX, TET	TF (DH10B)	*bla* TEM-1b	AMP, NEO,SMX, TET	IncB/O	q_1	140	IS*26*a (2 kb)	
faecal commensal	73-30819-1	A2	not rel.	AMP, STR, SMX, TMP, SXT	TF (DH10B)	*bla* TEM-1b	AMP, SMX, TMP	IncB/O	q	105	IS*26*a (2 kb) & IS*26*b (0.6 kb)	
faecal commensal	73-30622-3	B1	not rel.	AMP, NEO, STR, SMX, TET	TF (DH10B)	*bla* TEM-1b	AMP, NEO,SMX, TET	IncB/O	not readible	105	IS*26*a (2 kb) & IS*26*b (0.6 kb)	
diagnostic	74-30367-2	A1	not rel.	AMP, NEO, STR, SMX, TET	TF (DH10B)	*bla* TEM-1b	AMP, NEO,SMX, TET	IncB/O	p	105	IS26b (0.6 kb)	
diagnostic	74-13266-1	A2	not rel.	AMP, NEO, STR, SMX, TET, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP, NEO,SMX, TET	IncB/O	p_1	105	IS*26*a (2 kb) & IS*26*b (0.6 kb)	
diagnostic	74-12934-1	A1	Xba_2	AMP, NEO, STR, SMX, TET, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP, NEO,SMX, TET	IncB/O	p	105	IS*26*a (2 kb) & IS*26*b (0.6 kb)	
diagnostic	74-12933-1	A1	Xba_2	AMP, NEO, STR, SMX, TET, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP, NEO,SMX, TET	IncB/O	p	105	IS*26*a (2 kb) & IS*26*b (0.6 kb)	
diagnostic	74-12927-1	A2	not rel.	AMP, CHL, SPT,, STR, SMX, TET, TMP, [CEF]	TF (DH10B)	*bla* TEM-1a	AMP, SPT, SMX, TET, TMP	IncB/O	o	100	Tn*3*	
diagnostic	74-11919-1	A1	not rel.	AMP, SPT, STR, SMX, TET, TMP, [CEF]	TF (DH10B)	*bla* TEM-1a	AMP, SMX, TET, TMP	IncB/O	o	105	Tn*3*	
faecal commensal	74-30164-4	A2	not rel.	AMP, SPT, STR, SMX, [CEF]	TF (DH10B)	*bla* TEM-1a	AMP	IncI1	u_3	80	Tn*3*	
faecal commensal	74-30167-1	B1	not rel.	AMP,[APR], NEO, STR, SMX, TMP	TF (DH10B)	*bla* TEM-1b	AMP, NEO, SMX, TMP	IncI1	z	105	IS*26*a (2 kb)	
faecal commensal	74-30166-3	A1	not rel.	AMP, CHL, STR, SMX, TET	TF (DH10B)	*bla* TEM-1b	AMP	IncI1	α	105	IS*26*a (2 kb)	
faecal commensal	73-30816-2	A1	not rel.	AMP	TF (DH10B)	*bla* TEM-1b	AMP	IncI1	v	80	Tn*2*	
diagnostic	74-13309-1	A1	not rel.	AMP, NEO, STR, SMX	TF (DH10B)	*bla* TEM-1b	AMP	IncI1	y	80	IS*26*a (2 kb)	
diagnostic	74-13304-2	D1	not rel.	AMP, SPT, SMX, TET, TMP, CEF	TF (DH10B)	*bla* TEM-1b	AMP, SMX, TET, TMP	IncI1	x	150	IS*26*a (2 kb)	
diagnostic	74-12848-1	A1	not rel.	AMP, CIP, NAL, TET	TF (DH10B)	*bla* TEM-1b	AMP, TET	IncI1, IncP STnew[A]	not readible	100	IS*26*a (2 kb)	STnew[A]- rep1: ard2: trbA3:sogS9:pilL3
diagnostic	74-13303-1	A1	Xba_6	AMP,CIP, NAL, SPT, SMX, TET, TMP, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP	IncI1, IncFIB, IncFIC	s	80	Tn*2*	
diagnostic	74-13348-1	A1	not rel.	AMP, GEN, SPT, STR, TMP	TF (DH10B)	*bla* TEM-1b	AMP, GEN, TMP	IncFII	d ST FII35-closest match	70	IS*26*a (2 kb)	
diagnostic	74-13224-1	A2	not rel.	AMP, SPT, STR, SMX, TET, CEF	TF (DH10B)	*bla* TEM-1b	AMP	IncFII	l	80	Tn*2*	
diagnostic	74-13205-1	B1	not rel.	AMP, CHL, CIP, NAL ,STR, SMX, TET, TMP, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP, SMX, TET	IncFII	e	105 (possible co-int.)	Tn*2*	
faecal commensal	73-30620-6	D2	not rel.	AMP, STR, SMX, TET, TMP, SXT	TF (DH10B)	*bla* TEM-1b	AMP, SMX, TET, TMP	IncFII	f	70	IS*26*a (1.3 kb)	
diagnostic	74-13297-1	A1	Xba_6	AMP, STR, SMX	TF (DH10B)	*bla* TEM-1b	AMP, SMX	IncFII	d_2	70	IS*26*a (1.3 kb)	
diagnostic	74-11272-1	A1	not rel.	AMP, APR, GEN, SPT, STR, SMX, TET, TMP, [CEF]	TF (DH10B)	*bla* TEM-1a	AMP	IncFIB	not tested	80	Tn*3*	
diagnostic	74-12902-3	A2	not rel.	AMP, SPT, STR, SMX, TET, TMP	TF (DH10B)	*bla* TEM-1a	AMP	IncFII, IncFIB	not tested	105	Tn*3*	
faecal commensal	74-30162-3	D2	not rel.	AMP, SPT, STR, SMX, TET	TF (DH10B)	*bla* TEM-1a	AMP, SMX	IncFII, IncFIB	not tested	220	Tn*3*	
faecal commensal	73-30813-7	B1	not rel.	AMP	TF (DH10B)	*bla* TEM-1b	AMP	IncFII, IncFIB	not tested	160	Tn*2*	
diagnostic	74-13344-1	A2	not rel.	AMP, CIP, NAL, NEO, STR, SMX, TMP, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP, TMP	IncFII, IncFIB	not tested	150 (possible co-int.)	IS*26*a (1.3 kb)	
diagnostic	74-12904-1	A2	not rel.	AMP, NEO, STR, SMX, TET, TMP, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP, NEO, SMX, TMP	IncFII, IncFIB	not tested	140	IS*26*a (2 kb)	
diagnostic	74-11186-2	B2	not rel.	AMP, NEO, SPE, SMX, TET, TMP, CEF	TF (DH10B)	*bla* TEM-1c	AMP, AMC, TET	IncFII, IncFIB	not tested	180	Tn*2*	
diagnostic	74-12935-1	A1	not rel.	AMP, NEO, STR, SMX, TET	TF (DH10B)	*bla* TEM-1b	AMP, NEO,SMX, TET	IncFII, IncFIB	not tested	105	IS*26*a (1.3 kb)	
diagnostic	74-11566-3	A1	not rel.	AMP, APR, GEN, NEO, STR, SMX, TET, TMP, CEF	TF (DH10B)	*bla* TEM-1b	AMP, NEO, SMX, TET, TMP	IncFII, IncP	not tested	180	Tn*2*	
faecal commensal	73-30883-3	A1	not rel.	AMP, SPT, STR, SMX, TET	TF (DH10B)	*bla* TEM-1b	AMP, SPT, SMX, TET	IncFII, IncFIB, IncP	not tested	200	Tn*2*	
faecal commensal	73-30615-7	A2	not rel.	AMP, TET	TF (DH10B)	*bla* TEM-1b	AMP	IncFII, IncR	not tested	130	Tn*2*	
diagnostic	74-12901-2	A1	not rel.	AMP, CHL, CIP, NAL,NEO, SPT, STR, SMX, TET, TMP	TF (DH10B)	*bla* TEM-1b	AMP, CHL, NEO, SMX	HI1	not tested	105	unknown	
diagnostic	74-04083-1	A1	not rel.	AMP, NEO, STR, SMX, TET, CEF	TF (DH10B)	*bla* TEM-1b	AMP, AMC, NEO	NT	not tested	2 plasmids (180 & 30)	Tn*2*	
diagnostic	74-11738-1	D1	not rel.	AMP, APR, CIP, GEN, NAL, NEO, STR, SMX, TET, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP, NEO,SMX, TET	NT	not tested	105	IS*26*a (2 kb)	
diagnostic	74-11649-1	A1	not rel.	AMP, APR, CHL, CIP, GEN, NAL,SPT, STR, SMX, TET	TF (DH10B)	*bla* TEM-1b	AMP	NT	not tested	50	Tn*2*	
diagnostic	74-11493-1	A1	not rel.	AMP, STR, SMX, TMP, [CEF]	TF (DH10B)	*bla* TEM-1b	AMP, SMX, TMP	NT	not tested	50	unknown	
faecal commensal	74-30181-3	D2	not rel.	AMP, NEO, SPE, STR, SMX, TET, TMP	TF (DH10B)	*bla* TEM-1b	AMP	NT	not tested	10	Tn*3*	
faecal commensal	74-30182-1	D2	not rel.	AMP, CHL, SPT, STR, SMX, [CEF]	TF (DH10B)	*bla* TEM-1a	AMP	NT	not tested	10	Tn*3*	

Table S3 c. Summary of results for *E. coli* from poultry (Manuscript II).

Reservoir	POULTRY												
Species	*E. coli*												
wild type (WT)	Strain number	WT phylotype	PFGE relatedness of selected WT strains	WT Resistances	Type of recipient in which the plasmid was characterized	*bla* TEM allele	Resistances associated with blaTEM plasmid	Replicon (s) detected	RFLP (pMLST or RST types if available)	Plasmid size [kb]	Putative element upstrem the blaTEM gene	Selftrasmissibility
faecal commensal	7430125-1	A1	not rel.	AMP	TF (GeneHogs)	*bla* TEM-1b	AMP	IncI1	t	82	Tn*2*	
faecal commensal	7430287-1	A2	not rel.	AMP	TC (K12HEHA4)	*bla* TEM-1b	AMP	IncI1	t	90	Tn*2*	
faecal commensal	7430237-1	A2	not rel.	AMP	TF (GeneHogs)	*bla* TEM-1b	AMP	IncI1	t	85	Tn*2*	
faecal commensal	7430521-1	B1	not rel.	AMP, [CEF]	TC (MT101)	*bla* TEM-1b	AMP	IncI1	t_1 (1-2 bands difference)	85	Tn*2*	
faecal commensal	7430557-1	A1	not rel.	AMP	TF (GeneHogs)	*bla* TEM-1b	AMP	IncI1	t	85	Tn*2*	
faecal commensal	7430186-1	A1	not rel.	AMP	TF (GeneHogs)	*bla* TEM-1b	AMP	IncI1	t	82	Tn*2*	
faecal commensal	7430284-1	A2	not rel.	AMP, SMX	TC (K12HEHA4)	*bla* TEM-1b	AMP, SMX	IncI1	u_1	104	Tn*2*	
faecal commensal	7430605-1	A1	not rel.	AMP	plasmid DNA from S1-PI	*bla* TEM-1b	Not applicable	IncI1	Not applicable	85	Tn*2*	
faecal commensal	7430621-1	D	not rel.	AMP, CEF	TF (GeneHogs)	*bla* TEM-1b	AMP	IncI1	β	82	Tn*2*	
faecal commensal	7430641-1	B1	not rel.	AMP, [CEF]	TF (GeneHogs)	*bla* TEM-1b	AMP	IncI1	Not readible, possibly similar to α	85	Tn*2*	
diagnostic	7365811-1	A2	not rel.	AMP, SMX, STR, SXT, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, SXT, TET, TMP	IncI1+ IncP	θ	125	Tn*2*	
diagnostic	7275882-1	A2	not rel.	AMP, AMC, SMX, TET	TC (K12HEHA4)	*bla* TEM-1b	AMP, AMC	IncI1	Not readible	85	Tn*2*	
diagnostic	7370817-2	B1	not rel.	AMP, SMX, TET	TF (GeneHogs)	*bla* TEM-1b	AMP	IncI1	t_1 (1-2 bands difference)	85	Tn*2*	
faecal commensal	7430256-1	D	not rel.	AMP, TET	TF (GeneHogs)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	poul1	138	Tn*2*	
diagnostic	7276769-1	D	Xba_7	AMP, TET	TF (GeneHogs)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	poul2	135	Tn*2*	
diagnostic	7277386-1	D	Xba_7	AMP, [STR], TET	plasmid DNA from S1-PI	*bla* TEM-1c	not applicable	IncFII, IncFIB	not applicable; likely poul2]	138	Tn*2*	
diagnostic	7370940-1	A2	not rel.	AMP, CHL, SMX, STR, SXT, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, CHL, SMX, SXT, TET, TMP	IncFII, IncFIB	poull3	138	Tn*1*	
diagnostic	7285398-1	D	Xba_8	AMP, SMX, STR, SXT, TET, TMP	TC (K12HEHA4)	*bla* TEM-1b	AMP, SMX, SXT, TET, TMP	IncFII,IncFIB, IncP	poul4	138	IS*26* a (1.3 kb)	yes
diagnostic	7279663-1	D	Xba_8	AMP, SMX, STR, SXT, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, SXT, TET, TMP	IncFII,IncFIB, IncP	poul4	130	IS*26* a (1.3 kb)	
diagnostic	7370888-1	B2	not rel.	AMP, CHL, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, CHL, SMX, TET, TMP	IncFII,IncFIB, IncP	Not readible	138	IS*26* a (1.3 kb)	
diagnostic	7372121-1	B2	Xba_9	AMP, NEO, SMX, SPT, STR, TET	TC (K12HEHA4)	*bla* TEM-1b	AMP, $(NEO)^{REC}$, SMX, TET	IncN	poul5 ST3	45	Tn*2*	yes
diagnostic	7373304-1	B2	Xba_9	AMP, SMX, [STR], SXT, TET, TMP	TC (K12HEHA4)	*bla* TEM-1b	AMP, SMX, SXT, TET, TMP	IncN	poul5 ST3	45	Tn*2*	yes
faecal commensal	7430280-1	A2	not rel.	AMP	TF (GeneHogs)	*bla* TEM-1b	AMP	NT	poul6	130	Tn*2*	
diagnostic	7278662-1	not tested	not rel.	AMP, CIP, NAL, SUL, TET	No plasmid detected in W	*bla* TEM-1b	not applicable	not applicable	Not applicable	not detected	Tn*2*	

recipient HEHA4 was neo resistant

Table S3 d. Summary of results for *E. coli* from humans- faecal commensal isolates (Manuscript II).

Reservoir	HUMAN										
Species	*E. coli*										
wild type (WT)	Strain number	WT phylotype	PFGE relatedness of selected WT strains	WT Resistances	Type of recipient in which the plasmid was characterized	*bla* TEM allele	Resistances associated with *bla* TEM plasmid	Replicon (s)	RFLP (pMLST types if availal	Plasmid size [kb]	Putative element upstrem the *bla* TEM gene
faecal commensal	433-CO2	A	ALL NOT RELATED	AMP	TF (GeneHogs)	*bla* TEM-1a	AMP	IncB/O	p_2	95	Tn3
faecal commensal	1231-CO3	B2		AMP	TF (GeneHogs)	*bla* TEM-1b	AMP	IncB/O	n	85	Tn*2*
faecal commensal	97-CO2	B2		AMP, SMX, STR	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX	IncB/O	m_2	95	Tn*2*
faecal commensal	457-CO2	B2		AMP, NEO, SMX, STR, TET	TF (GeneHogs)	*bla* TEM-1b	AMP,NEO, SMX, TET	IncB/O, IncP	p	110	IS*26*a (2 kb) & IS*26*b (0.6 kb)
faecal commensal	1084-CO3	B1		AMP, STR, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, TET	IncK	RFLP _inck	66	Tn*2*
faecal commensal	438-CO2	B2		AMP, SMX, STR	TF (GeneHogs)	*bla* TEM-1c	AMP, SMX	IncB/O	m	88	Tn*2*
faecal commensal	1082-CO3	B2		AMP, SMX, STR	TF (GeneHogs)	*bla* TEM-1c	AMP, SMX	IncB/O	m	92	Tn*2*
faecal commensal	1297-CO3	NT		AMP, CHL, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1g	AMP, SMX	IncB/O	m	70	Tn*2*
faecal commensal	875-CO3	D		AMP, AMC, SMX, STR, TMP	TF (GeneHogs)	*bla* TEM-30	AMP, AMC, SMX	IncB/O	m	80	Tn*2*
faecal commensal	1307-CO3	B2		AMP, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TMP	IncFII	h	70	IS*26*a (1.3 kb)
faecal commensal	855-CO3	B2		AMP, AMC, CIP, NAL	TF (GeneHogs)	*bla* TEM-1b	AMP, (AMC)	IncFII	k	60	Tn*2*
faecal commensal	550-CO2	B2		AMP, AMC, SMX, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, (AMC), (SMX)	IncFII, IncFIA, IncFIB	not tested	130	Tn*2*
faecal commensal	604-CO2	A		AMP, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET, TMP	IncFII, IncFIB, IncP	not tested	90	IS*26*a (2 kb)
faecal commensal	955-CO3	D		AMP, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, TET	IncFII, IncFIB	not tested	155	IS*26b* (1.2 kb)
faecal commensal	785-CO3	D		AMP, CHL, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET, TMP, CHL	IncFII, IncFIB	not tested	175	Tn*2*
faecal commensal	190-CO2	B2		AMP, TET	TF (GeneHogs)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	not tested	130	Tn*2*
faecal commensal	982-CO3	B2		AMP, TET	TF (GeneHogs)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	not tested	135	Tn*2*
faecal commensal	904-CO3	B1		AMP, AMC, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1 j	AMP, (AMC), SMX, TET, TMP	IncFII, IncFIB	not tested	150	Tn*2*
faecal commensal	862-CO3	D		AMP, AMC	TF (GeneHogs)	*bla* TEM-1d	AMP, (AMC)	IncFII, IncFIB	not tested	85	Tn*1*
faecal commensal	257-CO2	D		AMP, AMC, SMX, STR, TMP	TF (GeneHogs)	*bla* TEM-40	AMP, AMC, SMX, TMP	IncFIB	not tested	75	IS*26b* (1 kb)
faecal commensal	545-CO2	D		AMP, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1c	AMP, SMX, TET, TMP	IncI1	w_1	105	Tn*2*
faecal commensal	1341-CO3	NT		AMP	TF (GeneHogs)	*bla* TEM-1b	AMP	IncI1	t	85	Tn*2*
faecal commensal	1033-CO3	NT		AMP, SMX	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX	IncI1	u	100	Tn*2*
faecal commensal	1112-CO3	A		AMP, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-135	AMP, SMX, TMP	IncI1	u_2	104	Tn*2*
faecal commensal	1336-CO3	A		AMP, AMC, SMX, STR	TF (GeneHogs)	*bla* TEM-1b	AMP, (AMC)	NT	not tested	180	Tn*2*
faecal commensal	1308-CO3	B2		AMP, AMC, SMX, STR, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, (AMC), SMX, TET, TMP	IncN	not tested/ ST6	58	Tn*2*
faecal commensal	1413-CO3	NT		AMP, NEO, SMX, STR, TET	TF (GeneHogs)	*bla* TEM-1a	AMP	IncX1A	not tested	45	Tn*3*
faecal commensal	1437-CO3	NT		AMP, CHL, SMX, STR, TET	Not applicable; no pl. detected in WT	*bla* TEM-1b	not applicable	not applicable	not applicable	not applicable	IS*26*a (2 kb)
faecal commensal	924-CO3	B1		AMP, SMX, STR	TF (GeneHogs)	*bla* TEM-1b	AMP	NT	not tested	35	Tn*2*
faecal commensal	399-CO2	B2		AMP	TF (GeneHogs)	*bla* TEM-1c	AMP	NT	not tested	40	Tn*2*
faecal commensal	564-CO2	B2		AMP, SMX, SPE, STR, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX	NT	not tested	50	Tn*2*

Table S3 e. Summary of results for *E. coli* from humans clinical isolates (Manuscript II).

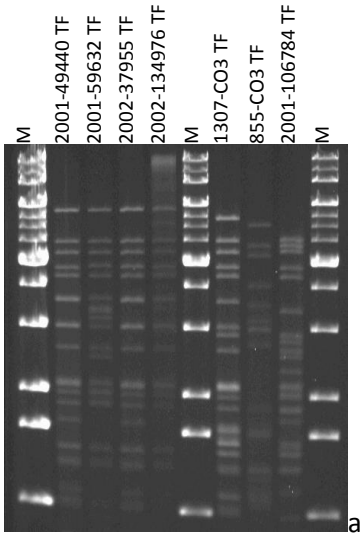
Reservoir	HUMAN										
Species	*E. coli*										
wild type (WT)	Strain number	WT phylotype	WT PFGE relatedness of WT strains	WT Resistances	Type of recipient in which the plasmid was characterized	*bla* TEM allele	Resistances associated with blaTEM plasmid	Replicon (s)	RFLP (pMLST types if available) Profiles designations correspond to the symbols used in Tables 3-5 in the manuscript	Plasmid size [kb]	Putative element upstrem the *bla* TEM gene product size is specified in kb)
clinical	2001-37255	A	not rel.	AMP, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET, TMP	IncB/O, IncP	p	125	IS*26*a (2 kb) & IS*26*b (0.6 kb)
clinical	2001-131351	A	not rel.	AMP, STR, SMX, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET	IncB/O, IncP	p	110	IS*26*a (2 kb) & IS*26*b (0.6 kb)
clinical	2001-25022	D	not rel.	AMP, STR, SMX	TF (GeneHogs)	*bla* TEM-1c	AMP, SMX	IncB/O	m_4	90	Tn*2*
clinical	2002-50	D	not rel.	AMP, SPT, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1c	AMP, SMX, SPT	IncB/O	m	80	Tn*2*
clinical	2002-14946	B2	Xba_ possibly rel.10	AMP, CIP, NAL, STR, SMX	TF (GeneHogs)	*bla* TEM-1c	AMP, SMX	IncB/O	m_2	80	Tn*2*
clinical	2002-110474	B2	not rel.	AMP, STR, SMX	TF (GeneHogs)	*bla* TEM-1c	AMP, SMX	IncB/O	m_1	70	Tn*2*
clinical	2000-37030	A	not rel.	AMC, AMP, NEO, STR, SMX, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TMP, NEO	IncFII, IncFIB	not tested	142	IS*26*a (2 kb)
clinical	2000-128256	B2	Xba_possibly rel.11	AMP, STR, SMX, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX	IncFII, IncFIB	not tested	145	IS*26*b (0.6 kb)
clinical	2002-61144	B2	Xba_possibly rel.11	AMC, AMP, CIP, STR, SMX, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX	IncFII, IncFIB	not tested	153	IS*26*b (0.6 kb)
clinical	2002-121167	D	not rel.	AMC, AMP, STR, SMX	TF (GeneHogs)	*bla* TEM-1b	AMP, AMC(I), SMX	IncFII, IncFIB	not tested	155	IS*26*a (2 kb)
clinical	2000-2592	A	not rel.	AMP, AMC, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, AMC, SMX, TET, TMP	IncFII, IncFIB	not tested	110	Tn*2*
clinical	2000-90435	B2	not rel.	AMP, TET	TF (GeneHogs)	*bla* TEM-1b	AMP, TET	IncFII, IncFIB	not tested	112	Tn*2*
clinical	2002-94787	B2	not rel.	AMC, AMP, TET	TF (GeneHogs)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	not tested	125	Tn*2*
clinical	2001-93504	A	not rel.	AMP, TET	TF (GeneHogs)	*bla* TEM-1c	AMP, TET	IncFII, IncFIB	not tested	130	Tn*2*
clinical	2001-118896	B2	not rel.	AMP, SPT, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1a	AMP, SMX, TET, TMP, SPT	IncFII, IncFIB, IncP	not tested	145	Tn*3*
clinical	2001-1662	B2	not rel.	AMC, AMP, CHL, TET, TMP	TF (GeneHogs)	*bla* TEM-1d	AMP, TMP, CHL, TET	IncFII, IncFIB, IncFIA	not tested	92	unknown
clinical	2002-48269	D	not rel.	AMC, AMP, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, AMC, SMX, TET, TMP	IncFIB, IncY	not tested	225	IS*26*a (2 kb)
clinical	2001-49440	D	not rel.	AMP, CIP, NAL, STR, SMX, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TMP	IncFII	g ST FII2	64	IS*26*a (1.3 kb)
clinical	2001-59632	B2	not rel.	AMP, CIP, GEN, STR, SMX	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, (GEN)	IncFII	i	70	IS*26*a (1.3 kb)
clinical	2001-106784	B2	not rel.	AMP	TF (GeneHogs)	*bla* TEM-1a	AMP	IncFII	j	70	Tn*3*
clinical	2002-37995	D	not rel.	AMC, AMP, STR, SMX, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP,SMX, TMP	IncFII	g	65	IS*26*a (1.3 kb)
clinical	2002-134976	B2	not rel.	AMP, STR, SMX	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX	IncFII	g _1 ST FII2	60	IS*26*a (1.3 kb)
clinical	2002-70903	non-typeable	not rel.	AMP, STR, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1b	AMP, SMX, TET, TMP	IncI1, IncP	w ST 97-related [A]	120	IS*26*a (2 kb) & IS*26*b (0.6 kb)
clinical	2000-14183	D	not rel.	AMC, AMP, APR, GEN, SPE, STR, SMX	TF (GeneHogs)	*bla* TEM-1a	AMP, AMC, (APR), (SPT)	[ColE1* -likely the small pl.]	not tested	possibly 3 pl. (125 kb, 20 kb; 4 kb)	Tn*3*
clinical	2001-71294	D	not rel.	AMC, AMP, CHL, SPE, STR, SMX	TF (GeneHogs)	*bla* TEM-1b	AMP, (AMC), (SMX)	[ColE1* -likely the small pl.]	not tested	multiple small pl. (20-30 kb 15kb, 10kb)	Tn*2*
clinical	2000-103495	D	not rel.	AMC, AMP, GEN, STR	TF (GeneHogs)	*bla* TEM-1c/1d	AMP, AMC, GEN	NT	not tested	82	Tn*3*
clinical	2001-13567	D	not rel.	AMC, AMP, SMX, TMP	TF (GeneHogs)	*bla* TEM-1a	(AMC), AMP, SMX	NT	not tested	possibly 3 pl. (150 kb; 30 kb; 9 kb)	Tn*3*
clinical	2000-27668	B2	Xba_ possibly rel.10	AMC, AMP, SMX, TET, TMP	TF (GeneHogs)	*bla* TEM-1c	AMP, (AMC), SMX	NT	not tested	possibly 3 pl. (150 kb, 20-30 kb; 8 kb)	Tn*2*
clinical	2000-49043	B2	not rel.	AMP, STR, SMX	TF (GeneHogs)	*bla* TEM-1c	AMP	NT	not tested	possibly 3 pl.(180 kb, 30 kb; 8 kb)	Tn*2*
clinical	2000-78651	B2	not rel.	AMC, AMP	TF (GeneHogs)	*bla* TEM-1c	(AMC), AMP	NT	not tested	possibly 3 pl. (90 kb, 30 kb; 9 kb)	Tn*2*
clinical	2002-27242	B2	not rel.	AMP, CHL, STR, SMX, TET	TF (GeneHogs)	*bla* TEM-1b	*bla* TEM-1 non transferable	not applicable	not applicable	not applicable	[WT tested - IS*26*a (0.4 kb)]

[A] one nucleotide difference for trbA16 locus

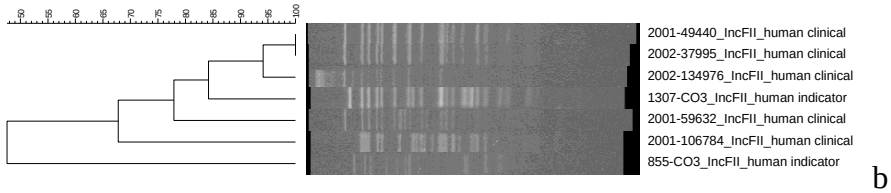
* ColE1 PCR (Garcia –Fernandez *et al*. 2009) was performed additionally on some of the non-typable transformants; however, the TFs that produced positive signals were suspected to harbour more than one plasmids and therefore further verification is needed to determine which of the plasmid harbours the $bla_{\text{TEM-1}}$ gene and which of the plasmids is actually the ColE1 type.

S4. Supplementary data for RFLP

RFLP analysis of IncFII plasmids from humans, cattle and pigs.



a



b

Figures 1 a and b. The HincII digestions of plasmids harbouring no other than IncFII replicons from human isolates (a) and analysis with BioNumerics software (b). M stands for 1 kb extended DNA ladder (Invitrogen).

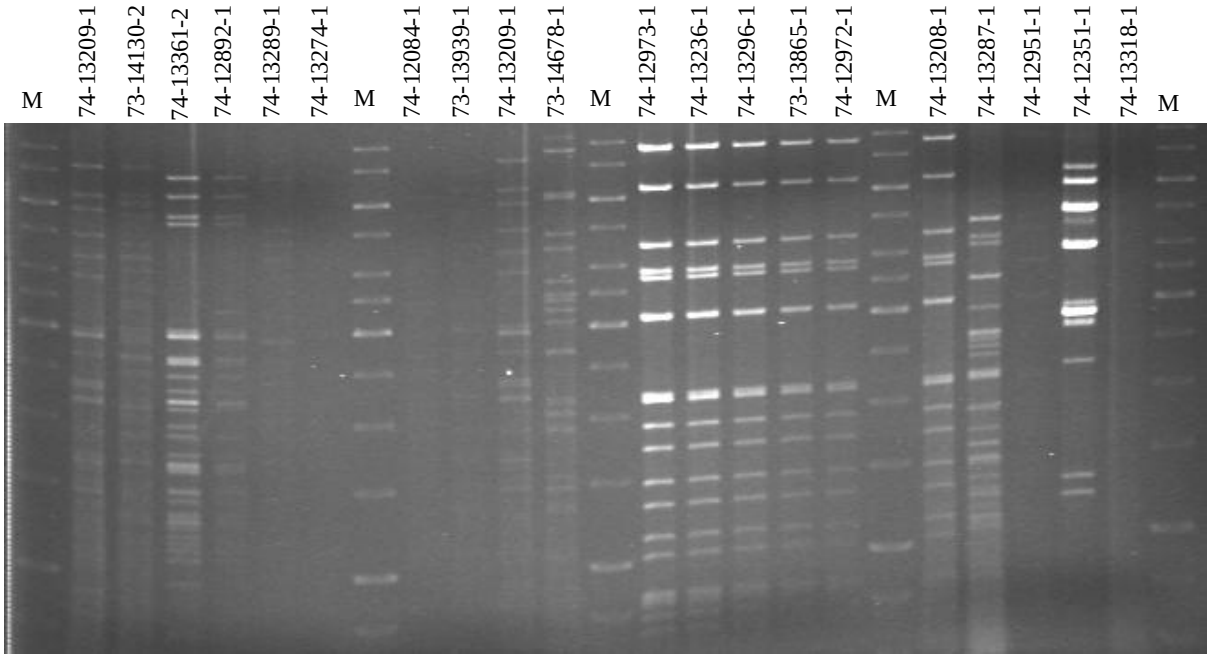


Figure 2. HincII digestions of selected IncF-family plasmids from cattle isolates. M, 1 kb extended DNA ladder (Invitrogen).

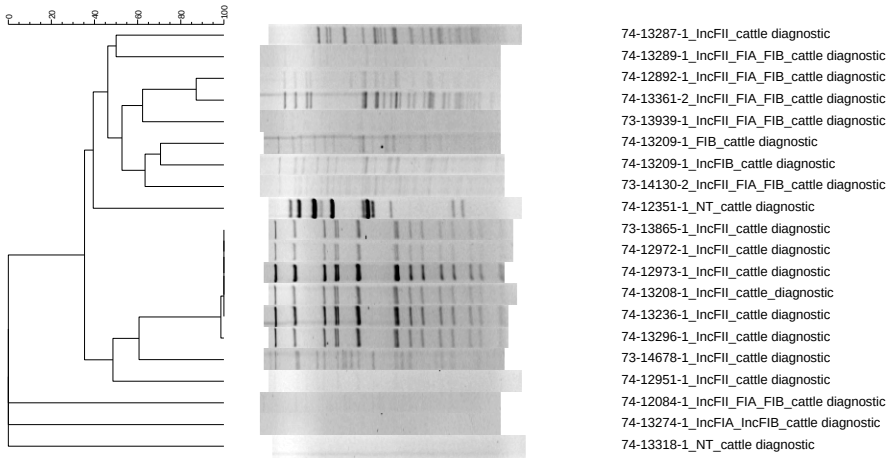


Figure 2 a . Analyses of HincII digestions of selected IncF-family plasmids from cattle isolates with the use of BioNumerics.

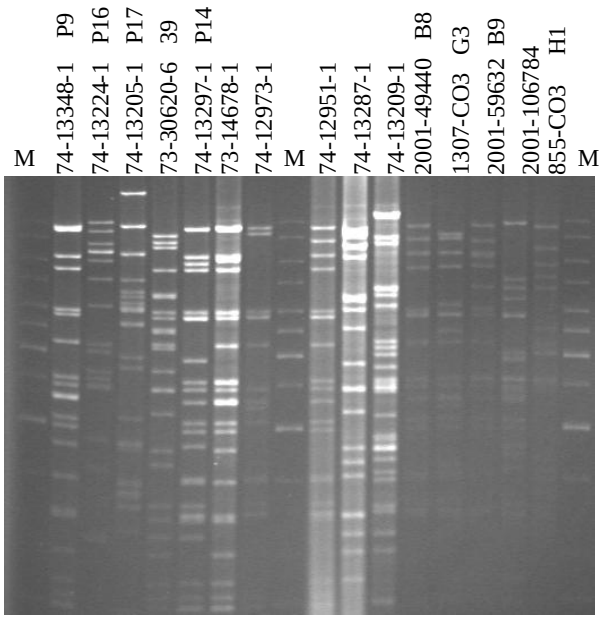


Figure 3. EcoRV digestions od IncFII plasmids from pigs and representative IncFII plasmids from humans and cattle. IncFIB plasmid from 74-13209-1TF (cattle) was included to test the DNA extraction; M, 1 kb extended DNA ladder (Invitrogen).

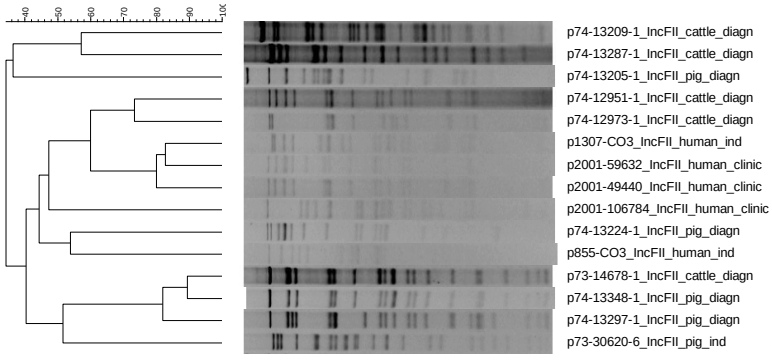


Figure 3a . Analysis of the RFLP profiles from Figure 6 with the use of BioNumerics.

HincII digestions of selected plasmids with multiple IncF-type replicons

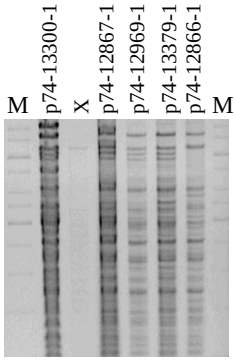
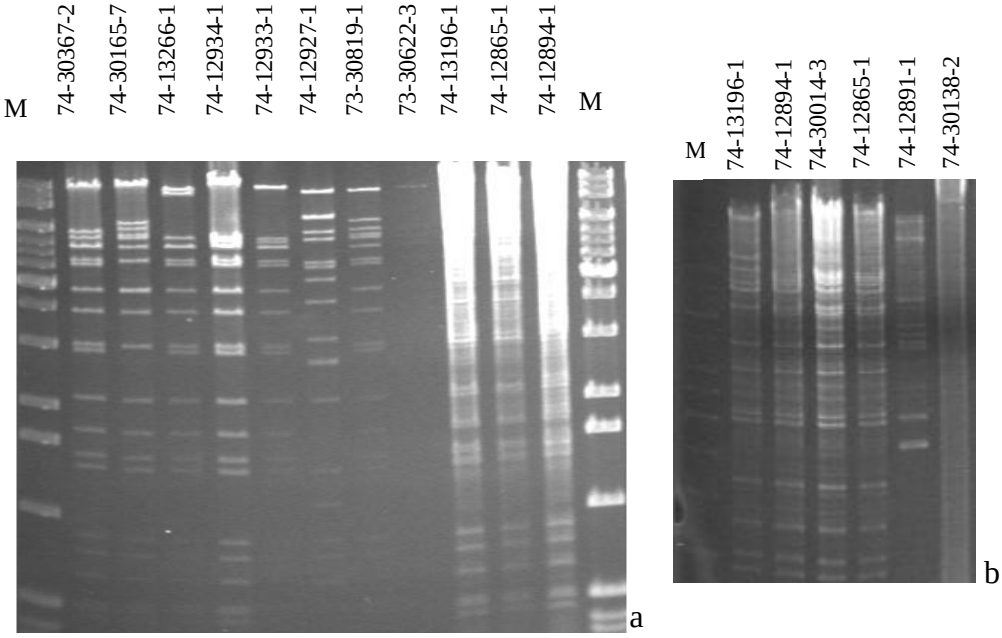


Figure 4. HincII digestions of IncFII& IncFIB plasmids from cattle; plasmid p74-13300-1 harboured also IncP replicon; x- lane not analysed; M, 1kb extended ladder (Invitrogen)

RFLP analysis of plasmids with IncB/O replicons, IncB/O & IncP and IncK from humans, pigs and cattle.



Figures 5 a and b. BamHI digestions of the IncB/O and IncB/O & IncP $bla_{\text{TEM-1}}$ plasmids from selected pig isolates and cattle isolates; M stands for 1 kb extended DNA ladder (Invitrogen).

The fingerprints of the IncB/O plasmid p74-13196-1, IncB/O & IncP plasmids p74-30014-3, p74-12865-1 and p74-12894-1 have been additionaly digested with HincII enzyme (Figure 5c below).

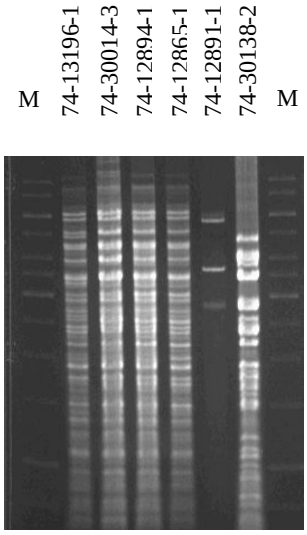


Figure 5 c. HincII digestion of IncB/O and IncP plasmids isolated from cattle.

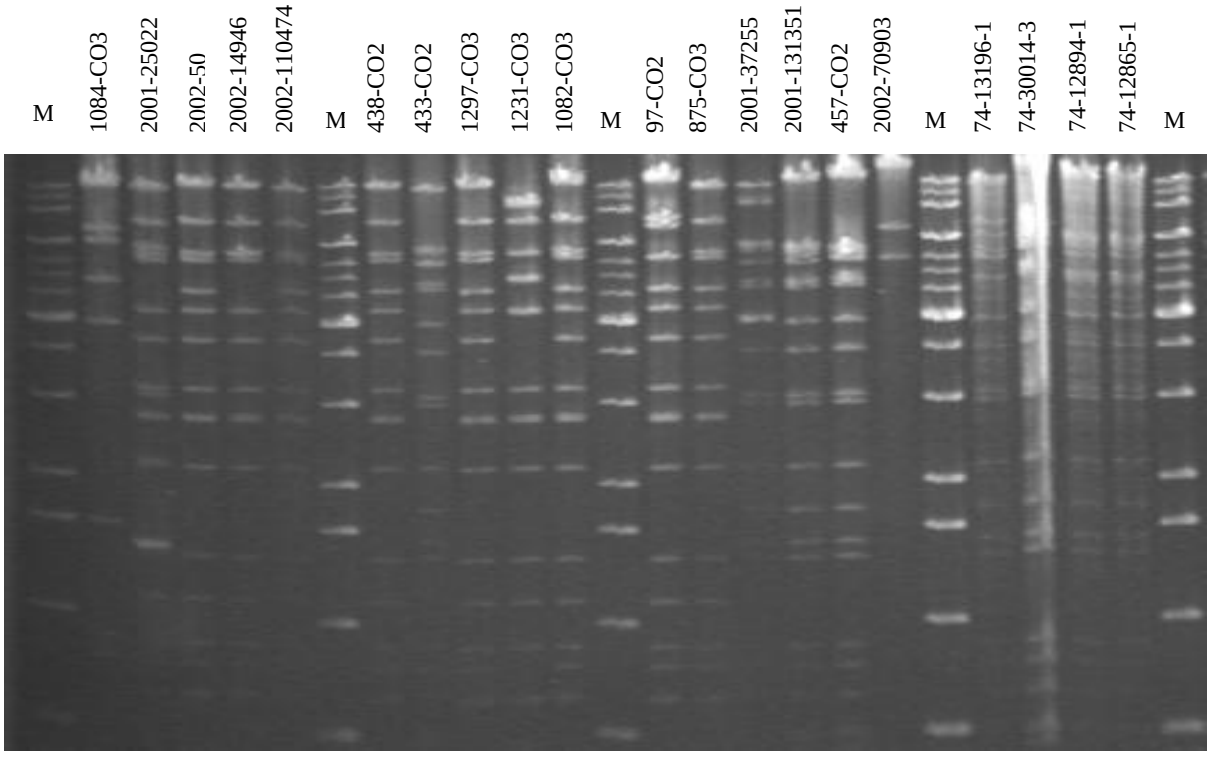


Figure 6.BamHI digestion of selected IncB/O, IncB/O & IncP and IncK plasmids from humans and from cattle; M stands for 1 kb extended DNA ladder (Invitrogen). Plasmid p2002-70903 (IncI1 & IncP) was also included in the analysis due to the presence of IncP replicon.

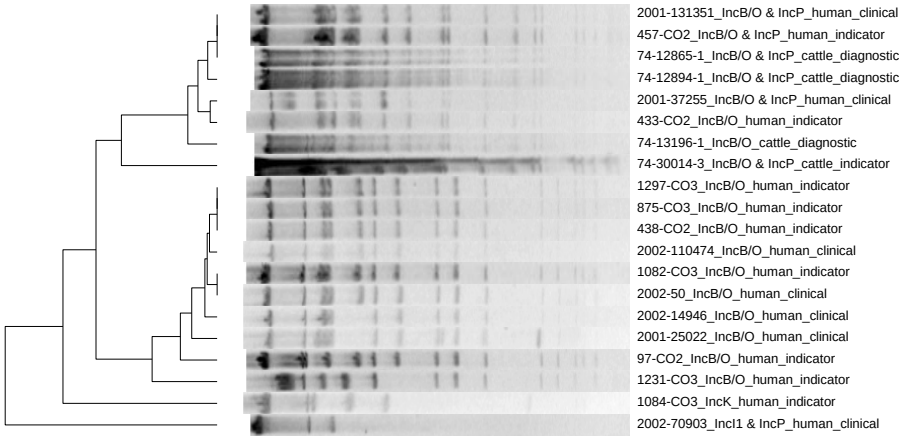


Figure 6a. Analysis of the RFLP fingerprints from Figure 2 with the use of BioNumerics software.

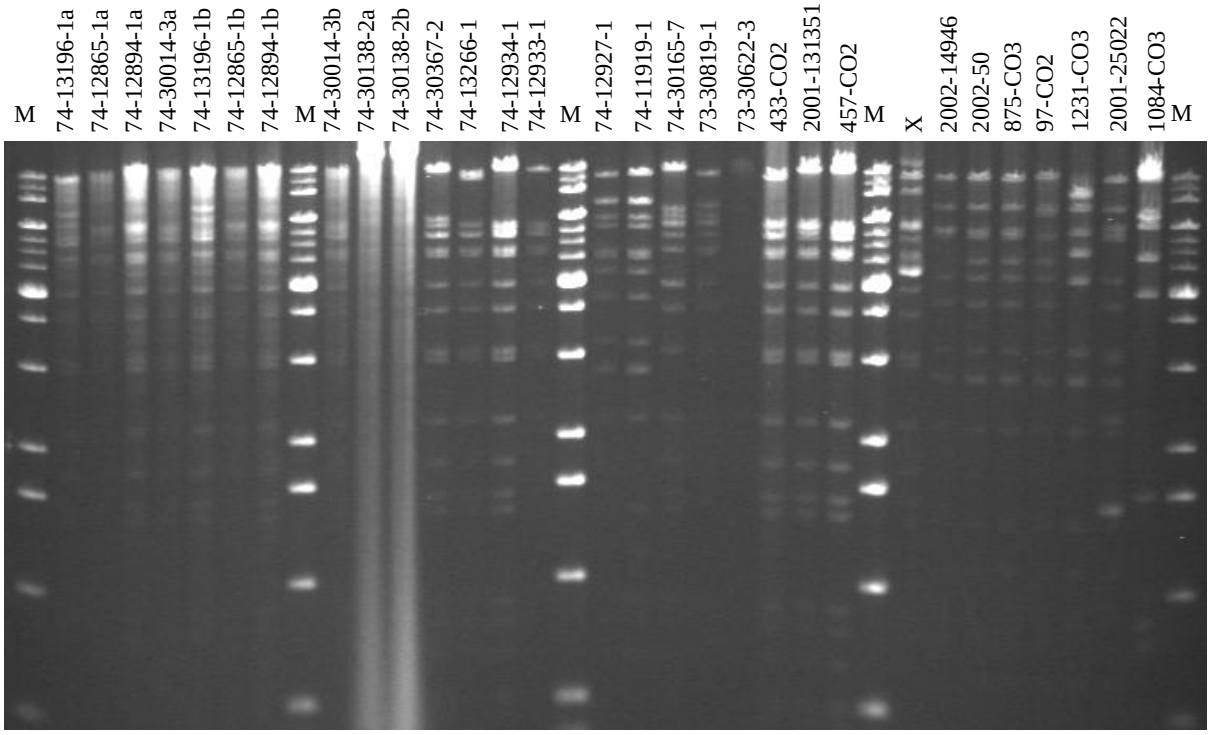


Figure 7. BamHI digestion of selected IncB/O, IncB/O & IncP and IncK plasmids from cattle, pigs and humans. Two different volumes of DNA purifications from the same plasmids from cattle were digested (indicated with 'a' and 'b' next to the strains names), X referes to the mistake at this position and this band was not included in the analysis; M stands for 1 kb extended DNA ladder (Invitrogen).

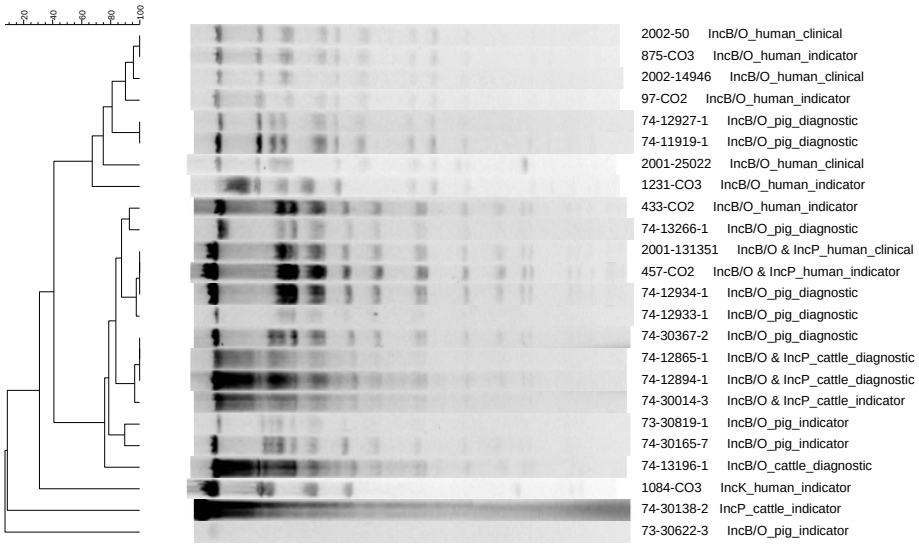


Figure 7a. Analysis of the RFLP fingerprints from the figure 3 with the use of BioNumerics.

<u>HincII and EcoRV digestions of IncI1 plasmids.</u>

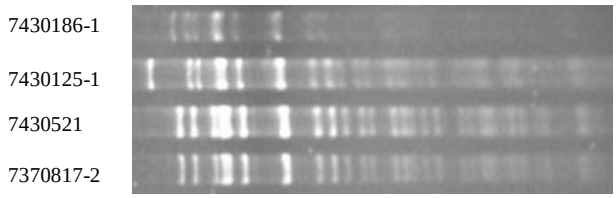


Figure 8. HincII digestion of selected IncI1 plasmids from poultry. Plasmids p7370817-2, p7430521 produced undistinguishable RFLP profiles and p7430125-1 seemed to be closely related to these two (2 bands difference).

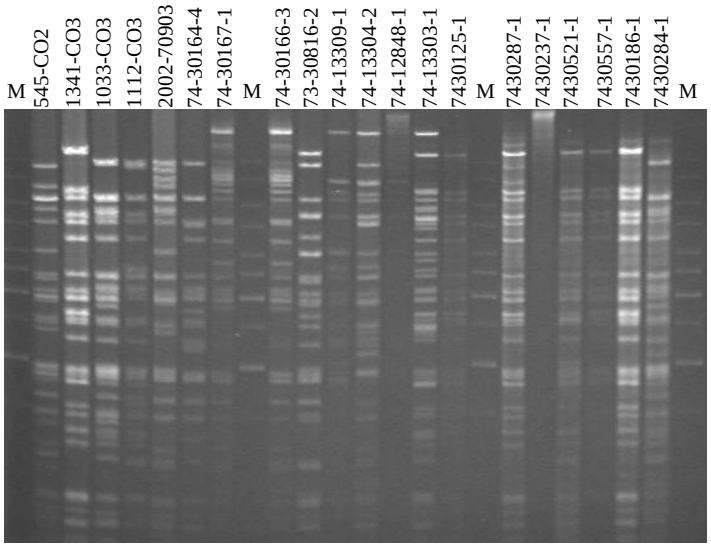


Figure 9. EcoRV digestions of selected IncI1 and IncI1 toghether with IncP plasmids.

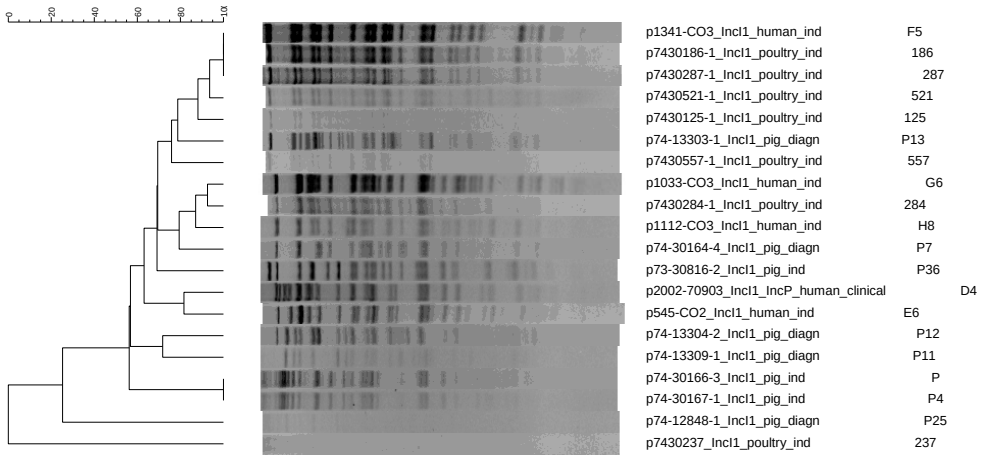


Figure 9a. Analysis of EcoRV digestions of selected IncI1 and IncI1 toghether with IncP plasmids from Figure 7a in BioNumerics.

MANUSCRIPT III

Typing of plasmids from *Klebsiella pneumoniae* from human infections and from the environment with a novel multiplex PCR

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Typing of plasmids from *Klebsiella pneumoniae* from human infections and from the envronment with a novel multiplex PCR.

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Running title: Novel mPCR for typing of plasmids from *K. pneumoniae*

Key words: replicases, clinical *vs* environmental isolates

Abstract

Klebsiella pneumoniae is a common opportunistic pathogen of humans. Plasmids found in this species can be platforms harbouring genes encoding resistance to antimicrobials and virulence factors. The objective was to compare the diversity of plasmids found in *K. pneumoniae* from human infections and from the environment. Novel multiplex PCR (mPCR) was designed in order to detect broader spectrum of plasmids that are currently non-typable (NT) by the available PCR-based replicon typing methods (PBRT). Sequenced replicons of plasmids from *K. pneumoniae* available in public databases were first collected and aligned with each other. Based on these alignments the *rep* genes (encoding replicases) were grouped according to the similarities; eight homology groups (designated with numbers from repI to repVIII) were defined for these genes. The mPCR was designed to target seven of these groups (from repI to repVII). Group repVIII comprised of the IncN replicases targeted by the PBRT.

Plasmids from a collection of *K. pneumoniae* bacteraemia (n=20), urinary tract infections (n=20) and surface waters (n=10) isolates were then typed by means of the novel mPCR and by the PBRT methods. N=67 individual plasmids (≥30 kb) were retrieved and typed. Replicases from repI, -III and -IV groups were most often observed on the plasmids from the tested collection. Often the classical IncFII, $IncFII_k$, IncR replicons and also NT - replicons were found on the plasmids with the diverse replicases belonging to repI-VII groups. Differences in distribution of replicases were observed between plasmids originating from the human and environmental reservoirs. A higher ratio of repIII, repIV and NT plasmids was found in the blood-stream isolates; repIV and repV appeared to be characteristic for plasmids from the environmental isolates. Moreover, the repIV-type replicase may constitute a novel replicon related to the classical IncFIB and IncFIA.

The novel mPCR method proved to be an efficient tool for replicase typing and also to deliver accessory information about the replicons when applied together with the standard PBRT methods.

1. Introduction

Klebsiella pneumoniae is a member of the family *Enterobacteriaceae*. It is commonly found in natural environments but may also cause infections typically in the immune-compromised individuals (Stahlhut et al., 2009). Among others urinary and respiratory tract infections and also life threatening infections like liver abscess, meningitis and sepsis are reported in humans (Chen et al., 2004; Chen et al., 2006; Chen et al., 2007b; Gootz et al., 2009; Jiang et al., 2010; Leavitt et al., 2010; Soler Bistue et al., 2008; Wu et al., 2009; Zhao et al., 2010). Plasmids often contribute to virulence of *K. pneumoniae* by encoding a range of virulence factors, most common examples being iron sequestering systems, adhesins and phospholipases (Chen et al., 2004; Gootz et al., 2009; Wu et al., 2009). Moreover, many of the plasmids found in *K. pneumoniae* harbours genes encoding resistances to antimicrobials and also to heavy metals which increases the capabilities of this bacterium to survive in the diverse niches (Chen et al., 2004; Chen et al., 2006; Gootz et al., 2009; Jiang et al., 2010; Zhao et al., 2010). Studying the epidemiological relationships of the *K. pneumoniae* plasmids is therefore of high importance in order to obtain more detailed overview of the mobile genetic elements driving the transmission of the resistance and virulence genes in this species.

Plasmids tend to have modular structures and the modules encoding the replication and the control of replication functions are together termed replicons (Carattoli et al., 2005). Replicon is an indispensable part of plasmid backbone therefore it constitutes a good target for epidemiological studies (Carattoli et al., 2005). It has been shown that two plasmids sharing similar replicons often could not be stably maintained in the same cells without the external selection against them. Such replicons were designated as incompatible and classified to the same incompatibility (Inc) groups (Carattoli et al., 2005). Currently there are more than 27 Inc groups recognized in *Enterobacteriaceae*. PCR-based replicon typing (PBRT) methods are available for rapid detection and classification of plasmids representing the most commonly encountered Inc groups (Carattoli et al., 2005; Garcia-Fernandez et al., 2009; Villa et al., 2010). The PBRT targets the key incompatibility determinants found on plasmids. These targets are i) the replication control elements encoding antisense RNAs found on IncFII, IncFII variants (FII_k, $_{-s\ -y}$), IncI1, IncB/O and IncK replicons ii) iterons (IncFIA, IncP, IncHI2) iii) genes encoding partition functions (IncHI1) iv) origins of replication (IncX2) and v) the *rep* genes encoding replicases (IncL/M, IncN, IncFIB, IncFIC, IncA/C, IncT, IncW,

IncY, IncR) (Carattoli et al., 2005; Garcia-Fernandez et al., 2009; Praszkier et al., 1991; Villa et al., 2010).

In previous studies mainly the IncFII related replicons (RepFIIA) and also IncA/C, IncN, IncL/M and IncR replions were detected on the diverse resistance plasmids from *K. pneumoniae* (Andrade et al., 2011; Athanasopoulos et al., 1995; Carattoli et al., 2010; Ktari et al., 2011; Mataseje et al., 2011; Zhu et al., 2009). We noticed that the previously sequenced strain of *K. pneumoniae* MGH78578 harboured two $IncFII_k$ plasmids (Villa et al., 2010). However, these plasmids encoded different secondary replication initiator proteins (replicases). These additional replicons possibly enabled the two plasmids to overcome the incompatibility phenomenon. We further noticed that many of the sequenced plasmids from *K. pneumoniae* deposited in GenBank share a similar *rep* sequence coding for a replicase that could not be assigned to any of the known incompatibility groups. This replicase is also found as a part of independent replicons on plasmids pCK41 from *Edwardsiella tarda* (HQ332785) and pSC138 from *Salmonella enterica* (AY509004; Chiu et al., 2005). Moreover, the latter replicons share similarities with the classical IncFIB and IncFIA replicons found on plasmids form *E. coli* (according to analysis performed in this study). Plasmid pKP187 from *K. pneumoniae* Kp342 harbours this IncFIA/FIB –like replicase as well as another replicon encoding the RepB2 initiator protein. None of these two replicons of pKP187 would be detected by the currently available PCR based methods for plasmid replicon typing (PBRT) (Carattoli et al., 2005; Villa et al., 2010).

As the PBRT targets diverse elements of the plasmid replicons we decided to design a multiplex PCR method (mPCR) targeting the diverse *rep* sequences of the plasmids from *K. pneumoniea*. Among others the secondary replicases of the aforementioned $IncFII_k$ plasmids from *K. pneumoniae* MGH78578 and the two replicases of pKP187 were included as targets in the mPCR. We observed that the majority of the fully sequenced plasmids smaller than 30 kb and originating from *K. pneumoniae* belonged to the promiscuous ColE1 family (Cao et al., 2002; Gootz et al., 2009; Riley et al., 2001; Sarno et al., 2002; Zhu et al., 2009; Zioga et al., 2009). Therefore the mPCR was designed mainly to detect replicases of the large plasmids.

The aim of this study was to access the diversity of plasmids in *K. pneumoniae* from human infections and from the environment not selected based on the resistance markers. This was done by typing of plasmids from a collection of previously published *K. pneumoniae* isolates from both human infections and from surface waters (Stahlhut et al., 2009) by the standard

PBRT and by the above described novel mPCR. The mPCR allowed for detection of diverse replicases found on plasmids that may simultaneously carry classical incompatibility determinants other than the replicase.

The *in silico* analysis in combination with the results of plasmid typing performed *in vitro* enabled us to draw conclusions whether the *K. pneumoniae* causing infections in humans harbour similar range of plasmids as the isolates from the natural environments. Additionally, we deliver the new mPCR method developed to detect and characterize plasmids which replicons would not be detected by means of the other so far available PBRT schemes.

2. Materials and methods

2.1 Designing of the mPCR

All work involving DNA sequence editing and analysis was performed using Vector NTI Suit 11 (Invitrogen, Inc.). Sequences of all known plasmid replicons (except the ColE1 replicons) from *K. pneumoniae* available in public databases (ACLAME, GenBank®) were collected. These sequences were aligned with each other. Based on these alignments eight homology groups were defined for the genes encoding a version of the replication initiation protein (*rep*A, -A2 -B, -B2 and –E etc.). These homology groups were designated as repI, -II, -III, -IV, -V, -VI, -VII, -VIII (Table 1). Additionally, primers available for replicon typing of plasmids belonging to currently recognized incompatibility groups in *Enterobacteriaceae* (Carattoli et al., 2005; Garcia-Fernandez et al., 2009; Villa et al., 2010) were blasted against the sequenced replicons retrieved from the public databases. This allowed us to determine *in silico* the presence of other classical incompatibility determinants on these sequenced plasmid replicons.

Replicases belonging to the six of the aforementioned groups (repI, -II, -III, -IV, -V, -VI) turned out not to be included as targets in any of the previously published PBRT PCRs. Although, a pattern was observed that the repI on the majority of sequenced plasmids corresponded the *rep*FII_k (on these replicons the *cop*B gene, not the replicase *rep*$AFII_k$ gene, is targeted by the PBRT), while repIII typically corresponded to the *rep*FIA (the iterons of IncFIA replicons are targeted by the PBRT).

As expected, some of the previously sequenced replicons or full plasmids with the diverse combinations of the replicases from repI-repVII groups also harboured the classical incompatibility determinants (Table 1). Namely two groups, repVII and repVIII corresponded to replicases of IncR and IncN type replicons, respectively. These two *rep* sequences, *rep*R

and the *rep*N are targeted by the standard PBRT(Carattoli et al., 2005; Garcia-Fernandez et al., 2009). The IncN family is reviewed elsewhere therefore these IncN plasmids were not included in the mPCR design (Table 1; Chen et al., 2007b; Garcia-Fernandez et al., 2011; Gootz et al., 2009). None of the previously published studies focused on the IncR plasmids. In order to learn more about the distribution of this group of replicons the repVII replicases have been also incorporated as targets into the mPCR. The position of the target for repVII PCR compared to the segment of the IncR replicon targeted by PBRT is indicated on the Figure 1 (generated with the use of sequences previously described by Osborn et al., 2000; Petty et al., 2010; Zhao and Dang, 2011).

The sequences of the replicases of repI –repVII (repVIII excluded) were BLASTed against GenBank database in order to identify similar sequences originating from hosts other than *K. pneumoniae* and which would be likely detected by the primers used in the mPCR. The following plasmids with repIII type sequences were retrieved: pHCM1/ IncHI1 from *S. enterica* (Parkhill et al., 2001), pEK499/ IncFII, -FIA from *E. coli* (Woodford et al., 2009), pRSB10/ IncFII, -FIB from an unspecified environmental species (Szczepanowski et al., 2005), pEC_L8/IncFII, -FIB and pEC_L46/ IncFII, -FIA, -N from *E. coli* (Smet et al., 2010) and pU302L/ IncFIA, -FIB from *S. enterica* (Chen et al., 2007a). The following plasmids from species other than *K. pneumoniae* and with repIV type replicases were retrieved: pSA1 from *S. enterica* (Mulec et al., 2002) and pECL_A EC from *Enterobacter cloacae* (Ren et al., 2010); and with repVII (IncR): pEFER from *Escherichia fergusonii* (CU928144), pLV1403 from *Pantoea agglomerans* (Osborn et al., 2000) and pK727 from *E. coli* (Bielak et al., 2011). These plasmids are also indicated in the Table 1.

For seven of the rep groups specific primer pairs were designed to be used in the corresponding seven simplex PCR reactions targeting respectively the repI, - II, -III, -IV, V, -VI and -VII of plasmids listed in the Table 1. Primer sequences, the *rep* targets on the reference plasmids and the expected PCR product sizes are listed in Table 2. Primers from the simplex PCRs were further combined and used in the mPCR.

Some of the replicases found on plasmids from *K. pneumoniae* could not be allocated to any of the eight groups Our *in silico* analysis indicated that other targets on plasmids with these remaining replicases can still be detected either by the standard PBRT or by PCR targeting the repIV-type sequences (Table 1).

2.3 Bacterial strains and positive controls

Three strains with previously sequenced plasmids were used as positive controls for the simplex and then for the mPCRs described in the preceding subsection. *E. coli* HB101 transconjugant with plasmid pMET1 kindly provided by Marcelo Tomalsky (California State University) was used as control for repII; pMET1 was originally isolated from *K. pneumoniae* strain from human infection (Soler Bistue et al., 2008). *K. pneumoniae* Kp342 with plasmids pKP91 and pKP187 was used as a control for repI, repIII, repIV and repV (Fouts et al., 2008). *K. pneumoniae* MGH78578 harbouring pKPN3, pKPN4 and pKPN5 was used as control for repI, repIV, repVI and repVII (Stahlhut et al., 2009). These two strains originated from a plant (Kp342) and from human infection (MGH78578), respectively. Associations of the repI-VII groups with the each of the individual reference plasmids are indicated in Table 1.

Plasmids from previously published collection of fifty *K. pneumoniae* strains (Stahlhut et al., 2009) were further typed by the novel mPCR and by the classical PBRT methods (Carattoli et al., 2005; Garcia-Fernandez et al., 2009; Villa et al., 2010). The strains were isolates from diverse reservoirs, namely natural surface waters (Germany, n=10), urinary tract infections (Denmark, n=19 and USA, n=1) and blood infections (Denmark, n=20). Strains were isolated in the period from 1992 to 2006 as described in (Stahlhut et al., 2009).

2.4 Verification of plasmids' sizes and number per strain

Presence and sizes of the large plasmids were verified for all strains by means of S1-PFGE (suitable primarily for detection of plasmids larger than 30 kb; Barton et al., 1995); the running conditions were as described previously by (Bielak et al., 2011).Presence of plasmids smaller than 30 kb was verified by extracting plasmidic DNA using QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. These plasmid purifications were subjected to electrophoresis at the following conditions: 0.8% agarose gel (SeaKem Agarose/Lonza) at 45V for 19 h. DNA was visualised by the standard ethydium bromide staining method.

2.5 Running conditions for the novel mPCR

The following conditions were applied both in the simplex PCR reactions targeting the repI-VII groups and for the mPCR: initialization step for 5 min. at 94°C followed by 25 cycles of i) denaturation for 15 s at 94 °C ii) annealing for 15 s at 55 °C iii) extension step for 60 s at 72 °C and final elongation step for 5 min. at 72 °C. DreamTaqTEM Green Polymerase Master Mix (Fermentas) was used according to manufacturer's instructions. Concentration of

each primer used per reaction was 0.5 µM in all of the seven simplex PCR reactions and then in the multiplex PCR set-up.

Results of the multiplex PCR were visualized on 1.8 % agarose gel (LA Agarose/ Lonza) after electrophoresis for minimum 1h at 130V and subsequent standard staining in ethydium bromide.

2.6 DNA templates

Boiled lysates from the wild type strains were used as DNA templates for initial testing of the control strains and then for typing of the *K. pneumoniae* collection containing the unknown plasmids. Whenever possible, the individual bands representing the plasmids were extracted from the agarose gels produced either in S1-PFGE or in electrophoresis performed to visualised the smaller plasmids (<30 kb). The extracted plasmidic DNA was purified on GFX columns (Amersham) according to manufacturer's instructions. This procedure was performed for all wild type strains in which at least one plasmid was detected. These GFX purifications were used as templates for both the mPCR and then the classical PBRT in order to assign specific rep types and the classical incompatibility determinants to individual plasmids.

2.7 Typing of a collection of *K. pneumoniae* isolates from diverse reservoirs

Initially the simplex and later the multiplex PCR were performed on the three control strains listed above containing the reference plasmids (Fouts et al., 2008; Soler Bistue et al., 2008). To verify the utility of the DNA templates extracted from the agarose gels as described in the previous subsection the individual reference plasmids were extracted using the same method. The mPCR was performed on these plasmids in order to verify the specificity of this novel method.

Subsequently, a collection of fifty *K. pneumoniae* isolates underwent the typing using the described method. After initial typing of the wild type strains from the collection, typing with the novel method was further performed on the individual plasmids extracted to assign specific rep groups to each of the plasmids.

Further, all the wild type strains in which at least one large plasmid (≥30 kb) was detected underwent the classical PBRT as described by (Carattoli et al., 2005; Garcia-Fernandez et al., 2009; Villa et al., 2010). If necessary, the individual plasmids were also subjected to the screening with the use of the classical PBRT methods.

2.8 Sequencing of the selected targets

Only few sequences of plasmids with no other than the repIV type replicon (pCK41, pSC138) or harbouring the repV replicon (pKP187; harbours repIV & repV) are currently deposited in GenBank. Therefore the repIV and repV PCR products from the selected plasmids that produced positive signals to these repIV and repV replicases were sequenced by the standard capillary sequencing method (Macrogen Inc., Korea).

3. Results

3.1 Development of the novel mPCR method for replicase typing

In order to detect replicases of group repI to repVII *in vitro* rather than *in silico*, a multiplex PCR was developed and tested on a set comprising of three control strains (*K. pneumoniae* Kp342 and MGH78578 and *E. coli* HB101 transconjugant) containing plasmids covering all seven replicon groups. Initially, the control strains underwent the S1-PFGE. In *K. pneumoniae* Kp342 two plasmids of approximate sizes 190 kb and 90 kb were detected, corresponding to the reported sizes of pKP187 (187 kb) and pKP91 (91 kb), respectively; in *K. pneumoniae* MGH78578 three large plasmids were detected of the following approximate sizes: 175kb, 110 kb, 90 kb corresponding respectively to pKPN3 (175 kb), pKPN4 (107 kb), pKPN5 (87 kb). *E. coli* HB101 transconjugant was confirmed to harbour a plasmid sized to 40 kb corresponding to the pMET1 (42 kb).

Positive and correctly sized PCR products were observed in the seven simplex PCRs and in the mPCR performed initially on the boiled lysates from *K. pneumoniae* Kp342, *K. pneumoniae* MGH78578 and *E. coli* HB101/pMET1. For each of the individual plasmids extracted from the aforementioned control strains the products observed in the mPCR corresponded well with the results predicted by *in silico* analysis of the plasmid sequences (Table 1). The pKP187 produced positive signals to repIV and repV; pKP91 was positive to repI & repIII, pKPN3 was positive to repI & repIV replicases; pKPN4 produced signals to repI & repVI and pKPN5 was positive to repVII. The 40 kb plasmid from *E. coli* HB101 (pMET1) produced positive signal to the repII replicase. No other replicase types than the aforementioned ones were detected in the mPCR on these reference plasmids.

3.2 Typing of plasmids from *K. pneumoniae* clinical and environmental isolates by the novel mPCR approach and by the classical PBRT methods

Fifty isolates of *K. pneumoniae* underwent the described mPCR as well as the standard PBRT methods available for replicon typing. Furthermore, individual plasmids, if detected, were isolated from the agarose gels and replicon typing was performed on these using the same PCR schemes as for testing of the wild type isolates. The summary of the results is presented in the Table 3. Detailed results of S1 PFGE, PBRT and the mPCR performed on the wild type strains are listed in the Table S1 (supplementary material).

In nine isolates no plasmid was detected by any of the applied methods, while in one isolate the only plasmid detected was 3.8 kb in size (Sp33, blood-stream isolate). The plasmid free isolates originated from blood (n=5), surface waters (n= 3) and urinary tract infection (n=1). These ten isolates did not produce a positive signal in the any of the tested PCR schemes.

In the remaining strains a total of n=118 individual plasmids of various sizes were detected by S1-PFGE or the standard gel electrophoresis. Among these n=67 were large plasmids ≥30 kb in size. Four of the detected plasmids were of an intermediate range of size, from 23≤ to < 30 kb. The remaining n=47 plasmids detected were smaller than 18 kb. The intermediate size and the small plasmids did not produce a PCR signal neither in the mPCR nor in the classical PBRT tested in this study (for details see Table S1).

3.2.1 Plasmids carrying two replicases, repI and repIV

Twenty- two large plasmids tested in the mPCR produced signal to the combination of repI and repIV (UTI, n=16; bloodstream isolates, n=4; isolates from surface waters, n=2). All of these repI,-IV positive plasmids except one gave also a positive signal for FII_k type replicon (85 kb plasmid from UTI isolate Cas671 harboured IncFII determinant); two plasmids from isolate Cas681 (UTI isolate; plasmids sized to 220kb and 85 kb) produced additionally positive signals for the IncFII determinant. Overall, the repI,-IV, FII_k plasmids fell into the size range of 85 kb to 230 kb.

3.2.2 Plasmids producing signal for no other than repIV type replicase

Eleven of the individually tested plasmids produced a signal in the mPCR for no other than repIV group (UTI, n=5; bloodstream isolates, n=3; water isolates, n=3). Overall, these repIV type plasmids fell into the size range from 33 kb to 230 kb. Three of these plasmids appeared positive for the FII_k incompatibility determinant (plasmids sized from 180kb to 229 kb and

located in strains C3091, Cas664 and Cas674; UTI isolates). One of the repIV positive plasmids was found to carry the FII determinant (100 kb, Cas681; UTI isolate).

In the remaining (n=7) cases the repIV positive plasmids did not produce positive PCR signals for any of the classical incompatibility determinants tested. These plasmids represent the group that would possibly remain undetected if screening was performed only by means of the classical PBRT methods.

3.2.3 Plasmids producing signal to individual repI, -IV, - VI or –VII$_{(IncR)}$ replicases; or diverse combinations of these

The following other combinations of replicons were observed on five large plasmids based on the positive PCR results. In Cas673 the 121 kb plasmid harboured repVII$_{(IncR)}$ & FII$_k$ replicons; in Cas119 a 128 kb plasmid harboured repI, -VII$_{(IncR)}$ & IncFII$_k$ replicon; in Cas664 a 68 kb plasmid produced signals to repIII , -VII$_{(IncR)}$ replicases; two plasmids sized to 267 kb (Cas664) and 87 kb (Cas669) produced signals to the combination of three replicases repIII, -IV, -VII$_{(IncR)}$ & FII$_k$ determinant.

Three large plasmids sized to 95 kb (Sp15), 123 kb (Sp15) and 103 kb (Cas122) produced positive signals to repIII replicases in the novel mPCR. The aforementioned 95 kb and 103 kb, repIII plasmids were also positive to FII and FII$_k$, respectively.

Single cases were found of the large plasmids that produced signals in the mPCR to the individual replicases of repI type (68kb plasmid from Cas673, positive also to both FII and FII$_k$), repV type (377 kb plasmid from Cas122) and repVI (112 kb plasmid from Sp13, positive also to FII$_k$).

Otherwise the following combinations of replicases were also detected on the tested plasmids: repI, -III, -IV & IncFII (298 kb plasmid from Sp20) and repI, -III, -IV & IncFII$_k$ (180kb plasmid from Sp22) ; repI, -IV, -VI & IncFII$_k$ (170 and 174 kb plasmids from Sp34 and Sp41, respectively); repIII, -IV (92 kb, 142 kb and 153 kb plasmids located in Sp20) and repIV,-V (244 kb plasmid located in Cas127).

3.2.4 Sequencing of the selected replicases

The single plasmid in Sp30 (33 kb) and the largest of the three plasmids in Sp10 (113 kb) produced signals to no other than repIV PCR. These two rep IV PCR products were sequenced. The repIV of the plasmid from Sp10 shared 99% identity with the *rep*A sequence

of pCK41 (HQ332785); the repIV of the plasmid from Sp30 shared 99% identity with pKCTC2242 (CP002911); both sequences shared from 90% to 98% similarity to the *rep*A of the reference plasmid pKPN3 (CP000648).

In silico analysis of the previously sequenced plasmids with the repIV replicase indicated, that the DNA segments containing the repIV replicases on these plasmids share similarities with the IncFIA and IncFIB replicons. On the majority of fully sequenced plasmids harbouring the repIV replicase the *sop*A and *sop*B of IncFIA replicon were found, although the repIV-*rep* genes shared only a low level of nucleotide sequence identity with the *rep*FIB (Figure 2). The repIV detected also on the diverse plasmids in this study may therefore constitute variants of either IncFIA or IncFIB replicons.

The repV sequences detected on a mega-plasmid in Cas122 (estimated size >300 kb) and on the largest of the two plasmids from Cas127 (244 kb, this plasmids was positive to both repIV and repV) were sequenced. These repV sequences both shared 99% identity with the repV of pKP187 (corresponding to the *rep*B2 of this reference plasmid). This repIV, -V/ 244 kb plasmid from Cas127 (isolate from surface waters) may be closely related to pKP187.

3.2.5 Plasmids that did not produce a signal in the novel mPCR

For fifteen of the plasmids, no signal was observed in the mPCR. Among these, five produced positive signals to other classical incompatibility determinants in PBRT. An approximately 48 kb plasmid from Cas119 produced a positive signal for FII_k determinant and a plasmid of the same estimated size located in Cas671 produced a positive signal for FII determinant. FII and/or FII_k determinants were also detected on 110 kb plasmid from Cas123 (FII_k), 30 kb plasmid from Cas671 (FII and FII_k), 85 kb plasmid from Cas676 (FII).

In the remaining ten cases the plasmids turned out to be non-typable both by means of the mPCR and by the standard PBRT methods. None of the tested plasmids or wild type strains produced a positive signal to repII in the mPCR.

4. Discussion and conclusions

The novel mPCR allowed us for rapid detection of a broad spectrum of the different replicases found in this study on IncFII, $IncFII_k$ plasmids and various plasmids non-typable by other classical PBRT methods from *K. pneumoniae*. Several combinations of replicases that were not observed *in silico* were detected in this study during screening performed by the mPCR method. Namely, repI, III, IV & $IncFII_{(k)}$ ($Sp20_{FII}$ and $Sp22_{FIIk}$); repI, -IV, -VI & $IncFII_k$ (Sp34 and Sp41) and also repIII, -IV, -VII $_{(IncR)}$ (Cas664). Interestingly, these aforementioned multi-replicon scaffolds were detected only in clinical isolates (blood-stream and urinary tract). Moreover, we detected individual cases of repV (377 kb, Cas122, water isolate) and repVI (112 kb, Sp13, blood isolate) type replicons on two of the tested plasmids. This is an interesting finding considering that plasmids that would harbour only these individual replicons are not found in the public databases. Nevertheless, these two replicase types seem to be present rather sporadically on the plasmids from *K. pneumoniae*. The repV was also found on a 244 kb plasmid (Cas127, water isolate) in combination with repIV type replicase. This pattern was observed *in silico* on pKP187 located in *K. pneumoniae* isolated from a plant. The two above mentioned plasmids producing signals for repV replicase in the mPCR (Cas122 and Cas127) and the pKP187 were all located in the environmental isolates of *K. pneumoniae*. It is therefore tempting to suggest that the repV replicons could be typical for large plasmids found in the external natural environments rather than in *K. pneumoniae* causing infections in humans. However, more replicon typing data on plasmids from such environmental *K. pneumoniae* strains is needed to make a definite statement about the distribution of these repV type replicons.

Curiously, the repII-type replicases were not detected in this study on plasmids from the tested collection of *K. pneumoniae*. The two previously sequenced plasmids harbouring the repII type replicases were originally isolated from strains from Argentina (pMET1, clinical isolate of *K. pneumoniae*) and China (pCRY, animal isolate of *Y. pestis*) (Soler Bistue et al., 2008). This family of replicons is thus either rare or might be characteristic to some specific reservoirs or geographical locations that were not included in this study, which mostly covered Danish isolates.

In the study we observed also that on some of the repIV- positive plasmids detected by the mPCR no classical determinants were detected by the standard PBRT methods. Sequence analysis of the plasmids found in GenBank indicated, that the repIV may constitute an independent replicon being an equivalent or variant of either IncFIA or IncFIB classical

replicons. Importantly, the mPCR proved to be a useful tool for detection of these repIV replicases.

In the study we also detected possibly epidemic plasmids sized to 230 kb, positive for repI,-IV & IncFII$_k$ replicons that were found among two of the blood isolates (Sp7 and Sp13) and nine of the urinary tracts isolates (Cas663,-665,-666,-668,-670,-671,-672,-677,-679) . The urinary tract *K. pneumoniae* isolates harbouring these similar plasmids could be clonally related (Stahlhut et al., 2009). However, the two blood isolates represented different capsule serotypes and therefore belonged to other clonal lineages than the urinary tract isolates (Stahlhut et al., 2009). This indicates that these repI,-IV & IncFII$_k$, 230 kb plasmids might have been circulating in different *K. pneumoniae* strains causing infections in Denmark.

Another observation drawn from the study is that majority of the repVII$_{(IncR)}$ replicons were found to be hybrids with FII$_k$ replicons. This pattern was observed both *in silico* and among the plasmids typed in the study by means of the mPCR and the standard PBRT methods. IncR plasmids seem to be broad host range links between *K. pneumoniae* and other *Entrobacteriaceae* and are apparently capable of either forming multi-replicon scaffolds with F-related plasmids or functioning as independent replicons. IncR plasmids do not encode any of the previously characterized conjugative relaxases (Garcillan-Barcia et al., 2011). Previous study indicated that an IncR plasmid from *E. coli* pK727 was not capable of self-transfer from a donor strain containing no other plasmids than this one (Bielak et al., 2011). It cannot be excluded, that other proteins encoded by the IncR scaffolds that were so far not characterized may function as mobilization relaxases. Alternatively, IncR plasmids could be somehow mobilized by relaxases encoded by other co-residing plasmids. In fact, (Schjorring et al., 2008) demonstrated *in vivo* transmission of IncR pKPN5 plasmid from its host *K. pneumoniae* MGH78578 to a plasmid free recipient.

Generally, plasmids harbouring one or more of the repI-VIII type replicases which sequences were found in databases were located both in *K. pneumoniae* as well as in other species of enteric bacteria. These plasmids were isolated from bacteria originating from many different countries (USA, China, Vietnam, Taiwan, Canada, Georgia, Slovenia, United Kingdome, Korea, France, Germany and Australia) and from different human and non-human sources (human infections, lake and sea water, plant and animals). This study showed that the plasmids with repIII, -IV, -V, -VI and -VII$_{(IncR)}$ are also prevalent in *K. pneumoniae* from infections Denmark and from the water samples from Germany (Stahlhut et al., 2009). It is thus evident that *K. pneumoniae* responsible for causing infections in humans may contain

plasmids similar to those found in *Enterobacteriaceae* from the natural environments. These similar plasmids may be found in different geographical locations.

When looking on the classical determinants IncFII and $IncFII_k$, in this study these replicons were found in all three reservoirs, i.e. blood stream, urinary tract and surface waters; typically with repI (covering mainly $RepFII_k$), repIII (covering mainly RepFIA or related) or repIV replicases. Although, there were noticeable differences observed between replicases distribution found in different reservoirs (isolates from bacteraemia, urinary tract infections and surface waters). Namely, the highest ratio of repIII replicases or repIII combined with other rep- types was found on plasmids from blood infection isolates. As indicated above, in this reservoir also the highest number of replicase combinations not observed *in silico* was found on individual plasmids. Also the highest ratio of the blood originating plasmids turned out to be non-typable by any of the applied PCR methods. The blood was the only reservoir in which the repVI type replicases were detected on two of the plasmids. The individual repIV- type replicases were most often detected on the plasmids from the surface waters, while the highest frequency of occurrence of repI combined with repIV was observed among plasmids from the urinary tract infections.

It should be kept in mind that the sequence data available in the public databases might be biased due to the focus put on plasmids encoding antibiotic resistance or conferring virulence factors. This might be the reason why a certain pattern was observed *in silico* for replicases found on the sequenced virulence and resistance plasmids from *K. pneumoniae*. This distribution pattern of replicases can apparently be different among plasmids selected on criteria other than the specific resistance or virulence conferred by these plasmids.

Overall, the majority of plasmids from human and environmental *K. pneumoniae* isolates examined in this study belonged to the IncFII, IncFIIk and to lesser extend IncR family of classical replicons (Garcia-Fernandez et al., 2009; Villa et al., 2010). These plasmids often carried a set of different replicases which most probably allow them to overcome the incompatibility phenomenon in cases, when other co-resident plasmids are equipped with similar incompatibility determinants (Villa et al., 2010). Also the plasmids belonging to the broad host range families of IncR, IncN and to lesser extend also to IncL/M replicons seem to be characteristic for *K. pneumoniae* as these replicons were found on the sequenced plasmids available in public databases (Athanasopoulos et al., 1995; Garcia-Fernandez et al., 2011; Zhao et al., 2010; Zhu et al., 2009). This study underlined that there are differences between the distribution of replicases on plasmids originating from diverse reservoirs (blood

infections, urinary tract infections and surface waters). Also many of the replicons still remain non-typable (and thus undetectable) both by the classical PBRT methods as well as by the novel mPCR. This problem might be solved in the future by applying the full genome sequencing on a larger scale instead of or in combination with the PCR-based methods for plasmids detection.

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Transparency declaration

None to declare

Description of tables and figures

Table 1. Summary of the *in silico* analysis of *K. pneumoniae* which replicon sequences are available in public databases and plasmids harbouring the similar replicases originating from other species (23 September 2011, date last accessed); allocation of the detected replicases into repI-VIII homology groups and selected features found on the listed plasmids.

a- refers to the experimentally confirmed features or presence of genes on the sequenced plasmids encoding the putative functions; b- based on (Garcilian-Barcia et al., 2011); c- multiple mismatches observed between the plasmid sequence and both primers designed for the given rep group; d- individual mismatches observed *in silico* between one of the primers for mPCR and its target on the given plasmid; e- marked sequences share 100% identity with each other at the amino acid sequence level; ab^r- antibiotic resistance, $dis.^r$ - resistance to disinfectants, $met.^r$- resistance to heavy metals, vir.- virulence factors; the groups highlighted with grey are targeted by the novel mPCR; KP- *K. pneumoniae*, EC- *E. coli*, EF- *Escherichia fergusonii*, ECL- *Enterobacter cloacae*, SE- *S. enterica*, KY- *Kluyvera intermedia*, CR- *Citrobacter rodentium*, PA- *Pantoea agglomerans*, YP- *Yersinia pestis*, ET- *Edwardsiella tarda*; EA- *Enterobacter asburiae*, n. a.-data not available; n. d. – not detected *in silico*; inf. – infection, UTI-urinary tract infection, L- Liver abscess, M- meningitis, sed.-sediment, tr. – treatment, bac. – bacteraemia, inc.- incompatibility; replicase genes of the classical Inc groups are abbreviated as *rep* with corresponding capital letters used for naming of that group (for example *repA* of IncN plasmids is designated as *repN* etc.)

Table 2. Primers used in the novel multiplex PCR and the target sequences on the reference plasmids.

Table 3. Summary of the results of replicon typing of plasmids from *K. pneumoniae* clinical and water isolates

nd – not detected

[a] the signal detected in the given PCR was significantly weaker that the control signal for that PCR

Table S1. Detailed results of detection and typing of plasmids from a collection of *K. pneumoniae* clinical and surface waters isolates.

[a] results correspond to the given reference plasmids; [b] excluding PCR targeting ColE-type replicons (Garcia-Fernandez et al., 2009); [c] the signal observed in the PCR was significantly weaker than the corresponding signal on the control; U- urinary tract, B- blood, W- water, P- plant, S- sputum, N- neonatal infection; nd- signal not detected; nt-not tested.

Figure 1. Schematic representation of the IncR replicons and genetic elements downstream of the replicons detected on the fully or partially sequenced IncR plasmids. Black arrows represent open reading frames (ORFs) predicted in this study with the Vector NTI Suite 11 software (Invitrogen, Inc.), grey arrows represent two *cis* origins of replication predicted for pGSH500 by (Silva-Tatley and Steyn, 1993), rectangle filled with slanting lines represents element proposed previously to be the cop/inc region of pGSH500 (Silva-Tatley and Steyn, 1993). The figure was generated based on the sequences available in GenBank.

Figure 2. Schematic overview of repIV-type replicons and their comparison with the classical IncFIA and IncFIB replicons. Black arrows represent open reading frames (ORFs) predicted in this study with the Vector NTI Suite 11 software (Invitrogen, Inc.), black rectangles represent regions containing direct repeats, grey arrows filled with vertical or slanting lines represent IncFIA and IncFIB features, respectively, that were not detected in this study on the repIV-type replicons. The figure was generated based on the sequences available in GenBank.

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Table 1. List of large plasmids found in *K. pneumoniae* which replicon sequences are available in public databases and plasmids harbouring the similar replicases originating from other species (23 September 2011, date last accessed); allocation of the detected replicases into repI-VIII homology groups and selected features found *in silico* on the listed plasmids.

Plasmid name	Host	Isolation source	Country or region of isolation	Size [kb]	Types of detected in silico *rep* sequences									Other classical inc. determinants detected in silico	Features conferred by the plasmid (a)	Genes encoding key relaxases detected on the plasmid (b)	Accession	References
					repI	repII	repIII	repIV	repV	repVI	repVII (RepR)	repVIII (RepN)	Remainig reps					
pKPN3	KP	Hum.	USA	176	+			+						IncFIIk	ab r, met. r	*tra* I_KP	CP000648	
pOU7519	SE	Hum.	Taiwan	127	*rep* FIIAs [a]			+						IncFIIs, FIBs	ab r, vir.	traI_SE	EU219534	
pc15-k	KP	Hum.	China	96	+			+						IncFIIk	ab r	*tra* I_KP	HQ202266	-
pKPN4	KP	Hum.	USA	108	+					+				IncFIIk	ab r, met. r	*tra* I_KP	NC009650	-
pKpQIL	KP	Hum. inf.	Israel	114	+					+				IncFIIk	ab r, met. r	*tra* I_KP	NC_014016	(Leavitt et al., 2010)
pKP91	KP	Plant	USA	91	+		+							IncFIIk	n. d.	traI_KP (truncated)	NC_011281	(Fouts et al., 2008)
pKP048	KP	Hum.	China	151	+						+			IncFIIk	ab r, met. r	*tra* I_KP	FJ628167	(Jiang et al., 2010)
pLV1402	KY	Lake water sed.	UK	n.a.	*rep* FIIA α						+ β			repFIIA (α)	n. a.	n. a.	AJ009981 (α) AJ009986 (β)	(Osborn et al., 2000)
pGSH500	KP	Hum. inf.	South Africa	107	+ α						+ β			IncFIIk	abr	n. a.	AJ009980 (α) Z11775.1 (β)	(Osborn et al., 2000)
pKF3-94	KP	Hum. inf.	China	94	+									IncFIIk	ab r	*tra* I_KP	FJ876826	(Zhao et al., 2010)
pRMH760	KP	n. a.	Australia	n. a.	+									n. a.	ab r, met. r	n. d.	GU553923	-
pSLMT	KP	Hum. inf.	UK	21	+									IncFIIk	ab r	n. d.	HQ589350	-
pMET1-FC1	KP	Hum. inf.	Argentina	42		+								n. d.	ab r	*mob* B, -C (Ti/ ICE related)	EU383016	(Soler Bistue et al., 2008)
pCRY	YP	Animal	China	22		+								n. d.	n. d.	*mob* B, -C (Ti/ ICE related)	AE017044	(Soler Bistue et al., 2008)
pO111_1	EC	Hum. inf.	Japan	205			+							IncHI1	ab r, met. r	*tra* I_HI1	AP010961	
R27	SE	n. a.	Canada	181			+							IncHI1	ab r	*tra* I_HI1	AF250878	(Barton et al., 1995)
pAKU_1	SE	Hum. inf.	Pakistan	212			+							IncHI1	ab r	*tra* I_HI1	AM412236	(Barton et al., 1995)
pHCM1	SE	Hum. inf.	Vietnam	218			+							IncHI1	ab r	*tra* I_HI1	NC_003384	(Parkhill et al., 2001)
pKF3-140	KP	Hum. inf.	China	147	(*rep* FIIA)		*rep* FIA +	*rep* FIB						IncFII, repFIIA, IncFIA, FIB	ab r, putative dis. r	*tra* I_F	FJ876827	(Zhao et al., 2010)
pIP1206	EC	n. a.	France	168	(*rep* FIIA)		*rep* FIA +	*rep* FIB						repFIIA , IncFIA, FIB	ab r, putative dis. r	*tra* I_F	AM886293	-
pU302L	SE	Hum. inf.	USA	85			*rep* FIA +	*rep* FIB						IncFIA, FIB	ab r	n. d.	AY333434	(Chen et al., 2007a)
pEK499	EC	Hum. inf.	USA	118	(*rep* FIIA)		*rep* FIA +							IncFII, repFIIA, IncFIA	ab r, putative dis.r	n. d.	EU935739	(Woodford et al., 2009)
pEC_L8	EC	Hum. UTI	Belgium	119	(*rep* FIIA)		*rep* FIA +							IncFII, repFIIA, IncFIA	ab r	*tra* I_F	GU371928	(Smet et al., 2010)
pEC_L46	EC	Hum. UTI	Belgium	145	(*rep* FIIA)		*rep* FIA +					*rep* N		IncFII, repFIIA IncFIA, IncN	ab r	*tra* I_N	GU371929	(Smet et al., 2010)
pSA1	SE	Animal/ Hum.	Slovenia	n. a			*rep* FIA +							n. a	n. a.	n. a	AF239689	(Mulec et al., 2002)
pSFO157	EC	Hum. inf.	USA	121	(*rep* FIIA)		*rep* FIA +	*rep* FIB						IncFII, repFIIA, IncFIA, FIB	vir.	traI_F	AF401292	-
pRSB107	n. a.	Sewage tr. plant	Germany	121	(*rep* FIIA)		*rep* FIA +							IncFII, repFIIA, IncFIA	vir., met. r	*tra* I_F (truncated)	AJ851089	(Szczepanowski et al., 2005)
pKP245	KP	Hum. UTI	Taiwan	98			+				+			n. d.	ab r	n. d.	DQ449578	(Chen et al., 2006)

Table 1.- Continued.

Plasmid name	Host	Isolation source	Country or region of isolation	Size [kb]	Types of detected in silico *rep* sequences									Other classical inc. determinants detected in silico	Features conferred by the plasmid (a)	Genes encoding key relaxases detected on the plasmid (b)	Accession	References
					repI	repII	repIII	repIV	repV	repVI	repVII (RepR)	repVIII (RepN)	Remainig reps					
pKP187	KP	Plant	USA	188				+	+					n. d.	met. r	n. d.	NC_011282.1	(Fouts et al., 2008)
pKCTC2242	KP	n. a.	Korea	203				+					*rep* A [e]	n. d.	vir., met. r	n. d.	CP002911	-
pLVPK	KP	Hum. inf.	Taiwan	219				+					*rep* A [e]	n. d	vir., met. R	n. d.	AY378100	(Chen et al., 2004)
pK2044	KP	Hum. inf.	Taiwan	224				+					*rep* A [e]	n. d	met. r	n. d.	AP006726	(Wu et al., 2009)
pECL_A	ECL	Hum. inf.	USA	200				(d)					*rep* $A2_{(pECL\_A)}$	n. d.	met. r	*tra* I ECL_A =ENTAS	CP001919	(Ren et al., 2010)
pENTAS01	EA	n. a.	n. a.	167				+					*rep* $A1_{(pENTAS)}$	n. d.	putative dis.r	*tra* I ECL_A =ENTAS	CP003027	
pCVM19633	SE	Hum. inf.	USA	110				+					*rep* $A2_{(pCVM19633)}$	n. d.	ab r, putative dis.r	*tri* K, *tri* L (similar to ICEkp1)	CP001125	-
pCK41	ET	Fish	Korea	73				+						n. d.	ab r, putative dis.r	*tri* K, *tri* L (similar to ICEkp1)	HQ332785	
pSC138	SE	Hum. inf.	Taiwan	139				+						n. d.	ab r, met. R	*nik* AB (similar to R64)	AY509004	(Chiu et al., 2005)
pKPN5	KP	Hum.	USA	89							+			n. d.	ab r, putative dis.r	n. d.	NC009651.1	
pEFER	EF	Hum.	USA	55							+			n. d.	ab r	n. d.	CU928144	
pLV1403	PA	Lake water sed. UK	n. a.							+				n. a.	n. a.	n. a.	AJ009954	(Osborn et al., 2000)
pQ19_1	KP	Sea water	China	n. a.							+			n. d.	ab r	n. a.	HM371193	(Zhao et al., 2011)
pK727	EC	Hum.	France	40							+			n. a.	ab r	n. a.	JF708954	(Bielak et al., 2011)
pCROD1	CR	Mouce inf.	n. a.	55	(*rep* FIIA)						(+ [c])			IncFII, repAFII, IncR	vir.	n. d.	FN543503	(Petty et al., 2010)
pKF3-70	KP	Hum. inf.	China	70	(*rep* FIIA)						+			IncFII, repAFII	ab r	*tra* I_F	FJ494913	(Zhao et al., 2010)
pKP96	KP	Hum. inf.	China	68								*rep* N		IncN	ab r, putative dis.r	*tra* I_N	EU195449.1	(Garcia- Fernandez et al., 2011)
Plasmid 12	KP	Hum. inf.	USA	76								*rep* N		IncN	ab r	*tra* I_N	NC_011385.1	
Plasmid 9	KP	Hum. inf.	USA	71								*rep* N		IncN	ab r	*tra* I_N	NC_011383.1	
pNL194	KP	Hum.	Greece	79								*rep* N		IncN	ab r, putative dis.r	*tra* I_N	GU585907	
pLJ1	KP	Hum.	Australia	65								*rep* N		IncN	ab r, putative dis.r	*tra* I_N	EU880929	
pCTX-M360	KP	Hum.	China	68									*rep* L/M	IncL/M	ab r	*mob* ABC, *tra* I_L/M	NC_011641	(Zhu et al., 2009)
pMU407.1	KP	Hum.	Australia	n. a.									*rep* L/M	IncL/M	ab r	n. a.	U27345.1	(Athanasopoulos et al., 1995)
pEI545	KP	n.a.	n. a.	n. a.									*rep* Z	IncZ	n. a	n. a	M93064.1	(Praszkier et al., 1991)

[a]- based on the presence of genes on the sequenced plasmids encoding the putative functions; [b]- based on (Garcilian-Barcia *et al*., 2011); [c]- multiple mismatches observed between the plasmid sequence and both primers designed for the given rep group; [d]- individual mismatches observed *in silico* between one of the primers for mPCR and its target on the given plasmid; [e]- marked sequences share 100% identity with each other at the amino acid sequence level; ab[r]- antibiotic resistance, dis. [r]- resistance to disinfectants, met.[r]- resistance to heavy metals, vir.- virulence factors; the groups highlighted with grey are targeted by the novel mPCR; KP- *K. pneumoniae*, EC- *E. coli*, EF- *Escherichia fergusonii*, ECL- *Enterobacter cloacae*, SE- *S. enterica*, KY- *Kluyvera intermedia*, CR- *Citrobacter rodentium*, PA- *Pantoea agglomerans*, YP- *Yersinia pestis*, ET- *Edwardsiella tarda*; EA- *Enterobacter asburiae*, n. a.-data not available; n. d. – not detected *in silico*; inf. – infection, UTI- urinary tract infection, sed.-sediment, tr. –treatment, bac. – bacteraemia, inc.- incompatibility; replicase genes of the classical Inc groups are abbreviated as *rep* with corresponding capital letters used for naming of that group (for example *repA* of IncN plasmids is designated as *repN* etc.)

Table 2. Primers used in the novel multiplex PCR and the target sequences on the reference plasmids

Primer name	Primer sequence	Target sequence		Product size [bp]	Plasmid accession
repIP$_{fw}$	5'-AAGCTGATGGCGAAAGC-3'	*rep*A	of pKPN3	207	CP000648
repI P$_{rv}$	5'-ACACTCGATGGCCATATTG-3'				
repII P$_{fw}$	5'-CCGGCACAACAAGTATTG-3'	*rep*A	of pMET1	264	EU383016
repII P$_{rv}$	5'-GCGTCATAAGGATTGAAGG-3'				
repIII P$_{fw}$	5'-GGCGGCATATAGTCTCTCC-3'	*rep*E	of pKP91	367	NC_011281
repIII P$_{rv}$	5'-CTGCGTAAACCGGTTCTG-3'				
repIV P$_{fw}$	5'-TGTTAAGCTGGATGTCAACAG-3'	*rep*B1	of pKP187	417	NC_011282.1
repIV P$_{rv}$	5'-CCTTTGGTCTCGCTCTG-3'				
repV P$_{fw}$	5'-TTTCTCGTGGACGTCTG-3'	*rep*B2	of pKP187	499	NC_011282.1
repV P$_{rv}$	5'-TACTTCAGGGAGGGATTTCTC-3'				
repVI P$_{fw}$	5'-CTCAGGAAGGCTCTCAATG-3'	*rep*A	of pKPN4	601	NC009650
repVI P$_{rv}$	5'-CATGCCTTCCAGAAACG-3'				
repVII P$_{fw}$	5'-TGGGAAAGAGGTCAAGTTC-3'	*rep*B	of pKPN5	768	NC009651.1
repVII P$_{rv}$	5'-CAGGTTCCAGGAAGGTAC-3'				

Table 3. Summary of the results of replicon typing of plasmids from clinical and surface waters isolates of *K. pneumoniae*

Number of individual plasmids ≥30kb detected (n=67)				
rep group (s) detected in the novel multiplex PCR	Isolate name	Isolation source	Size of the individual plasmid detected [kb]	Other incompatibility determinants detected on the plasmid
repI (n= 1)				
	Cas673	U	68	FII, FII_k[a]
repIII (n= 3)				
	Sp15	B	95	FII
	Sp15	B	123	nd
	Cas122	W	103	FII_k
repIV (n= 11)				
	C3091	U	180	FII_k
	Sp10	B	113[b]	nd
	Sp28	B	159	nd
	Sp30	B	33[b]	nd
	Cas 121	W	220	nd
	Cas 122	W	188	nd
	Cas 125	W	195	nd
	Cas 664	U	180	FII_k
	Cas 674	U	229	FII_k
	Cas 678	U	229	nd
	Cas 681 (repIV)[a]	U	100	FII
repV (n= 1)				
	Cas122	W	377 [C]	nd
repVI (n= 1)				
	Sp13	B	112	FII_k
repVII (n=1)				
	Cas673	U	121	FII_k, IncR
repI, -IV (n= 22)				
	Sp7	B	229	FII_k
	Sp13	B	229	FII_k
	Sp29	B	124	FII_k
	Sp32	B	180	FII_k
	Cas119	W	180	FII_k
	Cas123	W	224	FII_k
	Cas663	U	229	FII_k
	Cas665	U	229	FII_k
	Cas666	U	229	FII_k

Table 3- continued

rep group (s) detected in the novel multiplex PCR	Isolate name	Isolation source	Size of the individual plasmid detected [kb]	Other incompatibility determinants detected on the plasmid
repI, -IV continued	Cas668	U	229	FII_k
	Cas670	U	229	FII_k
	Cas671	U	229	FII_k
	Cas671	U	85	FII
	Cas672	U	229	FII_k
	Cas675	U	230	FII_k
	Cas676	U	220	FII_k
	Cas677	U	229	FII_k
	Cas678	U	217	FII_k
	Cas679	U	229	FII_k
	Cas680	U	220	FII_k
	Cas681	U	220[d]	FII, FII_k
	Cas681 (repI & IV)[a]	U	85[d]	FII, FII_k
repI,-VII (n= 1)				
	Cas119	W	148	FII_k, IncR
repI, -III, -IV (n= 2)				
	Sp20	B	298	FII
	Sp22	B	180	FII_k
repI, -IV, -VI (n= 2)				
	Sp34	B	174	FII_k
	Sp41	B	170	FII_k
repIII, -IV (n= 3)				
	Sp20	B	153[d]	FII[a]
	Sp20	B	142[d]	FII[a]
	Sp20	B	92[d]	FII[a]
repIII, -VII (n= 1)				
	Cas664	U	68	IncR
repIII, -IV, -VII (n= 2)				
	Cas664	U	267	FII_k[a], IncR
	Cas669	U	87	FII_k, IncR
repIV -V (n= 1)				
	Cas127	W	244[c]	nd

Table 3- continued

Cases when no signal was produced in the novel multiplex PCR on the detected, large (≥ 30 kb) plasmids; (n=15)			
Isolate name	Isolation source	Size of the individual plasmid detected [kb]	Other incompatibility determinants detected on the plasmid
Sp10	B	104	nd
Sp13	B	95	nd
Sp14	B	60	nd
Sp28	B	50	nd
Sp28	B	70	nd
Sp39	B	110	nd
Cas119	W	48	FII_k
Cas123	W	110	FII_k
Cas126	W	110	nd
Cas127	W	104	nd
Cas669	U	104	nd
Cas671	U	30	FII; FII_k
Cas671	U	48	FII
Cas676	U	85	FII
Cas680	U	60	nd

Isolates in which no plasmid was detected (n=10, including Sp33 where the only plasmid detected was 3.8 kb)	
Isolate name	Isolation source
Sp3	B
Sp19	B
Sp25	B
Sp31	B
Sp33 (small 4 kb plasmid detected)	B
Sp37	B
Cas120	W
Cas124	W
Cas128	W
Cas667	U

nd – not detected

[a] the signal detected in the given PCR was significantly weaker that the control signal for that PCR; [b]- the repIV PCR product was sequenced from the indicated plasmid; [c] repV PCR product was sequenced from the indicated plasmid; [d]- this was a screening study and the presence of the same set of incompatibility determinants on multiple plasmids in the same isolate should be further verified by sequenced based methods

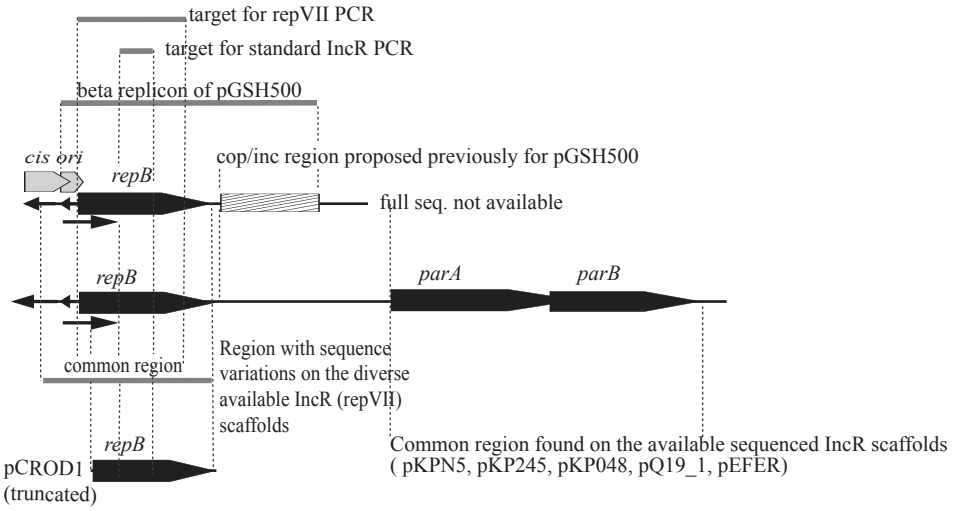

Figure 1. Schematic representation of the IncR replicons (corresponding to repVII in this study) and genetic elements downstream of the replicons detected on the fully or partially sequenced IncR (repVII) scaffolds. Black arrows represent open reading frames (ORFs) predicted in this study with the Vector NTI Suite 11 software (Invitrogen, Inc.), grey arrows represent two *cis* origins of replication predicted for pGSH500 by (Silva-Tatley and Steyn, 1993), rectangle filled with slanting lines represents element proposed previously to be the cop/inc region of pGSH500 (Silva-Tatley and Steyn, 1993). The figure was generated based on the sequences available in GenBank.

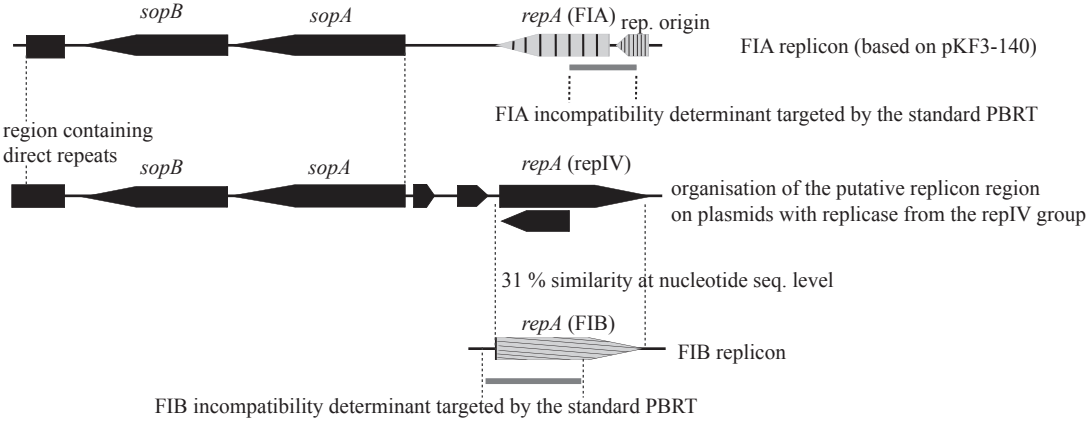

Figure 2. The schematic overview of repIV-type replicons and their comparison with the classical FIA and FIB replicons. Black arrows represent open reading frames (ORFs) predicted in this study with the Vector NTI Suite 11 software (Invitrogen, Inc.), black rectangles represent regions containing direct repeats, grey arrows filled with vertical or slanting lines represent IncFIA and IncFIB features, respectively, that were not detected in this study on the rep3-type replicons. The figure was generated based on the sequences available in GenBank.

Table S1 Results of detection and typing of plasmids from a collection of K. pneumoniae clinical and surface waters isolates

Isolate name	Isolation source	Replicases detected by mPCR	Replicons detected by PBRT	Sizes of plasmids detected in the isolate	Replicases detected by mPCR on the individual plasmids	Replicases detected by PBRT on the individual plasmids
C3091	U	repIV	FIIk	180	repIV	FIIk
Sp3	B	nd	nt	nd	nt	nt
Sp7	B	repI, repIV	FIIk	229	repI, repIV	FIIk
Sp10	B	repIV	nd	113	repIV	nd
				104	nd	nd
				4	nd	nt
Sp13	B	repI, repIV, repVI	FIIk	229	repI, repIV	FIIk
				112	repVI	FIIk
				95	nd	nd
				11	nd	nd
				8	nt	nt
				7	nt	nt
				6	nt	nt
				3	nt	nt
Sp14	B	nd	nd	60	nd	nd
Sp15	B	repIII	FII	123	repIII	nd
				95	repIII	FII
				4	nd	nt
Sp19	B	nd	nd	nd	nt	nt
Sp20	B	repI, repIII, repIV	FII	298	repI, repIII, repIV	FII
				153[d]	repIII, repIV	FII[c]
				142[d]	repIII, repIV	FII[c]
				92[d]	repIII, repIV	FII[c]
				29	nd	nd
				26	nd	nd
				24	nd	nd
Sp22	B	repI, repIII, repIV	FIIk	180	repI, repIII, repIV	FIIk
Sp25	B	nd	nt	nd	nt	nt
Sp28	B	repIV	nd	159	repIV	nd
				70	nd	nd
				50	nd	nd
				6	nd	nt
S29	B	repI, repIV	FIIk	124	repI, repIV	FIIk
				2	nd	nt
Sp30	B	repIV	nd	33	repIV	nd
				6	nd	nt
Sp31	B	nd	nt	nd	nt	nt
Sp32	B	repI, repIV	FIIk	180	repI, repIV	FIIk
Sp33	B	nd	nt	4	nd	nt
Sp34	B	repI, repIV, repVI	FIIk	174	repI, repIV, repVI	FIIk
Sp37	B	nd	nt	nd	nt	nt
Sp39	B	nd	nd	110	nd	nd
Sp41	B	repI, repIV, repVI	FIIk	170	repI, repIV, repVI	FIIk

Table S1- continued

Isolate name	Isolation source	Replicases detected by mPCR	Replicons detected by PBRT	Sizes of plasmids detected in the isolate	Replicases detected by mPCR on the individual plasmids	Replicases detected by PBRT on the individual plasmids
Cas119	W	repI, repIV, repVII	FIIk, IncR	180	repI, repIV	FIIk
				148	repI, repVII	FIIk , IncR
				48	nd	FIIk
				5	nd	nt
				3	nd	nt
Cas120	W	nd	nt	nd	nt	nt
Cas121	W	repIV	nd	220	repIV	nd
				23	nd	nd
				17	nd	nd
				13	nd	nd
				10	nd	nd
				8	nd	nd
				4	nd	nt
				3	nd	nt
Cas122	W	repIII, repIV, repV	FIIk	377	repV	nd
				188	repIV	nd
				103	repIII	FIIk
				14	nd	nd
				7	nd	nd
				5	nd	nt
				3	nd	nt
Cas123	W	repI, repIV	FIIk	224	repI, repIV	FIIk
				110	nd	FIIk
				3	nd	nt
Cas124	W	nd	nt	nd	nt	nt
Cas125	W	repIV	nd	195	repIV	nd
				7	nd	nt
Cas126	W	nd	nd	110	nd	nd
Cas127	W	repIV, repV	nd	244	repIV, repV	nd
				104	nd	nd
Cas128	W	nd	nt	nd	nt	nt
Cas663	U	repI, repIV	FIIk	229	repI, repIV	FIIk
				7	nd	nt
				4	nd	nt
Cas664	U	repIII, repIV, repVII	FIIk , IncR	267	repIII, repIV, repVII	FIIk[c], IncR
				7	nd	nt

Table S1- Continued

Isolate name	Isolation source	Replicases detected by mPCR	Replicons detected by PBRT	Sizes of plasmids detected in the isolate	Replicases detected by mPCR on the individual plasmids	Replicases detected by PBRT on the individual plasmids
				7	nd	nt
Cas667	U	nd	nt	nd	nt	nt
Cas668	U	repI, repIV	FIIk	229	repI, repIV	FIIk
				7	nd	nt
Cas669	U	repIII, repIV, repVII	FIIk , IncR	104	nd	nd
				87	repIII, repIV, repVII	FIIk, IncR
Cas670	U	repI, repIV	FIIk	229	repI, repIV	FIIk
				7	nd	nt
Cas671	U	repI, repIV	FII, FIIk	229	repI, repIV	FIIk
				85	repI, repIV	FII
				48	nd	FII
				30	nd	FII, FIIk
				7	nd	nt
				5	nd	nt
Cas672	U	repI, repIV	FIIk	229	repI, repIV	FIIk
Cas673	U	repI, repVII	FII, FIIk , IncR	121	repVII	FIIk , IncR
				68	repI	FII, FIIk [c]
				7	nd	nt
				6	nd	nt
				4	nd	nt
				3	nd	nt
Cas674	U	repIV	FIIk	229	repIV	FIIk
Cas675	U	repI, repIV	FIIk	240	repI, repIV	FIIk
				7	nd	nt
Cas676	U	repI, repIV	FII, FIIk	220	repI, repIV	FIIk
				85	nd	FII
				7	nd	nd
				5	nd	nt
Cas677	U	repI, repIV	FIIk	229	repI, repIV	FIIk
				7	nd	nt
Cas678	U	repI, repIV	FIIk	229	repIV	nd
				217	repI, repIV	FIIk
				7	nd	nt
Cas679	U	repI, repIV	FIIk	229	repI, repIV	FIIk
				7	nd	nt
Cas680	U	repI, repIV	FIIk	220	repI, repIV	FIIk
				60	nd	FIIk
				7	nd	nt
Cas681	U	repI, repIV	FII, FIIk	220 [d]	repI, repIV	FII, FIIk
				100	repIV [c]	FII
				85 [d]	repI [c] , repIV [c]	FII, FIIk
				7	nd	nt
				5	nd	nt
				4	nd	nt

Table S1- continued

Isolate name	Isolation source	Replicases detected by mPCR	Replicons detected by PBRT	Sizes of plasmids detected in the isolate	Replicases detected by mPCR on the individual plasmids	Replicases detected by PBRT on the individual plasmids
Kp342	P	repI, repIII, repIV, repV	FIIk	188	repIV, repV	FIIk (pKP187a)
			FIIk	91	repI, repIII	FIIk (pKP91a)
MGH78578	S	repI, repIV, repVI, repVII	FIIk, IncR	176	repI, repIV	FIIk (pKPN3a)
				108	repI, repVI	FIIk (pKPN4a)
				89	repVII	IncR (pKPN5a)
				4	nd	nt (pKPN6a)
				3	nd	nt (pKPN7a)
Tomalsky	N	repII	nd	42	repII	nd (pMET1a)

[a] results correspond to the given reference plasmids;

[b] excluding PCR targeting ColE-type replicons (Garcia-Fernandez et al., 2009);

[c] the signal observed in the PCR was significantly weaker than the corresponding signal on the control;

[d] presence of the same set of incompatibility determinants on multiple plasmids in the same isolate should be further verified

U- urinary tract, B- blood, W- water, P- plant, S- sputum, N- neonatal infection; nd- signal not detected; nt- not tested.

Sequencing of the selected PCR products:

1. repIV sequence from a 113 kb plasmid from Sp10

TGTCAACAGCACCAGTACCGTGCAGCCAGTAGCGCTCATGAGACTCGGTCTCTTCGTCCCAACT
CTGAAATCAACAGGGAAGAGCAAGGCGAACCGGAAAAACGTTACAGACGCGACTGAGGAGCT
TGTACAGCTGTCCATTGCCAAAAGCGAAGGATACACTGACGTTAAGATCACCGGTTCGCGTCTT
GATATGGACACGGATTTTAAAGTCTGGCTGGGGATAATTCGCTCCATGTCGGAGTATGGGGTAA
AAAGTGACACCCTGGAACTGTCGTTCGTCGAATTCGTTAAGATGTGCGGATTTGACTCCCGTCG
TTCAAATAAAAAAATGCGCGATCGCATCAGTAATTCCCTGTTTAAACTCGCCTCGGTTACGCTG
AAGTTCCAGAGC

2. rep IV sequence from a 33 kb plasmid from Sp30

GCAACAGTACCAGTACTGTGCAGCCGGTAGCGCTCATGAGGCTCGGTCTCTTCGTTCCAACCCT
GAAATCAACCTCCAGGAGTAAGGCAAACCGTAAAAACGTCACAGATGCCACTGAGGAGCTCGT
ACAGCTGTCCATCGCCAAAAGCGAAGGGTACACCGACGTTAAGATCACCGGTTCGCGTCTTGAT
ATGGACACGGATTTTAAAGTCTGGCTCGGGATCATTCGCTCGATGTCTGAGTATGGGGTAAAAA
GTGACACCCTGGAACTGTCGTTCGTTGAATTCGTTAAGATGTGTGGATTTGACTCACGGCGCTC
AAACAAGAAAATGCGGGATCGCATCAGCAATTCCCTGTTTAAACTCGCCTCGGTTACCCTGAAG
TTCCAGAGCGAAAACCAAAG

3. repV sequence from a plasmid > 300 kb from Cas122

TTCTCTTGATGATTTGAAACGCTATCTTGTTGCCTTCCTTAAGCTCCTGGTATTCAAGGTATCCA
ATGTCCTCCAGTTGCTTAAGTGCTTTTCTTACCGTTGAATTCTGAAGAGCAGGTCTGGAAGTGA
GGGACAAACGCTTCCTGAACCTCTCAATGCTGACAGGGATCGGATTAGGCGGCAGAGCCTCAA
TGAACGTATAAAGGGCCTGGGCTGACTCTTTCCTTGCAAGGGCTTTAAGGGCTCTCAGCTTAAG
TAGGATCTTGTATTCACCAACATACAGATCCTTAAGCGTTTTCGACGGAGTGAGGATAACTTTG
TCATTACCAATGTCCACGGTTGACTCACCAATGAGCTGAACAAATTTAGTGACTTCAATGTTTG
TGCCTCTGTCCTTGGAGAATTCAACAACATTAGACATCAGTTTCCTGAGCGAATCTTTGATTTTT
TCCCGAAATTCA

4. repV sequence from a plasmid from a 244 kb plasmid from Cas 127

GAGGGATTTCTCTTGATGATTTGAAACGCTATCTTGTTGCCTTCCTTAAGCTCCTGATATTCAAG
GTATCCAATATCCTCCAGTTGCTTAAGTGCTTTTCTTACCGTTGAATTCTGAAGAGCAGGTCTGG
AAGTGAGGGACAAACGCTTCCTGAACCTCTCAATGCTGACAGGGATCGGATTAGGTGGCAGAG
CCTCAATGAACGTATAAAGGGCCTGGGCTGACTCTTTCCTTGCAAGGGCTTTAAGGGCTCTCAG
CTTAAGCAGGATCTTGTATTCACCAACATACAGATCCTTAAGCGTTTTCGACGGAGTGAGGATA
ACTTTGTCATTACCAATGTCCACGGTTGACTCACCAATGAGCTGAACAAATTTAGTGACTTCAA
TGTTTGTGCCTCTGTCCTTGGAGAATTCAACAACATTAGACATCAGTTTCCTGAGCGAATCTTTG
ATTTTTTCCCGAAATTCAGCCTTCAGA

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