universität wien

DISSERTATION

Titel der Dissertation

„Characterization and detection of β-lactamases“

Verfasser

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angestrebter akademischer Grad

Doktor der Naturwissenschaften (Dr.rer.nat.)

Wien, 2013

Studienkennzahl lt. Studienblatt:	A 091 490
Dissertationsgebiet lt. Studienblatt:	Doktoratsstudium Molekulare Biologie
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Another brick in the wall.

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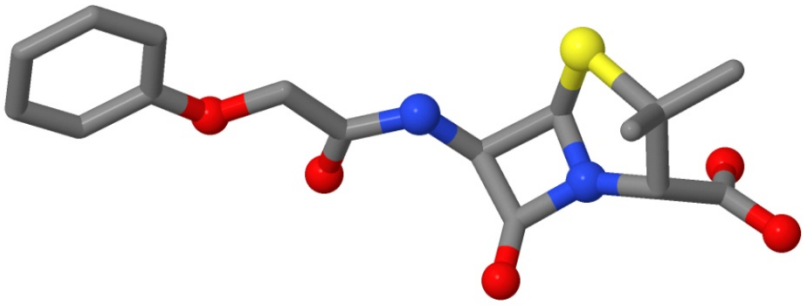
1 Introduction

The discovery of penicillin by Alexander Fleming in 1928 was a vital milestone in human history (Figure 1). It triggered a golden age giving mankind the opportunity to fight deadly diseases using antibiotics. Bacterial infections could be controlled easily and some optimistic minds saw this plague put in its place. The new bacterial control reagents also allowed modern surgery to develop rapidly because open wounds no longer represent perfect growth sites for bacteria. These factors, combined with artificial fertilizers, led to exponential human growth rates after World War II (1).

Fleming's finding had not only an impact on human medicine but also on agriculture and other fields. The productivity of livestock increased massively through the application of antibiotics. Furthermore, antibiotics helped to shape our modern view of life. As almost every biotechnological experiment uses antibiotics at a certain point to obtain the desired biological material, it is difficult to imagine how our understanding of life and biotechnology would have developed without antibiotics.

Figure 1. 3D-structure of penicillin. The chemical structure was drawn with Jmol.



Unfortunately, the reality in hospitals did not catch up with the optimistic expectations regarding the application of antibiotics. Only a few years after the discovery of penicillin, it became clear that bacteria can develop resistance mechanisms. Even more concerns were raised when Japanese researchers realized that bacteria can transfer these resistance mechanisms to different bacterial species. Although this discovery was

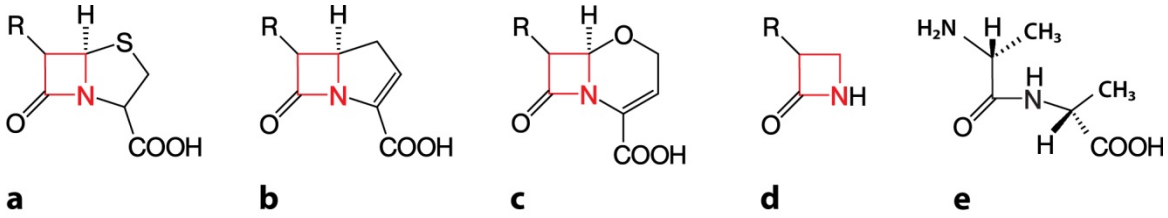
disappointing for clinicians, it was very exciting for biologists. It was the first demonstration that genetic information can be transferred between different species, independently of sexual or asexual reproduction.

The importance of this healthcare problem attracted many researchers to unravel the secrets behind antibiotic resistance mechanisms and their dissemination. Progress was rapid and the first mechanism deactivating penicillin was identified in 1940. An enzyme that hydrolyzed penicillin was isolated and many years later classified as an AmpC β-lactamase. It belonged to the very diverse group of β-lactamases.

1.1 β-Lactams

β-Lactam antibiotics share a common core structure, the β-lactam ring. They act as substrate analogs of D-alanyl-D-alanine, which is an important component of bacterial peptidoglycans. The highly reactive CO-N bond in the β-lactam ring shows structural similarity to the CO-N peptide bond in D-alanyl-D-alanine (Figure 2). This dipeptide, along with a transpeptidase, is involved in the cross-linking of peptidoglycan during bacterial cell wall synthesis. Due to their structural similarity, β-lactam antibiotics attach to the substrate-anchoring site of the transpeptidase responsible for cross-linking and bind covalently, thus inhibiting the enzyme. This process disables peptidoglycan cross-linking and activates a series of autolytic cell wall enzymes, muramidases, resulting in bacterial cell lysis (2;3).

Figure 2. β-Lactam core structures with the β-lactam ring in red. (a) Penam core (b) Carbapenem core (c) Oxacephem core (d) Monobactam core (e) D-alanyl-D-alanine



Based on four common chemical structures of the β-lactam ring, a nomenclature was established to classify the wide range of β-lactams. New β-lactam antibiotics can be designed by chemical modifications of the side chains of the β-lactam ring structure. The oldest antibiotics, the penicillins have a penam core and are further classified based

on their antimicrobial spectrum as narrow-, moderate-, broad-, or extended-spectrum penicillins (Figure 2a). Today, many pathogens have enzymes to degrade penicillins.

Carbapenems have a broad antimicrobial spectrum and are the last resort against microbial infections as antibiotic resistances are distributed in many pathogens. This class of β-lactams is highly resistant against degradation by β-lactamases (Figure 2b).

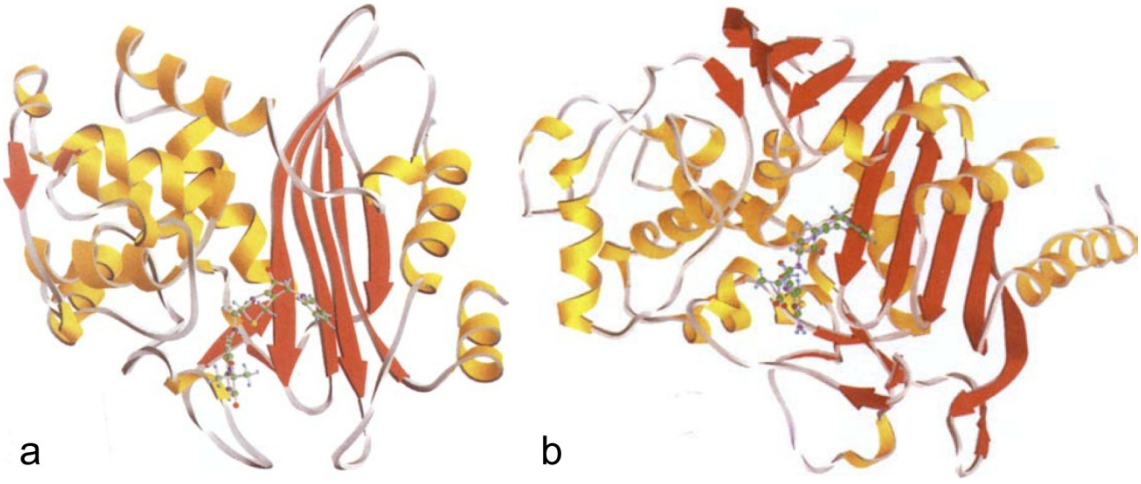
Cephalosporins have an unsatured six-membered ring and are more stable against enzymatic degradation than penicillins (Figure 2c). Four generations of these antibiotics have been developed so far.

Monobactams lack a ring that is fused to the β-lactam ring. They are stable against many β-lactamases but can only kill Gram-negative bacteria (Figure 2d) (4).

1.2 β-Lactamases

Enzymes that can degrade β-lactams are classified as β-lactamases. Many different unrelated β-lactamases have evolved and more than 950 unique naturally occurring enzymes exist (5). The first and partially still used classification schema of β-lactamases is based on the antibiotics that can be destroyed by these enzymes. For instance, enzymes that can cleave a carbapenem are designated as carbapenemases. This classification system is useful for clinicians but less practicable for scientific purposes, thus a more detailed classification was developed by Ambler (6). It is based on amino acid sequence similarity and comprises four classes (A-D). The class A, class C, and class D β-lactamases have a catalytic center with a serine playing an important role in the enzymatic reaction, whereas the catalytic mechanism of the class B β-lactamases is zinc-dependent. The amino acid sequences of the three classes of serine-based β-lactamases differ widely but they share similar 3D-structures (Figure 3) (7). Thus, Hall and Barlow were able to analyse the phylogenetic diversity of serine β-lactamases based on the protein structure similarity (8). The high sequence diversity of the serine β-lactamases is illustrated in Figure 4.

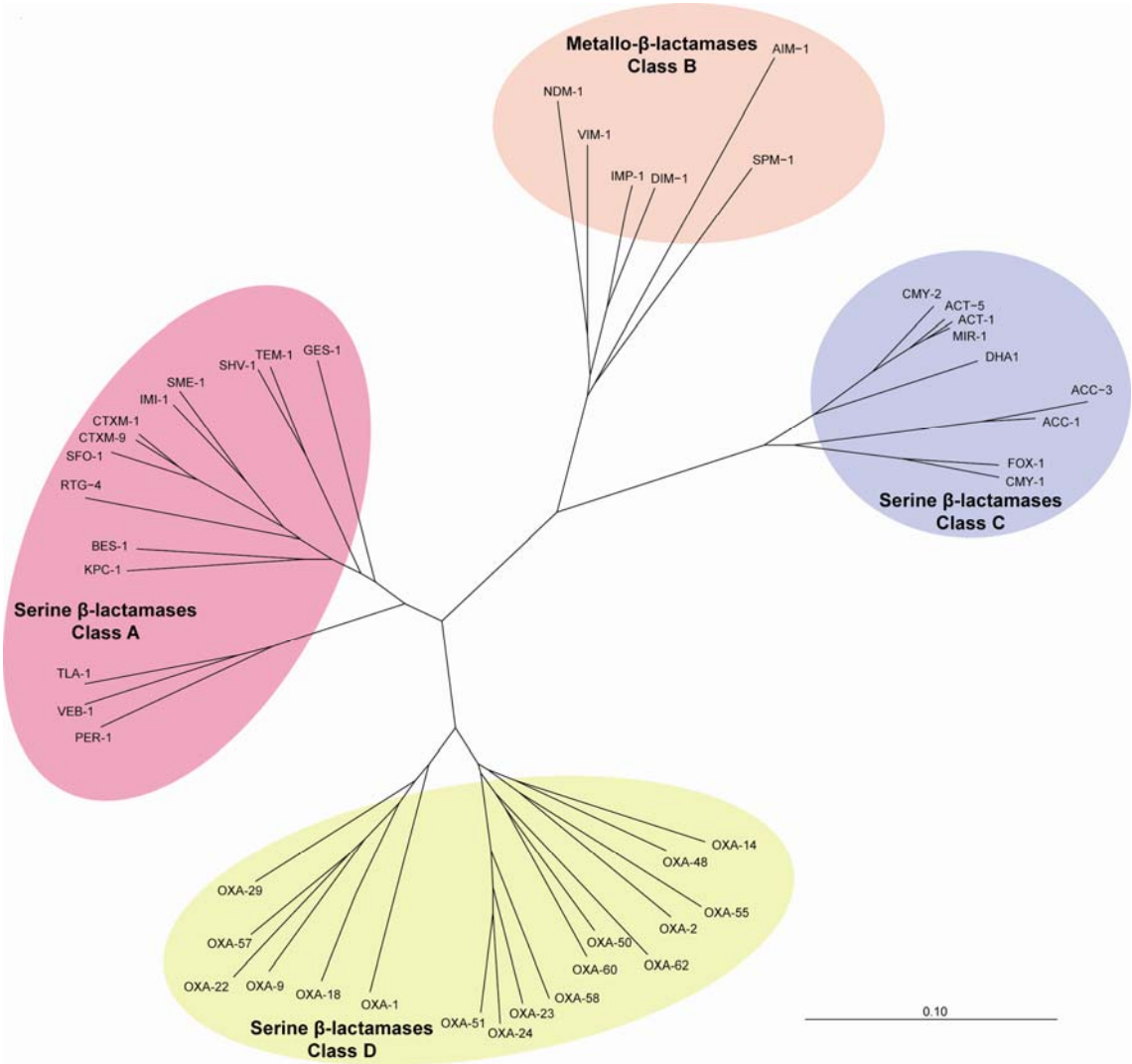
Figure 3. Ribbon diagrams of tertiary structures of β-lactamases. The helices are illustrated in yellow and β-strands in red. The β-lactam antibiotic ceftazidime is modelled in the enzyme binding sites. (a) The class A β-lactamase TEM-1 is shown. (b) The class C β-lactamase AmpC is illustrated. Diagrams were modified from Medeiros *et al.* (7).



The majority of the identified β-lactamases are of low clinical significance. It is believed that most antibiotic resistance genes are disseminated in the soil microbiom (9). Antibiotics and antibiotic resistance genes represent important parts in the inter- and intra-domain communication of microorganisms in various ecosystems (10). However, resistance genes can be transferred from environmental bacteria to human pathogens via horizontal gene transfer (HGT). These events can be hardly monitored in the environment in real time but are subsequently observed in patients infected with new drug resistant pathogens. It is obvious that the wide application of antibiotics in agriculture facilitates the HGT of resistance genes to human pathogenic bacteria because these species are also found in the intestine of other warm-blooded organisms. The treatment of animals with antibiotics and the application of their manure and slurry to fields have influenced the natural microbiological environments dramatically. The emergence of marine fish farming has led to the release of irresponsible amounts of antibiotics into marine environments (11). Furthermore, waste water from human settlements and especially from hospitals contributes to the enrichment of antibiotics in the environment. Thus, antimicrobials have been detected in groundwater as deep as >10m in regions with agricultural activity (12). Rivers and other surface waters are heavily contaminated with antibiotics (13). Consequently, the presence of pathogens,

antibiotics and the environmental microbiom in the same habitat catalyzes the HGT of antibiotic resistance genes to human pathogens. As humans and animals have intense contact to agricultural products and various types of waters, the uptake of these altered pathogenic bacteria cannot be avoided.

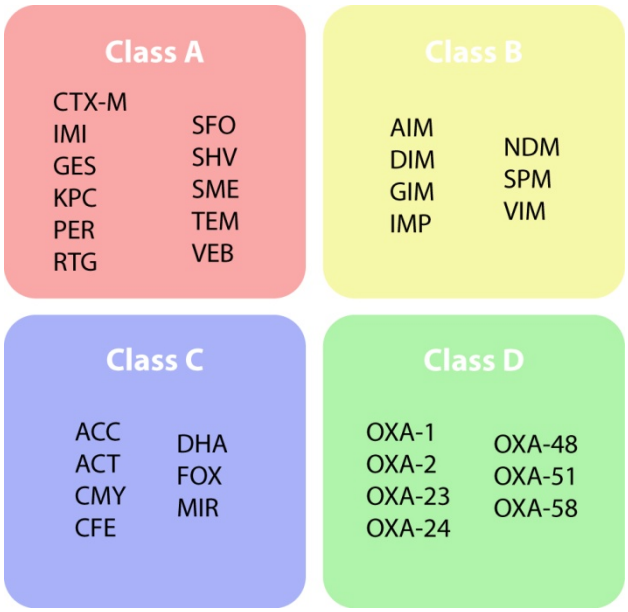
Figure 4. Phylogenetic tree based on the distance matrix based algorithm Neighbour Joining. Amino acid sequences of the clinically most relevant β-lactamases were used for the calculation. The thin bar indicates 10% sequence similarity. The non-homologous metallo-β-lactamases, which share no sequence similarity, were incorporated as out-group into the serine-β-lactamase alignment to illustrate the huge sequence diversity between and within the serine β-lactamases.



1.3 Substrate spectrum of β-lactamases

β-Lactamases have a wide substrate spectrum due to the high number of different enzymes, their huge diversity and the permanent mutations caused by selection pressure. However, β-lactamases within a distinct class have comparable enzymatic characteristics due to their structural and sequence similarity. The enzymatic activity is best understood for the clinically most relevant β-lactamases (Figure 5). Other β-lactamases are mainly of interest for environmental scientists. Thus, their enzymatic properties are less investigated. However, this changes until their emergence in human pathogens.

Figure 5. Clinically important β-lactamases.



The enzymes of class A are the most frequently encountered β-lactamases in bacterial isolates in hospitals. The genes of CTX-M, TEM or SHV are present in almost all β-lactam resistant isolates (14). The successful dissemination of these genes was possible due to their transfer to very mobile plasmids. Typically, bla_{SHV} is a chromosomal gene of the very frequent human pathogen *Klebsiella pneumoniae* (15). Furthermore, a very high sequence diversity is observed within this class leading to the widest substrate spectrum of all classes. Class A enzymes can be deactivated by the addition of β-lactamase inhibitors such as clavulanic acid (16). All β-lactam antibiotics can be deactivated by class A β-lactamases, including carbapenems which are often the last

resort to treat multidrug resistant infections. The class A KPC enzymes belong to the most frequently encountered carbapenemases in clinics. Treatment options for *bla*$_{KPC}$ associated infections remain limited. Infections with KPC producers lead to death rates higher than 50% (17;18). Genes belonging to class A have been identified on plasmids, transposons and chromosomes.

Metallo-β-lactamases (Class B) differ in many ways from the other classes. They have a different molecular structure, phylogeny and mode of action. All β-lactams except aztreonam are hydrolyzed by enzymes of this class (19). Metal chelators such as EDTA inhibit their activity whereas serine-β-lactamases remain unaffected by EDTA. Resistance against β-lactamase inhibitors are important features of metallo-β-lactamases. The large majority of class B enzymes have carbapenemase activity. The clinically most relevant enzymes are IMP, VIM and NDM (20). Death rates associated with metallo-β-lactamase positive pathogens range from 18% to 67% (21).

Class C enzymes are often referred to as AmpC β-lactamases. These serine-β-lactamases were initially only identified on chromosomes and not subject to rapid dissemination. They are phylogenetically very diverse and found in Gram-positive and Gram-negative bacteria. Thus, it is thought that they developed billions of years ago. Cephalosporins are the main substrate for these enzymes but they can also hydrolyze penicillins, cephamycins and monobactams at a rate of <1% of that of benzylpenicillin (22). However, the hydrolysis rate is not the only important factor but also the enzyme affinity to a substrate. Class C β-lactamases have thousand fold higher substrate affinities than other β-lactamases, which is important at a low antibiotic concentration. Carbapenemases have not been identified within this class so far. During the 1990s, class C β-lactamases with high spreading rates were identified. CMY and DHA are the clinically most relevant ones (23;24). The genes of these two enzymes are plasmid-encoded.

The ability to degrade isoxazolyl β-lactams like oxacillin was the reason to name the class D β-lactamases oxacillinases. This class shares the serine driven catalytic center with class A and C. Some class D enzymes can be inhibited by clavulanic acid (16). Today, a wide substrate spectrum has been reported, including carbapenems. However, hydrolysis of aztreonam was not reported yet. Although the sequence diversity is very

high within this class, all genes are named OXA. The clinically most relevant enzymes are OXA-1, OXA-23, OXA-24, and OXA-48 (25;26).

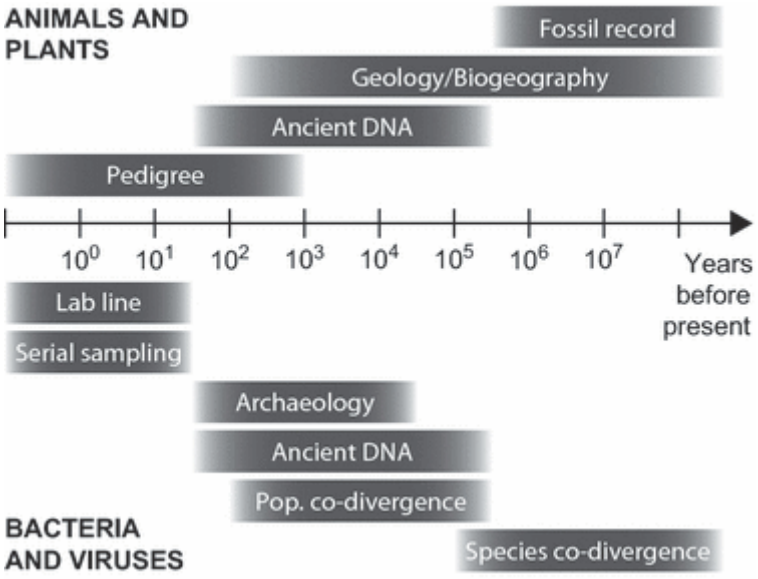
1.4 Phylogeny and molecular clocks

Today, the phylogeny of genes and organisms is determined with molecular tools. Marker genes, which are very old, and thus, present in all three domains of life (*Bacteria*, *Archaea* and *Eukaryota*) are used for this purpose. In many cases, they code for very important RNAs or proteins that are necessary for the survival of organisms. The most important marker genes code for rRNAs and proteins of the ribosome complex. Given their essential roles in metabolism, these genes evolved at a lower rate as mutations in these genes could affect the fitness of an organism more dramatically than those in non-essential genes. The low mutation rate is useful for researchers to identify sequence and structure homologies. Theoretically, the more similar gene sequences are, the more related are the organisms that harbour them. Various computer algorithms have been developed to calculate the similarity of sequences and visualize the information on phylogenetic trees (27).

However, the phylogenetic relationship is not the only evolutionary information hidden in DNA sequences. It is also possible to estimate the time point when two different sequences or organisms started to evolve from a common ancestor (28). Every DNA molecule underlies the environmental impacts that drive evolution, and thus, mutates. Given that on a long-term scale the number of mutations per time is constant, the starting point of the divergent evolution of two sequences can be calculated by counting the number of mutations. However, the main problem to perform these calculations is the lack of a reliable reference mutation rate (29). Every species has a different evolution rate due to a species-specific selection pressure and different environmental parameters. Furthermore, it needs a wide range of interdisciplinary data to calibrate these mutation rates (Figure 6). The comparison of different interdisciplinary records enables the optimization of the substitution rate for every species. Thus, the phylogenetic age of animals and plants can be calculated reliably. In contrast, fossil records of prokaryotes conserving species specific information are rare or lacking making it more difficult to estimate substitution rates. However, geochemical and isotopic data can also used to calibrate prokaryotic substitution rates. Sulfate reducing

prokaryotes are a good example to illustrate how the combination of geochemical, isotopic and phylogenetic data is used to calibrate the molecular clock. 3.47 billion years old microbial sulfur reduction was identified using geochemical and isotopic data (30). Phylogenetic analyses revealed that homologous enzymes performing the sulfur reduction are present in both prokaryotic domains; *Bacteria* and *Archaea* (31). Thus, the probability is high that the ancient sulfur reduction was catalyzed by a common precursor (32). The mutation rate of the sulfur reducing enzymes can be calculated based on the combined data.

Figure 6. The age ranges of several data types necessary to calibrate DNA mutation rates are shown. Illustration from Ho *et al.* (33).



Unfortunately for phylogenists, genetic information is not only transferred from one generation to the next but also between different unrelated species. This is especially in prokaryotes a frequently observed event and questions the significance of certain DNA based phylogenetic calculations. Different substitution rates in different organisms can also obscure the degree of relationship of organisms. The combination of a wide range of different genes allows the balancing of these errors and enabled the reconstruction of the tree of life (34;35).

1.5 Phylogeny of β-lactamases

Phylogenetic calculations can be performed with almost all DNA sequences because every gene is evolving. Functional genes that catalyze metabolic or katabolic reactions are also used for these analyses to investigate their roles in the environment. In the case of antibiotic resistance genes, it was necessary to learn more about the dynamics that drive the development of new resistances in order to incorporate prevention strategies. Because of their outstanding importance in medicine, the evolution of β-lactamases has been investigated in most detail. At least two unrelated enzyme-groups developed the ability to hydrolyze β-lactams; serine-β-lactamases and metallo-β-lactamases (Figure 4). These two groups do not share structure, sequence or mode of action so it is easy to conclude that the observed hydrolysis of β-lactams is the result of convergent evolution. The analysis of the phylogeny of serine-β-lactamases was more difficult because the different classes share no sequence similarity but have a similar enzymatic reaction center. Hall et al. used 3D structures of the proteins to reconstruct the phylogeny of β-lactamases based on the assumption that structures of proteins are more conserved than their sequences. They showed that the divergence of class C β-lactamases predated the divergence of the class A and class D enzymes. The ancient enzymes from which they originate are believed to be more than two billion years old (8;36).

The metallo-β-lactamases were also analysed based on their protein structures because no detectable sequence homology exists between two groups within this class. Structurally homologous proteins of *Bacteria*, *Archaea* and *Eukaryota* were included into the phylogenetic calculations and it was estimated that common metallo-β-lactamase ancestors may be older than 2 billion years. These incredible long time periods, during which metallo- and serine-β-lactamases had time to evolve, are the main reason why we face such a huge diversity in these enzyme classes today (37;38).

The ancestor molecule of the serine β-lactamases was probably involved in the bacterial cell wall synthesis. Penicillin-binding proteins (PBPs) and serine β-lactamases share several highly conserved amino acid sequences and seems to be homologous (7). They form the superfamily of penicillin-recognizing enzymes. The PBPs use D-alanyl-D-alanine as natural substrate which is part of bacterial murein. As mentioned previously, D-alanyl-D-alanine and the β-lactam core have a similar structure. Interestingly, all PBPs can also catalyze β-lactams to a small content (39). However, PBPs are the target

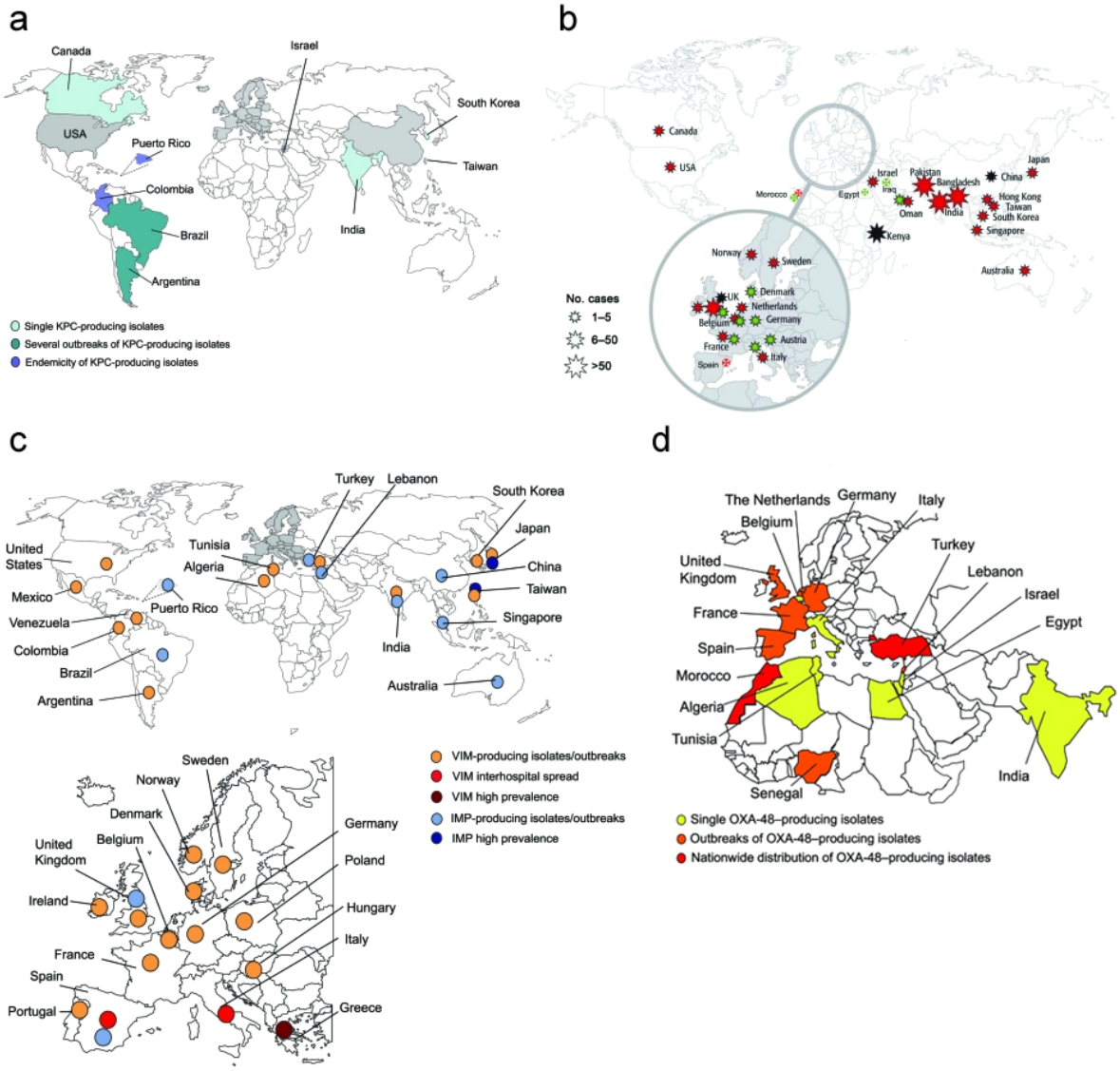
of action for β-lactam antibiotics. This phenomenon is an interesting example of the evolutionary dynamics behind the microbial interactions in various environments.

1.6 Clinically relevant β-lactamases

Enzymes of all four β-lactamase classes are present in human pathogens. Some of these enzymes have been integrated in chromosomes since millions of years, others were recently acquired. At the beginning of the antibiotic era and the subsequent spread of antibiotic resistance, it remained unclear whether the development of resistance was an ability that pathogens gained after admission of antibiotics or a widely distributed but dormant feature. The very first experiments showed that antibiotic sensitive bacteria could develop antibiotic resistance *in vitro*, so it was assumed that the global spreading resistance mechanisms rose due to the administration of antibiotics. With more detailed analyses of resistance mechanisms, it became clear that both assumptions were true. Bacteria could develop new antibiotic resistances and there was a large reservoir of phylogenetically old resistance genes that could be incorporated into pathogens via HGT. Analyses of resistance genes revealed that thousands of different resistance genes exist, in the case of the β-lactamases four unrelated groups with hundreds of members per group. However, although only a relative small fraction of all environmental β-lactamases has been identified so far, it has become clear that not all of them are clinically important. Most of the identified β-lactamases lack good spreading mechanisms, and thus, are only a local medical threat. In contrast, the few genes that managed to incorporate into efficient distribution vehicles are disseminated on all continents and challenge clinicians worldwide.

Today, the most spread β-lactamases associated with healthcare issues belong to class A. The globally most prevalent β-lactamases are TEM, SHV and the CTX-M group (40-42). The TEM and SHV genes exist in various variants differentiating only in a few amino acids. These variants of the firstly described TEM-1 and SHV-1 enzymes have changed substrate spectrums and are also referred to as extended spectrum β-lactamases (ESBL). In contrast, the CTX-M genes have a much higher sequence diversity. Hall et al. assume that the CTX-M genes diverged from a common ancestor 200-300 million years ago (43).

Figure 7. The worldwide distribution of the most prevalent carbapenemases is shown. Illustrations are taken from Nordmann *et al.* (44). (a) KPC producers are dominant in Latin America. (b) The dissemination of NDM-1 producers is shown. Red stars indicate pathogens traced back to India; Green stars show infections traced back to the Balkan states or the Middle East; and black stars of unknown origin. (c) The incidence of metallo-β-lactamase producers with carbapenem activity is shown. (d) OXA-48 producers are illustrated.



As many hospitals are turning to carbapenems due resistance to other β-lactams, carbapenemases are also becoming more distributed (Figure 7). The class A enzyme KPC has been isolated in Europe, North and South America and Asia. Carbapenemases of class B (IMP, VIM, and NDM-1) and class D (OXA-48) are also of high clinical importance. Especially NDM-1 has gained worldwide public interest because of its very

broad substrate spectrum and a very effective dissemination mechanism. Since its discovery in 2008 in Sweden from an Indian patient, it was identified within two years on all continents except South America (45).

CMY-2 and DHA-1 (Class C) are also regularly identified in clinical isolates but they represent a smaller medical threat due to their substrate spectrum. The same applies to a number of class D β-lactamases (OXA-1, OXA-23, OXA-24). Many other β-lactamases have been identified only in a few countries and are only of local interest. For the sake of completeness and the unpredictable evolution, these enzymes still have to be considered in analyses and molecular diagnostic tests (Figure 5).

Interestingly, the prevalence of resistance genes underlies dynamic processes which could be observed in the dissemination patterns of TEM and CTX-M-15 (46). During the 1990s, TEM was the dominant β-lactamase in β-lactam resistant pathogens. New antibiotics were administered due to increasing resistance but bacteria reacted soon and developed new β-lactamases with wider substrate spectrum. In the 2000s, CTX-M-15, which has a wider substrate spectrum than TEM-1 and also hydrolyses cephalosporins, was in many clinical studies the most prevalent β-lactamase. Now, most isolates have a gene cassette with different resistance genes that can deactivate a wide spectrum of antibiotics. Surprisingly, the presence of resistance genes correlates with reduced fitness of bacteria. This seems to be a reason why some resistance genes are lost again and the resistance genotypes change (47-49).

1.7 Detecting β-lactamase genes

The gold standard in clinical laboratories to characterize pathogens and determine their antibiotic resistance are cultivation based methods. The isolated bacteria are inoculated on agar plates or liquid media directly from the patient sample. In hospitals, disc diffusion tests on agar plates or fully automated commercial high throughput systems are used to test bacterial isolates whether antibiotic resistances are present. The advantages of the cultivation based methods are low cost and high sensitivity. Tremendous disadvantages of these methods are that they last rather long and give potentially false results due to false growth conditions and antimicrobial testing. The detection of mycobacterial growth causing tuberculosis for example needs between 4 to 8 weeks, skilled personal and a high complexity bacteriology laboratory (50). However, some infections need

rapid treatment and clinicians must be provided with the characteristics of the infections as fast as possible (51). Furthermore, studies have shown that also the cultivation automatization is error-prone. Blood cultures from automated culture detection systems were subsequently analysed manually and obtained in 3% to 40% of the cases contradictory results (52;53).

Diagnostic tests using molecular biological techniques retrieve data very fast and have in some cases higher sensitivity than cultivation based methods because they can also detect DNA from dead cells. PCR, real-time PCR and microarrays are the most widely used methods to characterize pathogens. Limitations of these techniques are lower specificity, higher cost and longer hands-on time. Commercial high-throughput systems can meet the demands regarding cost and hands-on time but specificity remains a problem.

A general problem is that DNA based tests can only identify resistance targets that have been described previously. Unfortunately, new antibiotic resistance genes are identified continuously. Antibiotic resistances are transferred rapidly on mobile genetic elements and plasmids between different species, and thus, evolve rapidly. The main genetic reservoir for this exchange system lies in the genomes of billions of environmental prokaryotes. Furthermore, the selection pressure caused by the application of antibiotics leads to mutations in existing resistance genes altering their substrate spectrum. However, even if the resistance genotype was correctly identified, it does not mean that the resistance phenotype is known. Whole genome sequencing of pathogens has revealed that the resistance genotype and phenotype are not necessarily identical. Various factors are involved in the expression of antibiotic resistance genes and due to limited technical possibilities in hospital laboratories, it remains unclear if and how strong the resistance genes are expressed.

The second appreciable problem regarding specificity of molecular techniques is of technical nature. More than 950 unique β-lactamases have been reported at present. Methods to detect this huge sequence diversity with a single test are rare and expensive. PCR based reactions cannot detect more than six different β-lactamase genes per reaction (54). Microarrays can detect thousands of different targets but DNA has to be pre-amplified in separate reactions (55). Thus, much effort is put into the development of new methods that can identify thousand of different genes in one reaction.

1.8 β-Lactamases in Austria

A very important discovery concerning the problem of antibiotic resistance was the description of HGT which explained the mechanism responsible for the spread of antibiotic resistances between different bacterial species (56). A further trigger for the revealing of antibiotic resistance mechanisms was the work of Ambler et al. (57), publishing amino acid sequences of a set of antibiotic resistance enzymes destroying penicillin, and introducing a classification scheme based on their sequence similarity. Although more than 950 unique, naturally occurring β-lactamases have been described so far, studies addressing this huge genetic diversity in clinical environments are rare (48). In Austria, research mainly focused on the presence of the β-lactamases TEM, SHV, CTX-M, and OXA-1, and recently on the carbapenemases KPC, VIM, and NDM identifying those enzymes in clinical isolates. Thus, the total β-lactamase dissemination in hospitals remained unclear from these reports because researchers were often focused on specific, interesting case reports.

Nevertheless, specific genetic information about the molecular mechanisms responsible for the resistances would be useful. Although the dissemination of ESBL and AmpCs all over the world is well documented, the majority of the studies have not sequenced the detected genes. For clinicians, this sequence information is of low interest, but the dynamics of evolution and distribution of β-lactamase genes can only be understood if a dense network of genetic information from important urban areas is available. Another more important factor for clinicians concerns molecular detection assays. As point mutations can alter the substrate spectrum of β-lactamases significantly, assay developers need detailed sequence information of the disseminated resistance genes in order to focus their assay design on the clinically most relevant targets.

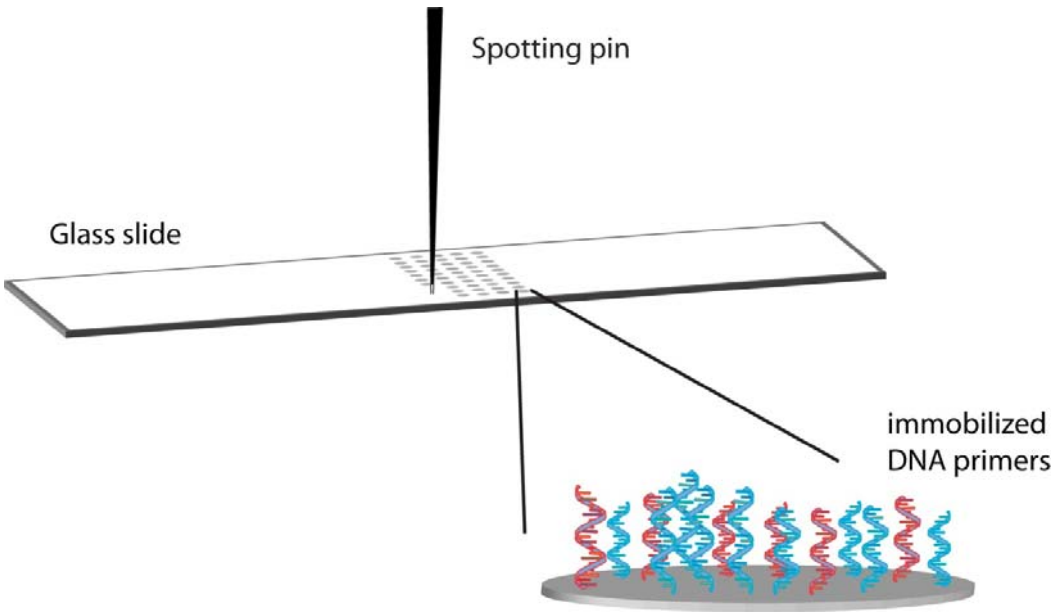
Molecular analyses detecting many genes in hundreds of isolates face the problem how to evaluate the significance of the obtained data. Ecologists have a similar problem when they analyse an environment with the need to estimate the number of different species in the sampled area. Thus, I introduced statistical models such as the species richness estimators ACE and Chao1 into the field of clinical microbiology in order to estimate the number of different β-lactamases in the investigated hospitals. These algorithms use the observed abundance of species to perform their calculations and can equally be used to calculate the incidence of genes. They have already been used to

estimate the number of a wide range of different genes and gene cassettes in various environments (58-60). Rarefaction curve analyses provide information about the significance of these estimations (61-63).

1.9 On-Chip PCR

Soon after the ingenious invention of PCR, researchers tried to immobilize this reaction on a solid support (64). This process was driven by two ideas; multiplex-PCR and PCR product identification. The main limitation of PCR is that it cannot amplify many different DNA products in a single reaction. Much effort has to be put into the development of multiplex PCR assays with more than ten DNA targets (65). Cross-reactions of primers targeting different genes lead to unspecific DNA amplificates, which out-compete the desired PCR products. On-chip PCR, also referred to as solid phase PCR, circumvents this problem because the primer pairs are physically isolated from each other. Every primer pair is immobilized on a distinct spot on a solid support making it impossible for the amplification product to interact with a primer pair targeting another gene (Figure 8). Thus, no unspecific primer can attach during the PCR to the immobilized amplification products.

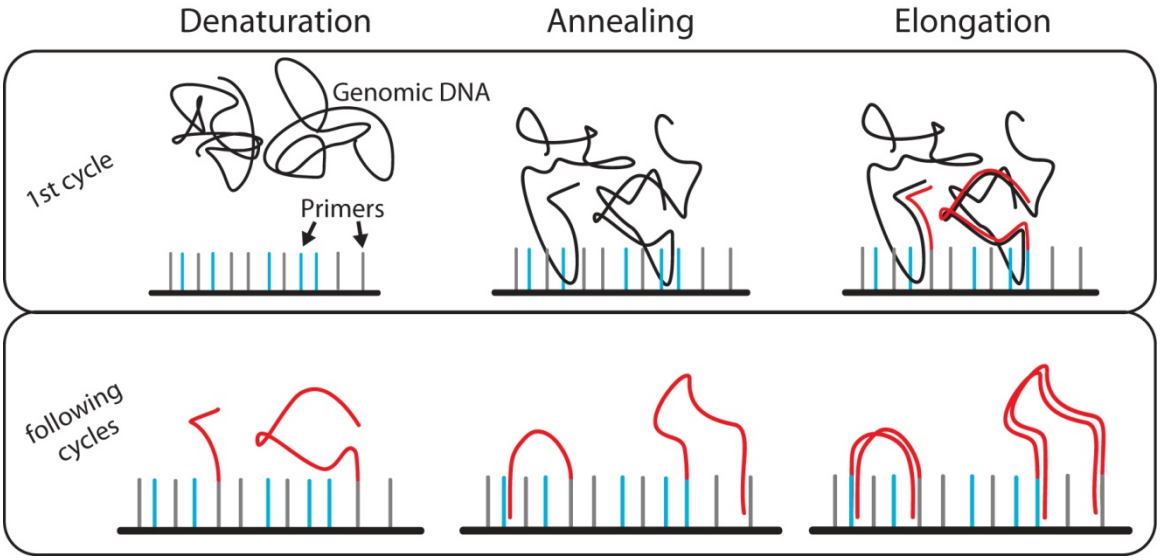
Figure 8. Schematic illustration of an on-chip PCR slide



The immobilization of primers has also another large advantage in comparison to liquid phase PCR. In most cases, the identification of the amplification products of a PCR is conducted with an agarose gel by size separation in a gel electrophoresis unit. This is time consuming work and thus, very unfavourable in clinical laboratories. Furthermore, amplification products of similar or equal size cannot be distinguished with ordinary gel electrophoresis. On-chip PCR uses immobilized primer pairs that are spotted to defined areas. The resulting amplification products can be labelled and identified according their position on the solid support after on-chip PCR. Microarray technology allows thousands of different amplification products to be analysed. These features make on-chip PCR a very promising technology.

The reaction mechanism of the on-chip PCR is analogous to the general liquid phase PCR. Thermal cycling is conducted to denaturate, anneal and elongate DNA (Figure 9). The elongation reaction of the on-chip PCR is also known as bridge-amplification and differs in some points from the elongation reaction of the liquid phase PCR. As the on-chip amplification reaction is immobilized on a planar surface, it is two dimensional. Thus, the amplification of DNA can only occur at the edges of a DNA colony if no free primers are left within the colony (66). Additionally, steric hindrance of immobilized amplification products further reduces the PCR efficiency.

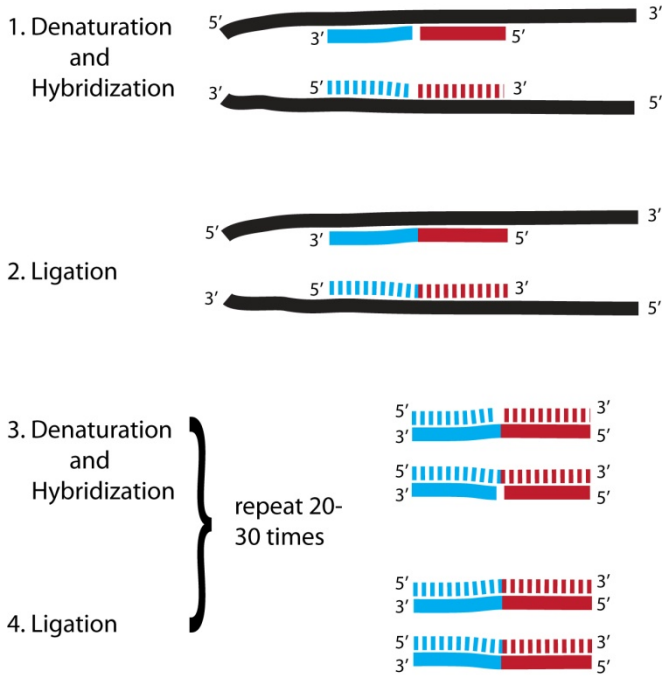
Figure 9. Schematic overview of the reaction mechanism of an on-chip PCR



1.10 Ligation mediated detection

A problem of PCR based detection methods is that unspecific PCR products are amplified after unspecific binding of DNA to primers. In some cases, the unspecific PCR products can out-compete the intended products for reaction reagents because they are thermodynamically more favoured and exponentially amplified. An alternative approach circumventing this problem is offered by ligation based detection methods (67). Ligases are implemented in these reactions and contribute to a higher specificity in comparison to PCR. These enzymes have a higher proof reading ability than the polymerases used in PCR. A wide range of methods using ligases for detection purposes has been developed. The Ligase Chain Reaction (LCR) and the padlock probes are the most prominent of these methods.

Figure 10. Schematic overview of reaction mechanism of ligase chain reaction.



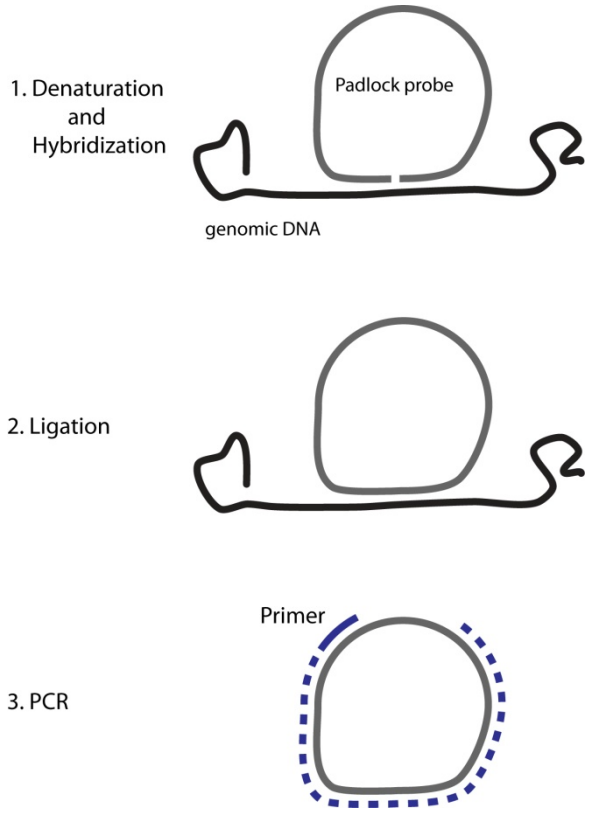
The LCR was shortly introduced after PCR but has been used rarely in diagnostic tests. The main mechanism of the reaction is based on the ligation of two probes annealing to the same DNA strand (Figure 10). After the first ligation step, DNA is denaturated again following a second annealing reaction. Complementary probes bind to the ligation product and are ligated as well. This process is repeated 20 to 30 times leading to an

exponential increase of the ligation product. Many variants of this technique (LDR, pLCR, G-LCR) were developed due its ability to detect short nucleotide polymorphisms (SNP), which are difficult to identify with PCR (68).

Another important ligation based detection method uses padlock probes and was published by Nilsson *et al.* in 1994 (69). These probes circularize when they bind to a DNA target (Figure 11). If they perfectly match to the DNA target, the padlock probes are ligated. The circularized probes are further amplified by PCR. An alternative amplification mode is rolling circle amplification (RCA) using the phi29-polymerase. Padlock probes are widely used for the detection of SNP (70).

The major disadvantage of ligation dependent methods is that complex enzymatic cascades are necessary to detect and amplify DNA targets. Furthermore, the sensitivity is lower compared to PCR.

Figure 11. Schematic overview of reaction mechanism using padlock probes.



1.11 Aims of this work

The dissemination of β-lactamases represents a serious health care problem in Austria and other countries. The investigation of recent trends in their evolution and distribution contributes massively to a better understanding of these phylogenetically very old classes of enzymes. In this work all in public DNA databases deposited genes of β-lactamases were downloaded and used for analyses. The aim was to establish a β-lactamase database and create a new nucleotide and amino acid sequence alignment. The design of primers and probes was based on this database. Re-evaluations of the phylogeny of β-lactamases, geographic dissemination and affiliations of distinct β-lactamases to particular species were also performed.

Besides the global trends regarding β-lactamases also the local resistance situation in Austria was of particular interest in this work. Very detailed screenings of bacterial patient samples from hospitals in Graz and Vienna were performed to reveal the actual presence of β-lactamases in these environments.

The main goal of this thesis was to develop a molecular method to detect β-lactamases. The determination of targets was based on the epidemiological data from clinics worldwide. The molecular method should comprise high sensitivity, high specificity, multiplex detection, fast data retrieval and a low hands-on-time in the clinical use. Since bacterial infections are very widespread in developing countries, which are thus the biggest markets for antibiotic resistance detection, low cost per analysis was also considered during development.

2 Material and Methods

2.1.1 Bacterial strains used for on-chip PCR

The bacterial strains *Klebsiella pneumoniae* DSM 16609 and *Escherichia coli* DSM 22316 were ordered from the Leibnitz-Institute DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Germany). Both strains had slightly different β-lactamases DNA sequences of bla_{TEM-1}. They were grown at 37°C in Caso-Bouillon medium (50 µg/ml ampicillin) overnight. The inoculations were used for DNA-extractions and gylcerol-stocks for long-time storage. One millilitre of the overnight culture was transferred in a cryo-tube and mixed with 500 µl glycerol. The tubes were stored at -80°C after 30 minutes of incubation at 37°C.

2.1.2 Bacterial isolates from the Medical University of Graz

At the Bacteriology and Mycology Laboratory of the Medical University Graz, 2177 ESBL-positive clinical isolates of *Enterobacteriaceae*, sent from different south-eastern Austrian hospitals to the university laboratory, were phenotypically identified in 2009 and 2010. They were further analysed for antibiotic susceptibility with in-house made Mueller-Hinton agar diffusion tests, the Vitek 2 system (bioMerieux, France), and the Etest (bioMerieux). For this study, 33 consecutive ESBL-positive bacterial strains (27 E.coli and 6 Klebsiella pneumoniae strains) were characterized. Prior DNA extraction, the isolates were grown at 37°C in LB-medium overnight.

2.1.3 Bacterial isolates from the General Hospital of Vienna

The Divison of Clinical Microbiology of the Medical University and General Hospital of Vienna collected 108 consecutive clinical isolates tested positive for ESBLs, AmpCs and carbapenemase between January and September 2011. Phenotypic antibiotic susceptibility testing of clinical isolates was performed using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) disc diffusion methodology and

EUCAST Clinical breakpoints (versions 1.2 and 1.3, The European Committee on Antimicrobial Susceptibility Testing - EUCAST 2011) on in-house made Mueller-Hinton agar plates. In case of resistance against third generation cephalosporins and/or cefoxitin in disc diffusion testing, *Enterobacteriaceae* known to possibly harbour plasmid-encoded AmpC enzymes were screened for the presence of AmpC β-lactamases by a combined-disc test (AmpC ID Confirm Kit; Rosco Diagnostica A/S, Denmark) according to manufacturer´s instructions. In organisms that produce an inducible chromosomal AmpC β-lactamase, identification alone was indicative of AmpC production (71). As in primary disc diffusion testing, extended-spectrum cephalosporins were put around a central amoxicillin-clavulanate disc in a way to enable inhibitor based synergisms. ESBL-producing *Enterobacteriaceae* could be recognized based upon observation of this defining criterion (72). In case of cefoxitin resistance and lacking synergism phenomena, a 20 mm-double-disc synergy test between an amoxicillin-clavulanate and a cefepime disc was performed to avoid missing ESBL phenotypes masked by concomitant AmpC production (73). According to EUCAST antimicrobial wild-type distributions of microorganisms (http://mic.eucast.org/Eucast2/SearchController/search.jsp?action=performSearch&BeginIndex=0&Micdif=dif&NumberIndex=50&Antib=177&Specium=1&Discstrength=10), the meropenem inhibition zone diameter ≤0.25 mm in primary disc diffusion testing was chosen to screen out isolates with elevated carbapenem minimal inhibitory concentrations (MIC), which had to undergo confirmatory phenotypic testing in cases where meropenem Etest (bioMérieux, Marcy-l'Étoile, France) revealed MICs ≥0.5 mg/l (http://mic.eucast.org/Eucast2/SearchController/search.jsp?action=performSearch&BeginIndex=0&Micdif=mic&NumberIndex=50&Antib=177&Specium=-1). Ambler class A β-lactamases, metallo-β-lactamases, or AmpC β-lactamases in combination with efflux pumps or reduced permeability were recognized phenotypically by KPC + MBL Confirm ID Kit (Rosco Diagnostica A/S), while Ambler class D β-lactamases where detected by the modified Hodge test (74).

For this study, 108 β-lactamase-positive clinical strains were genetically characterized. 56 ESBL-positive bacterial strains (35 *E. coli*, 16 *K. pneumoniae*, three *Klebsiella oxytoca*, one *Morganella morganii*, and one *Serratia marcescens*), as well as 47 isolates

phenotypically tested positive for AmpC β-lactamases (20 *E. coli*, 19 *K. pneumoniae*, three *Enterobacter cloacae*, two *Klebsiella oxytoca*, two *Proteus mirabilis*, and one *Citrobacter youngae*) and five carbapenem resistant strains (three *K. pneumoniae*, two *Enterobacter cloacae*).

Sample Number	Patient ID	Birthdate of patient	Bacterial species	Source	Phenotypic resistance characterization	Sample date
1	H 766	26/06/1945	*Escherichia coli*	Urine	ESBL	14/01/2011
2	A 3490	20/09/1987	*Escherichia coli*	Bronchial secretion	ESBL	21/04/2011
3	H 4720	18/01/1982	*Escherichia coli*	Urine	ESBL	17/03/2011
4	H 3984	29/01/1934	*Klebsiella pneumoniae*	Urine	ESBL	07/03/2011
5	H 4264	04/07/1947	*Escherichia coli*	Urine	ESBL	10/03/2011
6	h 226	29/03/1928	*Escherichia coli*	Urine	ESBL	05/01/2011
7	H 4464	19/04/1932	*Klebsiella pneumoniae*	Urine	ESBL	14/03/2011
8	A 3794	29/10/1992	*Serratia marcescens*	Urine	ESBL	30/04/2011
9	H 3584	12/08/1942	*Escherichia coli*	Urine	ESBL	28/02/2011
10	A 860	30/03/1942	*Klebsiella pneumoniae*	Bronchial secretion	ESBL	28/01/2011
11	RV 53196	21/05/1947	*Klebsiella pneumoniae*	Urethra	ESBL	30/03/2011
12	V 28	19/07/1941	*Escherichia coli*	Swab	ESBL	04/01/2011
13	H2701	15/11/1948	*Escherichia coli*	Urine	ESBL	14/02/2011
14	F 1757	10/02/1957	*Escherichia coli*	Stool	ESBL	16/03/2011
15	V 52368	16/11/1937	*Morganella morganii*	Stool	ESBL	09/03/2011
16	H 7117	27/07/1952	*Klebsiella pneumoniae*	Urine	ESBL	26/04/2011
17	F 125	19/10/1949	*Escherichia coli*	Stool	ESBL	11/01/2011
18	V 2462	19/12/1987	*Escherichia coli*	Swab	ESBL	21/02/2011
19	V 52410	25/05/1958	*Escherichia coli*	Stool	ESBL	09/03/2011
20	V 5352	24/02/1949	*Klebsiella pneumoniae*	Swab	ESBL	19/04/2011
21	V 53508	10/02/2009	*Escherichia coli*	Anal swab	ESBL	06/04/2011
22	F 705	06/01/2011	*Klebsiella pneumoniae*	Stool	ESBL	02/02/2011
23	V 1565	01/12/1981	*Escherichia coli*	Swab	ESBL	04/02/2011
24	F 2044	27/03/2006	*Escherichia coli*	Stool	ESBL	29/03/2011
25	V 4743	28/02/1943	*Escherichia coli*	Swab	ESBL	05/04/2011
26	RV 53776/2	15/02/1938	*Klebsiella oxytoca*	Stool	ESBL	15/04/2011
27	RV 53776/1	15/02/1938	*Escherichia coli*	Stool	ESBL	13/04/2011
28	H 629	13/02/2009	*Escherichia coli*	Urine	ESBL	13/01/2011
29	V 52028/1	29/07/2022	*Escherichia coli*	Anal swab	ESBL	28/02/2011
30	V 1145	26/11/1951	*Escherichia coli*	Drain fluid	ESBL	28/01/2011
31	H 1973	09/04/1925	*Escherichia coli*	Urine	ESBL	01/02/2011
32	H 6091	04/08/1944	*Escherichia coli*	Urine	ESBL	11/04/2011
33	A 283	31/12/1983	*Klebsiella pneumoniae*	Bronchial lavage	ESBL	19/01/2011
34	K 359	15/08/1943	*Escherichia coli*	Vena cava	ESBL	03/02/2011

35	V 1745	18/09/1979	*Escherichia coli*	Swab	ESBL	08/02/2011
36	B 4381	27/10/1939	*Escherichia coli*	Blood	ESBL	11/03/2011
37	RV 50505	02/12/1952	*Escherichia coli*	Urine	ESBL	18/01/2011
38	RV 54111	09/04/2011	*Escherichia coli*	Anal swab	ESBL	22/04/2011
39	RV 54111	09/04/2011	*Klebsiella pneumoniae*	Anal swab	ESBL	22/04/2011
40	V 5759	06/09/1941	*Escherichia coli*	Swab	ESBL	26/04/2011
41	F 2719	06/09/1941	*Klebsiella pneumoniae*	Stool	ESBL	26/04/2011
42	V 2072	09/05/1942	*Escherichia coli*	Swab	ESBL	14/02/2011
43	V 3649	21/09/1946	*Klebsiella pneumoniae*	Swab	ESBL	18/03/2011
44	F 21793	17/02/2011	*Klebsiella pneumoniae*	Stool	ESBL	21/03/2011
45	H 3176	27/05/1928	*Escherichia coli*	Urine	ESBL	22/02/2011
46	V 52686	23,061,953	*Klebsiella pneumoniae*	Stool	ESBL	21/03/2011
47	H 3365	01/05/1997	*Klebsiella oxytoca*	Urine	ESBL	25/02/2011
48	V 52157	24/07/1955	*Klebsiella pneumoniae*	Stool	ESBL	02/03/2011
49	H 992	09/05/1919	*Escherichia coli*	Urine	ESBL	18/01/2011
50	RV 50148/2	20/07/1931	*Klebsiella pneumoniae*	Stool	ESBL	12/01/2011
51	V 5102	11/01/1962	*Escherichia coli*	Swab	ESBL	11/04/2011
52	H 705	17/03/1927	*Escherichia coli*	Urine	ESBL	14/01/2011
53	H 1017	08/09/1940	*Klebsiella pneumoniae*	Urine	ESBL	24/01/2011
54	H 1317	08/09/1940	*Escherichia coli*	Urine	ESBL	24/01/2011
55	H 3491	21/04/1947	*Escherichia coli*	Sinus maxillaris	ESBL	25/02/2011
C1	V 12479	09/12/1938	*Klebsiella pneumoniae*	Swab	AmpC	07/09/2011
C2	V 12454	04/05/1947	*Klebsiella pneumoniae*	Swab	AmpC	07/09/2011
C3	F 5733	22/12/1921	*Enterobacter cloacae*	Stool	AmpC	05/09/2011
C4	H 14750	30/12/1938	*Klebsiella oxytoca*	Urine	Unclear	05/09/2011
C5	H 14684	21/02/2008	*Escherichia coli*	Urine	AmpC	01/09/2011
C6	V 12175	24/01/1969	*Klebsiella pneumoniae*	Swab	AmpC	31/08/2011
C7	H 14440	09/02/1964	*Escherichia coli*	Urine	AmpC	30/08/2011
C8	F 5514	19/10/1987	*Enterobacter cloacae*	Stool	Carbapenemase	26/08/2011
C9	A 7066	25/01/1923	*Klebsiella pneumoniae*	Bronchial lavage	AmpC	26/08/2011
C10	H 14214	27/03/1942	*Escherichia coli*	Urine	AmpC	25/08/2011
C11	F 5471	13/08/2011	*Escherichia coli*	Stool	AmpC	23/08/2011
C12	V 11693	28/02/1959	*Klebsiella pneumoniae*	Swab	AmpC	22/08/2011
C13	H13806	02/05/1954	*Escherichia coli*	Urine	AmpC	17/08/2011
C14	H13715	14/09/1951	*Escherichia coli*	Urine	AmpC	16/08/2011
C15	H13681	24/06/1988	*Escherichia coli*	Urine	AmpC	16/08/2011
C16	RV 58163	30/06/1933	*Escherichia coli*	Swab	AmpC	16/08/2011
C17	F 5304	01/06/1959	*Escherichia coli*	Stool	AmpC	15/08/2011
C18	H 13518	19/06/1940	*Escherichia coli*	Urine	AmpC	12/08/2011
C19	V 11232	12/03/1963	*Klebsiella pneumoniae*	Swab	Carbapenemase	11/08/2011
C20	F 5212	29/06/2011	*Klebsiella pneumoniae*	Stool	AmpC	10/08/2011
C21	V 11075	19/05/1943	*Klebsiella pneumoniae*	Dialysate	Carbapenemase	08/08/2011
C22	H 13116	04/09/1963	*Escherichia coli*	Urine	AmpC	05/08/2011
C23	V 10838/2	11/02/1941	*Klebsiella oxytoca*	Omentum	Unclear	04/08/2011

C25	A 6409	24/11/1958	*Klebsiella pneumoniae*	Tracheal secretion	AmpC	03/08/2011
C26	H 12814	15/10/1942	*Klebsiella pneumoniae*	Urine	AmpC	02/08/2011
C27	F 4967	28/01/1949	*Citrobacter youngae*	Stool	AmpC	01/08/2011
C28	H 12895	25/09/1928	*Proteus mirabilis*	Urine	AmpC	01/08/2011
C29	H 12889	24/04/1968	*Escherichia coli*	Urine	AmpC	01/08/2011
C30	B 12459	10/03/1959	*Klebsiella pneumoniae*	Blood	AmpC	29/07/2011
C31	A 6286	03/03/1959	*Klebsiella pneumoniae*	Bronchial secretion	AmpC	27/07/2011
C32	V 10239	04/04/1954	*Enterobacter cloacae*	Swab	Carbapenemase	21/07/2011
C33	V 10096	19/12/1967	*Klebsiella pneumoniae*	Swab	AmpC	19/07/2011
C34	V 10071	29/04/1925	*Klebsiella pneumoniae*	Drain fluid	AmpC	19/07/2011
C35	A 6061	31/03/1945	*Escherichia coli*	Bronchial lavage	AmpC	19/07/2011
C36	F 4752	22/03/1982	*Escherichia coli*	Stool	AmpC	15/07/2011
C37	H 12025	22/01/1940	*Escherichia coli*	Urine	AmpC	14/07/2011
C38	V 9799	16/03/1954	*Klebsiella pneumoniae*	Swab	AmpC	11/07/2011
C39	V 57045/2	12/05/1975	*Klebsiella pneumoniae*	Swab	Carbapenemase	08/07/2011
C41	H 11789	06/12/1963	*Escherichia coli*	Urine	AmpC	08/07/2011
C42	H 11574	27/06/1923	*Escherichia coli*	Urine	AmpC	06/07/2011
C43	V 9493	15/09/1972	*Klebsiella pneumoniae*	Swab	AmpC	05/07/2011
C44	H 9973	04/02/1928	*Klebsiella pneumoniae*	Urine	AmpC	13/06/2011
C45	V 9270	25/11/1963	*Klebsiella oxytoca*	Swab	ESBL	04/07/2011
C46	V 9304	24/10/1954	*Enterobacter cloacae*	Urine	Unclear	02/07/2011
C47	F 4446	22/03/1982	*Escherichia coli*	Stool	AmpC	01/07/2011
C48	V 56853/2	10/02/1922	*Proteus mirabilis*	Skin	AmpC	01/07/2011
C49	A 5573	13/05/1999	*Klebsiella pneumoniae*	Sputum	AmpC	30/06/2011
C50	A 5538	06/10/1945	*Klebsiella pneumoniae*	Sputum	AmpC	29/06/2011
C51	V 56753	11/06/1981	*Escherichia coli*	Swab	AmpC	29/06/2011
C52	K 2102	06/03/1951	*Klebsiella pneumoniae*	Blood	AmpC	29/06/2011
C53	H 11020	02/06/1976	*Klebsiella pneumoniae*	Urine	AmpC	28/06/2011
C54	A 5470	13/05/1940	*Escherichia coli*	Bronchial lavage	AmpC	28/06/2011
C55	A 5148	14/07/1965	*Enterobacter cloacae*	Sputum	Unclear	17/06/2011

Table 1. List of the clinical samples from the General Hospital of Vienna that were analysed in the study

2.1.4 DNA extractions

One millilitre of the bacterial overnight culture was transferred in a microcentrifuge tube. Bacterial cells were centrifuged at 13,000 rpm for five minutes. For the on-chip PCR experiments and the characterization of clinical isolates from the Medical University of Graz, genomic DNA was extracted using the DNA Isolation Kit for Cells and Tissue (Roche Applied Science, Germany) according to the manufacturer's instructions. DNA concentrations were measured using the Nanodrop

spectrophotometer (Thermo Scientific, USA). If not otherwise stated, a dilution of the DNA extraction in ddH_2O at a ratio of 1 to 10 was used in subsequent experiments.

For the on-chip ligation and padlock probe experiments, dilution series of *E. coli* and *K. pneumoniae* isolates with known cell numbers per millilitre were prepared. Genomic DNA from every dilution was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, Germany) according the manufactures instructions. DNA was quantified with an Epoch spectrometer (BioTek, USA).

Isolates used from the General Hospital of Vienna were grown on BD Columbia blood agar plates with 5% sheep blood (BD Diagnostic Systems, USA) at 37°C overnight. Cells were transferred with an inoculation loop into 1.5 ml microcentrifuge tubes with 500 µl ddH_2O and incubated at 100°C for ten minutes, followed by plunging into liquid nitrogen for one minute. The thermal cell lysis was repeated two more times. Subsequently, the lysed cell suspensions were centrifuged at 13,000 rpm for one minute. The supernatant was used for the subsequent characterization experiments as DNA template.

2.1.5 DNA samples

Beside genomic DNA from bacterial strains, also PCR products of β-lactamase genes were used as template DNA in the experiments. The PCR products were obtained with the primers listed in Table 4 and Table 5. The genomic DNA from the clinical isolates analysed in this study served as template strands. The final primer concentration was 1 µM in the PCR mix. Reagents of the Mastermix 16S Basic PCR kit (Molzym, Germany) were used at a total volume of 10 µl. One microlitre of genomic DNA was added per reaction. Thermal cycling was carried out by an initial denaturation step at 95°C for five minutes, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds (annealing temperatures listed in Table 4 and Table 5), elongation at 72°C for 80 seconds, and a final elongation cycle at 72°C for ten minutes. Negative controls without DNA template were used in all experiments. PCR products were analysed by ethidium bromide stained agarose (2%) gel electrophoresis and visualized with the video documentation system BioSpectrum 310 (UVP, USA).

Reactions with only one visible DNA lane in the agarose gel were further processed using the MinElute PCR Purification kit (Qiagen, Germany) according the manufactures instructions. If multiple lanes were present, the lane with the PCR product of the expected size was cut out and subsequently purified using the MinElute Gel Extraction kit (Qiagen) according the manufacturer's instructions. The DNA was eluated with both purification kits in ddH_2O instead the supplied eluation buffer. The purified PCR products were quantified with an Epoch spectrometer (BioTek). Dilution series were prepared based on copy numbers of PCR products per µl in order to determine sensitivity in subsequent experiments.

2.1.6 Primer design

Prior to primer design, a sequence database with the most important β-lactamase genes was constructed. Nucleotide sequences were downloaded from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) and imported into the software package ARB (75). The DNA sequences were analysed with the software tools Mfold and Freak (Institut Pasteur "Centre d'Informatique pour la Biologie", http://mobyle.pasteur.fr/cgi-bin/portal.py#welcome). Mfold calculates DNA secondary structures of a nucleotide sequence and illustrates them in several graphical data files. As simulation parameters for the secondary structure calculations, conditions occurring during the PCR annealing step were chosen. Temperature was set at 55°C. The Na^+ concentration was 0.05 M, Mg^{2+} was 0.002 M. Freak visualizes the varying GC content of a nucleotide sequence.

Nineteen primers were chosen for on-chip PCR based on the probe design function of ARB. The primer design was further optimized with the NetPrimer software (http://www.premierbiosoft.com/netprimer/index.html) by identifying DNA secondary structures such as hairpins, dimers, and cross dimers. DNA sequences, primers targets, and amplification product lengths of the on-chip PCR primers are listed in Table 2. Primers were thiol-modified on their 5' end and purchased from Microsynth (Switzerland).

Primer name	Gene targeted	Sequence 5' 3' [a]	Length	Product size
Solid TEM 20F	TEM variants	TTTTTTTTTTGTGTCGCCCTTATTCCCTTTTT	22	depending on primer combination
Solid TEM 789R		TTTTTTTTTTTTCATCCATAGTTGCCTGACTCCC	24	
Solid TEM 233R		TTTTTTTTTTGCCCGGCGTCAACACGGGATAATACCG	27	
Solid TEM 140 F		TTTTTTTTTTTGGATCTCAACAGCGGTAAGATCCTTGAGAG	31	
Solid TEM 204R		TTTTTTTTTTCCACATAGCAGAACTTTAAAAGTGCTCA	28	
Solid TEM SS 521 F		TATTATTATTACGACGAGCGTGACACCACGATGCC	25	
Solid TEM SS 681 R		TATTATTATTCCAGCCGGAAGGGCCGAGCGCA	22	
Solid TEM SS 528 F		TATTATTATTGCGTGACACCACGATGCCTGC	21	
Solid TEM SS 729 R		TATTATTATTCCGCGAGACCCACGCTCACC	20	
Solid CTX-M SS 437 F Solid CTX-M SS 637 R	Variants of CTX-M-1 group	TATTATTATTTTGGCGGCCCGGCTAGCGTC TATTATTATTACCAGCTGCGCCCGTTGGCT	20 20	200
Solid SHV SS 228 F Solid SHV SS 553 R	SHV variants	TATTATTATTAGTGCTGGCGCGGGTGGATG TATTATTATTGGCCATGCTGGCCGGGGTAG	20 20	325
Solid OXA SS 62 F Solid OXA SS 213 R	Variants of OXA-1 group	TATTATTATTACAGCAGCGCCAGTGCATCAACA TATTATTATTTCTGGTGCCATTTGCGTTGCACACT	23 25	151
Solid OXA-1 55F Solid OXA-1 472R	Variants of OXA-1 group	TTTTTTTTTTATTATCTACAGCAGCGCCAGTGCAT TTTTTTTTTTCGAGCCATGCTTCTGTTAATCCGTT	25 25	417
Solid 45F++ Solid 1386R+	16S rDNA	TTTTTTTTTTGCYTAAYACATGCAAGTCGARCG TTTTTTTTTTCCGGGAACGTATTCACCG	23 18	1341

[a]Sequences according IUPAC nomenclature

Table 2. On-chip PCR primers designed in this study

To evaluate the specificity of the used on-chip PCR primers, a conventional liquid phase PCR in tubes had to be performed. PCR primers with the same DNA sequences like the on-chip PCR primers but lacking the polyT-spacer targeting only the β-lactamase gene of TEM-1 were designed (Table 3).

Primer name	Sequence 5' 3'	Length	Product size with TEM 20F	Product size with TEM 140F	Annealing temperature
TEM 20F	GTGTCGCCCTTATTCCCTTT	20			56°C
TEM 140 F	TGGATCTCAACAGCGGTAAGA	21			56°C
TEM 789R	TTCATCCATAGTTGCCTGACTC	22	769 bp	649 bp	56°C
TEM 233R	GGCGTCAACACGGGATAATA	20	213 bp	93 bp	56°C
TEM 204R	CCACATAGCAGAACTTTAAAAGTG	24	184 bp	64 bp	56°C

Table 3. Liquid phase primers used in on-chip PCR

For the detection of β-lactamases in liquid phase PCR, 25 primer pairs were designed with ARB. These primers were used for the molecular characterization of the clinical isolates. DNA sequences, amplification product lengths, and annealing temperatures of those primers are listed in Table 4.

2.1.7 DNA amplification and sequencing

The genomic DNA from clinical samples was analysed using PCR as the method of choice. The clinical DNA extracts were amplified using primers listed in Table 4 and also those published recently (Table 5) to double-check PCR results. The final primer concentration was 1 µM in the PCR mix. Reagents of the Mastermix 16S Basic PCR kit (Molzym) were used at a total volume of 50 µl. One microlitre of extracted DNA was added per reaction. Thermal cycling was carried out by an initial denaturation step at 95°C for five minutes followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing for 30 seconds (annealing temperatures listed in Table 4 and Table 5), elongation at 72°C for 80 seconds, and a final elongation cycle at 72°C for ten minutes. Negative controls without DNA template were used in all experiments. PCR products were analysed by ethidium bromide stained agarose (2%) gel electrophoresis.

Reactions with only one visible DNA lane in the agarose gel were further processed using the MinElute PCR Purification kit (Qiagen). If multiple lanes were present, the lane with the PCR product of the expected size was cut out and subsequently purified using the MinElute Gel Extraction kit (Qiagen). DNA was eluated in 50 µl ddH_2O. The purified PCR products were Sanger sequenced (Microsynth).

Primer name	β-lactamase(s) targeted	Sequence 5' 3'	Length	Product size	Annealing temperature[a]
ACC F	ACC-1, ACC-4	GCAGAACACATTGAAGCTGTTATCCGT	27	1142 bp	60°C
ACC R		ATGAGCTCAGGATTTTATACGCCACCG	27		60°C
ACT F	ACT-1, ACT-2, ACT-8	TGACTAAATCCCTTTGCTGCGCCC	24	1136 bp	60°C
ACT R		ACAGCGCGCTCAAAATACGGT	21		60°C
CMY-1F	Variants of CMY-1 group, MOX-1	GCCGTGGCCACCCTGATGTG	20	1114 bp	60°C
CMY-1R		CGGCCAACTGCGCCAGGAT	19		60°C
CMY-2F	Variants of CMY-2 group	TGCGCTCTGCTGGCGTTTTCC	20	1116 bp	60°C
CMY-2R		TTCAAGAATGCGCCAGGCCG	20		60°C
CTX-M-1F	Variants of CTX-M-1 group	TCACTGCGCCAGTTCACGCT	20	849 bp	65°C
CTX-M-1R		GACGATTTTAGCCGCCGACGC	21		65°C
CTX-M-25F	Variants of CTX-M-25 group	AAGCGTAAGGCGGGCGATGT	20	859 bp	65°C
CTX-M-25R		ACCGTCGGTGACAATTCTGGCG	22		65°C
CTX-M-2F	Variants of CTX-M-2 group	TGACTCAGAGCATTCGCCGCT	21	854 bp	65°C
CTX-M-2R		GATTTTCGCCGCCGCAGCC	19		65°C
CTX-M-8F	Variants of CTX-M-8 group	ACATCGCGTTAAGCGGATGATGCT	24	858 bp	65°C
CTX-M-8R		TCGGTGACGATTTTCGCGGCA	21		65°C
CTX-M-9F	Variants of CTX-M-9 group	ACGGATGATGTTCGCGGCGG	20	854 bp	65°C
CTX-M-9R		ACAGCCCTTCGGCGATGATTCTC	23		65°C
DHA F	DHA-1	CGCTCTGCTGGCGTTTTCCG	20	1103 bp	65°C
DHA R		CCAGTGCACTCAAAATAGCCTGTGC	25		65°C
FOX F	FOX-1 to FOX-8	ACGACGTGCGTTCGCGCTAC	20	1125 bp	60°C
FOX R		CTCAGGATGGCGTGAGCCGC	20		60°C
GES F	GES variants	ACTGGCAGGGATCGCTCACTCT	22	836 bp	60°C
GES R		TGTCCGTGCTCAGGATGAGTTGTG	24		60°C
IMP-1F	Variants of IMP-1 group	ATGAGCAAGTTATCTGTATTCTTTATA	27	740 bp	55°C
IMP-1R		AGTTGCTTGGTTTTGATGGT	20		55°C
KPC F	KPC variants	GCTGTCTTGTCTCTCATGGCCGC	23	854 bp	60°C
KPC R		ACTGCCCGTTGACGCCCAATC	21		60°C
NDM-1F	NDM-1	CAGCGCAGCTTGTCGGCCAT	20	795 bp	60°C
NDM-1R		TATGCACCCGGTCGCGAAGC	20		60°C
OXA-1F	Variants of OXA-1 group	GAAAAACACAATACATATCAACTTCGC	27	806 bp	55°C
OXA-1R		TGGTGATCGCATTTTTCTTGGC	22		55°C
OXA-23F	Variants of OXA-23 group	AAATATTTTACTTGCTATGTGGTTGCT	27	784 bp	55°C
OXA-23R		TCAATAATTCATTACGTATAGATGCCG	27		55°C
OXA-24F	Variants of OXA-24 group	AGTTTCTCTCAGTGCATGTTCA	22	782 bp	55°C
OXA-24R		TGATTCCAAGATTTTCTAGCGACT	24		55°C
OXA-51F	Variants of OXA-51 group	ATGAACATTAAAACCCTCTTACT	23	825 bp	55°C
OXA-51R		CTATAAAATACCTAATTGTTCTAAGC	26		55°C
OXA-58F	Variants of OXA-58 group	TAAGCATAAGTATTGGGGCTTGTGCTG	27	800 bp	55°C
OXA-58R		ATGAAAAACACCCAACTTATCTAGCA	26		55°C
PER F	PER-1, PER-3, PER-4, PER-5	ACTGCCTCGACGCTACTGATGGT	23	894 bp	60°C
PER R		TGGGCTTAGGGCAGAAAGCTTTTTCAA	27		60°C
SHV F	SHV variants	ATGCGTTATATTCGCCTGTGTATTT	25	861 bp	60°C
SHV R		TTAGCGTTGCCAGTGCTCGAT	21		60°C
TEM F	TEM variants	ATGAGTATTCAACATTTCCGTGTC	24	861 bp	55°C
TEM R		TTACCAATGCTTAATCAGTGAGG	23		55°C
VEB F	VEB variants	AGCGGTAATTTAACCAGATAGGAGT	25	913 bp	55°C
VEB R		TCCACGTTATTTTTGCAATGTCTGA	25		55°C
VIM-1F	Variants of VIM-1 group	AGTTTATTGGTCTACATGACCGCGTCT	27	778 bp	60°C
VIM-1R		CGGCGACTGAGCGATTTTTGTGT	23		60°C

[a]Temperature used during PCR

Table 4. Primers designed in this study for liquid phase β-lactamase detection

Primer name	β-lactamase(s) targeted	Sequence 5' 3'[a]	Length	Product size	Annealing temperature[b]	Reference
PP ACC F	ACC-1, ACC-2	CACCTCCAGCGACTTGTTAC	20	346 bp	60°C	(54)
PP ACC R		GTTAGCCAGCATCACGATCC	20		60°C	(54)
PP ACT-1 F	ACT-1, MIR-1	CGGTAAAGCCGATGTTGCG	19	683 bp	60°C	(54)
PP ACT-1 R		AGCCTAACCCCTGATACA	18		60°C	(54)
PP CMY-1 F	Variants of CMY-1 group, MOX-1, MOX-2	GCAACAACGACAATCCATCCT	21	895 bp	60°C	(54)
PP CMY-1 R		GGGATAGGCGTAACTCTCCCAA	22		60°C	(54)
PP CMY-2 F	Variants of CMY-2 group, LAT-1 to LAT-3, BIL-1	CGAAGAGGCAATGACCAGAC	20	538 bp	60°C	(54)
PP CMY-2 R		ACGGACAGGGTTAGGATAGY	20		60°C	(54)
PP CTX-M-1 F	Variants of CTX-M-1 group	TTAGGAARTGTGCCGCTGYA	20	688 bp	60°C	(54)
PP CTX-M-1 R		CGATATCGTTGGTGGTRCCAT	21		60°C	(54)
PP CTX-M-2 F	Variants of CTX-M-2 group	CGACGCTACCCCTGCTATT	19	552 bp	60°C	(76)
PP CTX-M-2 R		CCAGCGTCAGATTTTTCAGG	20		60°C	(76)
PP CTX-M-25 F	Variants of CTX-M-8 group and CTX-M-25 group	GCACGATGACATTCGGG	17	327 bp	60°C	(76)
PP CTX-M-8 F		TCGCGTTAAGCGGATGATGC	20	666 bp	60°C	(76)
PP CTX-M-8U25R		AACCCACGATGTGGGTAGC	19		60°C	(76)
PP CTX-M-9 F	Variants of CTX-M-9 group	TCAAGCCTGCCGATCTGGT	19	561 bp	60°C	(54)
PP CTX-M-9 R		TGATTCTCGCCGCTGAAG	18		60°C	(54)
PP DHA F	DHA-1, DHA-2	TGATGGCACAGCAGGATATTC	21	997 bp	60°C	(54)
PP DHA R		GCTTTGACTCTTTCGGTATTCG	22		60°C	(54)
PP FOX F	FOX-1 to FOX-5	CTACAGTGCGGGTGGTTT	18	162 bp	60°C	(54)
PP FOX R		CTATTTGCGGCCAGGTGA	18		60°C	(54)
PP GES F	GES-1 to GES-9, GES-11	AGTCGGCTAGACCGGAAAG	19	399 bp	60°C	(54)
PP GES R		TTTGTCCGTGCTCAGGAT	18		60°C	(54)
PP IMP-1 F	Variants of IMP-1 group	TTGACACTCCATTTACDG	18	139 bp	55°C	(54)
PP IMP-1 R		GATYGAGAATTAAGCCACYCT	21		55°C	(54)
PP KPC F	KPC variants	CATTCAAGGGCTTTCTTGCTGC	22	538 bp	55°C	(54)
PP KPC R		ACGACGGCATAGTCATTTGC	20		55°C	(54)
PP OXA-1 F	Variants of OXA-1 group	GGCACCAGATTCAACTTTCAAG	22	564 bp	60°C	(54)
PP OXA-1 R		GACCCCAAGTTTCCTGTAAGTG	22		60°C	(54)
PP OXA-23 F	Variants of OXA-23 group	GATCGGATTGGAGAACCAGA	20	501 bp	52°C	(77)
PP OXA-23 R		ATTTCTGACCGCATTTCCAT	20		52°C	(77)
PP OXA-24 F	Variants of OXA-24 group	GGTTAGTTGGCCCCCTTAAA	20	246 bp	52°C	(77)
PP OXA-24 R		AGTTGAGCGAAAAGGGGATT	20		52°C	(77)
PP OXA-51 F	Variants of OXA-51 group	TAATGCTTTGATCGGCCTTG	20	353 bp	52°C	(78)
PP OXA-51 R		TGGATTGCACTTCATCTTGG	20		52°C	(78)
PP OXA-58 F	Variants of OXA-58 group	AAGTATTGGGGCTTGTGCTG	20	599 bp	52°C	(77)
PP OXA-58 R		CCCCTCTGCGCTCTACATAC	20		52°C	(77)
PP PER F	PER-1, PER-3	GCTCCGATAATGAAAGCGT	19	520 bp	60°C	(54)
PP PER R		TTCGGCTTGACTCGGCTGA	19		60°C	(54)
PP SHV F	SHV variants	CAAAACGCCGGGTTATTC	18	937 bp	54°C	(55)
PP SHV R		TTAGCGTTGCCAGTGCT	17		54°C	(55)
PP TEM F	TEM variants	TGAGTATTCAACATTTCCGTGT	22	861 bp	54°C	(55)
PP TEM R		TTACCAATGCTTAATCAGTGA	21		54°C	(55)
PP VEB F	VEB variants	CATTTCCCGATGCAAAGCGT	20	648 bp	60°C	(54)
PP VEB R		CGAAGTTTCTTTGGACTCTG	20		60°C	(54)
PP VIM-1 F	Variants of VIM-1 group	GATGGTGTTTGGTCGCATA	19	390 bp	55°C	(79)
PP VIM-1 R		CGAATGCGCAGCACCAG	17		55°C	(79)

[a]Sequences according IUPAC nomenclature

[b]Temperature used during PCR

Table 5. Previously published primers used for liquid phase β-lactamase detection

2.1.8 On-chip ligation oligonucleotides

DNA sequences of the on-chip ligation oligonucleotides used in this study were designed in ARB based on the β-lactamase sequence database generated. All DNA oligonucleotide were checked for secondary structures as previously described and are listed in Table 6 and Table 7. Oligonucleotide probes were designed for four different ligation based reaction mechanisms (Figure 12 to Figure 15)

Figure 12. Schematic overview of a ligation and detection process using linear probes immobilized on a glass slide. During the hybridization step a DNA target molecule binds to the immobilized probe. Subsequently, the specificity probe binds to the caught DNA target directly next to the immobilized probe and is ligated to it. The specificity probe has a detection sequence at its 3' end to which a detection oligonucleotide can bind.

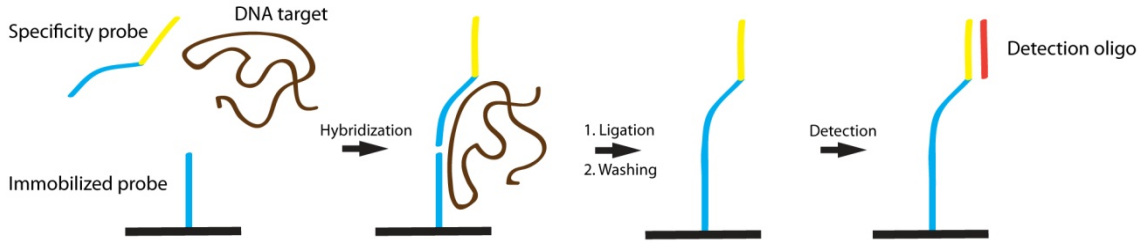
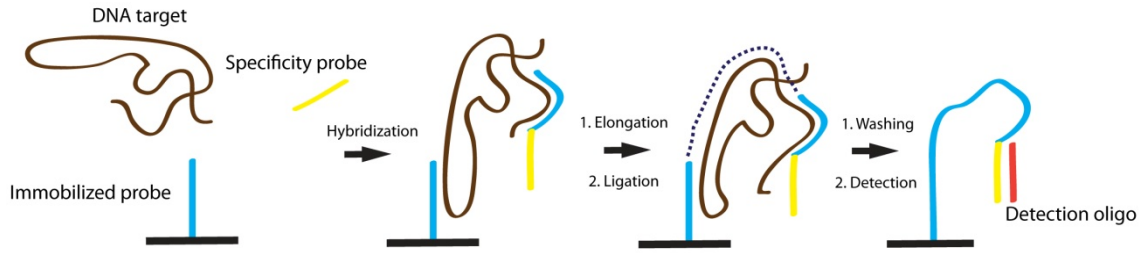


Figure 13. Schematic overview of a ligation and detection process using linear probes immobilized on a glass slide. During the hybridization step, a DNA target molecule binds to the immobilized probe. Subsequently, the specificity probe binds to the caught DNA target with several base pairs distance to the immobilized probe. A polymerase lacking exonuclease activity adds nucleotides to the immobilized probe until it reaches the hybridized specificity probe. Then a ligase binds the specificity probe to elongate the immobilized probe. The specificity probe has a detection sequence at its 3' end to which a detection oligonucleotide can bind.



Name	Sequence 5' 3'	Corresponding immobilized probe	Distance to immobilized probe	Length
Immobilized probe TEM 140 F	TTTTTTTTTTTGGATCTCAACAGCGGTAAGATCCTTGAGAG			41
Immobilized probe TEM 204R	TTTTTTTTTTCCACATAGCAGAACTTTAAAAGTGCTCA			38
Immobilized probe TEM 233R	TTTTTTTTTTGCCCGGCGTCAACACGGGATAATACCG			37
Immobilized probe TEM lig	TTTTTTTTTTGGATAATACCGCACC			25
Immobilized probe SHV lig	TTTTTTTTTTGGATCTGGTGGACTA			25
Immobilized probe OXA-1 lig	TTTTTTTTTTGTTGTTTTTTACTTTA			26
Immobilized probe CTX-M-1 lig	TTTTTTTTTTTAGCGTCACCGCGTT			25
Specificity probe TEM 140F	CGAAGAACGTTTTCCAATGATTTAAAACGACGGCCAGTGAGCTT	TEM 140F and TEM lig	9 bp resp. 0 bp	44
Specificity probe TEM 204R	CGCTGTTGAGATCCAGCTCTTTAAAACGACGGCCAGTGAGCTT	TEM 204R	45 bp	43
Specificity probe TEM 233R	ACATAGCAGAACTTTAAAAGTGCTTTTAAAACGACGGCCAGTGAGCTT	TEM 233R	4 bp	48
Specificity probe SHV 306	CTCGCCGGTCAGCGAAAATTTAAAACGACGGCCAGTGAGCTT	SHV lig	0 bp	42
Specificity probe OXA-1 144	CGATGCATCCACAAACGCTTTAAAACGACGGCCAGTGAGCTT	OXA-1 lig	0 bp	42
Specificity probe CTX-M-1 465	CGCCCGACAGCTGGGAGATTTAAAACGACGGCCAGTGAGCTT	CTX-M-1 lig	0 bp	42

Table 6. Oligonucleotides used for ligation based on-chip detection methods of β-lactamases presented in Figure 9 and Figure 10

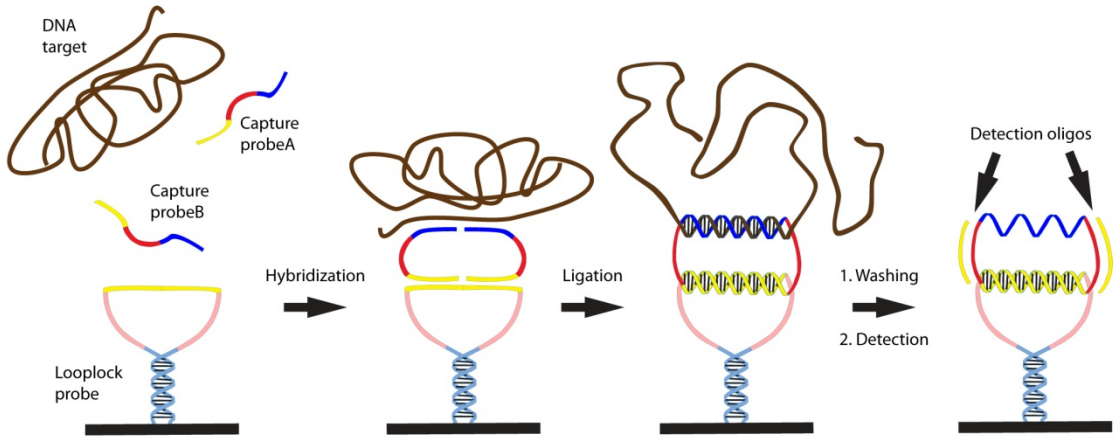
Looplock probes	5' Spacer	Spacer intern	Lock Region 1	Lock Region 2	Spacer intern	Spacer 3'	Length
LL CTX-M-1	GCGGGGCTAGTGGACGGGCTGCCAGGC	TTTTTTTTTT	AGCATCTACAGGCTGCTTCC	TGTTTTCTGCAGTGGTCTGG	TTTTTTTTTT	GCCTGGCAGCCCGTCCACTAGCCCGC	114
LL CTX-M-9	GCGGGGCTAGTGGACGGGCTGCCAGGC	TTTTTTTTTT	AGAAGCAGAGATCGCTGGAG	TCCAGAGCCACACCTTTCTC	TTTTTTTTTT	GCCTGGCAGCCCGTCCACTAGCCCGC	114
LL OXA-1	GCGGGGCTAGTGGACGGGCTGCCAGGC	TTTTTTTTTT	CACAGAGCTCAGGTGATCCA	CCACCTCCTCCAACTGTCTC	TTTTTTTTTT	GCCTGGCAGCCCGTCCACTAGCCCGC	114
LL SHV	GCGGGGCTAGTGGACGGGCTGCCAGGC	TTTTTTTTTT	ACTCTAACTGCCTCCCGACA	GGTGTCGAGCTGTTGTCCTT	TTTTTTTTTT	GCCTGGCAGCCCGTCCACTAGCCCGC	114
LL TEM	GCGGGGCTAGTGGACGGGCTGCCAGGC	TTTTTTTTTT	ACACGCAGATCAGTCAGCAT	GAGACAGTTGGAGGAGGGTGG	TTTTTTTTTT	GCCTGGCAGCCCGTCCACTAGCCCGC	114
LL c.pneu. P108	GCGGGGCTAGTGGACGGGCTGCC	25x T	TCCTTCCGGGCGTTG	CGAAGGGCGGGAACA	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL c.pneu. P646	GCGGGGCTAGTGGACGGGCTGCC	25x T	TTTGGGGCGCGCTAT	TTTTGTGCGCGGTCG	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL c.pneu. P191	GCGGGGCTAGTGGACGGGCTGCC	25x T	CGGGTGGAAGCCGAA	CCGCTAACGCGGAGA	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL b per. P737	GCGGGGCTAGTGGACGGGCTGCC	25x T	GTTGCGACACGACCG	TGGAAGGCGGCACTC	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL b per. P574	GCGGGGCTAGTGGACGGGCTGCC	25x T	CGAAGGGCGGGAACA	ACGGGCTGCTGAAGG	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL b per. P46	GCGGGGCTAGTGGACGGGCTGCC	25x T	AGTCTTTGTGCGCGG	CCTCGGGCGATCTCA	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL k.king. P79	GCGGGGCTAGTGGACGGGCTGCC	25x T	TGAGATCGCCGCAGG	CGACCGCGCACAAAA	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL k.king. P1008	GCGGGGCTAGTGGACGGGCTGCC	25x T	GCACTCTCTGCCCCC	TGGAAGGCGGCACTC	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL k.king. P455	GCGGGGCTAGTGGACGGGCTGCC	25x T	GCGGACAGCGGATCT	TTCCCGTCTGCTGGC	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL m.hom. P1001	GCGGGGCTAGTGGACGGGCTGCC	25x T	ACGGTTGCGAGCACT	CGCCCTCGCGGATAA	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL m.hom. P170a	GCGGGGCTAGTGGACGGGCTGCC	25x T	TTGCCAGCCAGTGSG	CCGTTACGTGCGCTG	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
LL m.hom. P170b	GCGGGGCTAGTGGACGGGCTGCC	25x T	GGGCGTCTGGAAGCA	GCGGCTGGTCTGACA	24x T	GGCAGCCCGTCCACTAGCCCCGC	125
Immobilized linear probes		**5' Spacer**	**Lock Region 1**	**Lock Region 2**			
SP CTX-M-1		TTTTTTTTTT	AGCATCTACAGGCTGCTTCC	TGTTTTCTGCAGTGGTCTGG			50
SP CTX-M-9		TTTTTTTTTT	AGAAGCAGAGATCGCTGGAG	TCCAGAGCCACACCTTTCTC			50
SP CTX-M-9 ddNTP		TTTTTTTTTT	AGAAGCAGAGATCGCTGGAG	TCCAGAGCCACACCTTTCTddCTP			50
SP OXA-1		TTTTTTTTTT	CACAGAGCTCAGGTGATCCA	CCACCTCCTCCAACTGTCTC			50
SP SHV		TTTTTTTTTT	ACTCTAACTGCCTCCCGACA	GGTGTCGAGCTGTTGTCCTT			50
SP SHV ddNTP		TTTTTTTTTT	ACTCTAACTGCCTCCCGACA	GGTGTCGAGCTGTTGTCCTddTTP			50
SP TEM		TTTTTTTTTT	ACACGCAGATCAGTCAGCAT	GAGACAGTTGGAGGAGGGTGG			50
SP TEM ddNTP		TTTTTTTTTT	ACACGCAGATCAGTCAGCAT	GAGACAGTTGGAGGAGGGTGddGTP			50
Capture probes A	**5' Lock Region 1**	**Spacer intern**	**Detection oligo**		**Spacer intern**	**Target Region 2**	
CP CTX-M-1 A	GGAAGCAGCCTGTAGATGCT	TTT	TTTAAAACGACGGCCAGTGAGCTT		TTT	CATACAGCGGCACACTTCC	69
CP CTX-M-9 A	CTCCAGCGATCTCTGCTTCT	TTT	TTTAAAACGACGGCCAGTGAGCTT		TTT	CTGTTGCGGCTGGGTAAA	68
CP OXA-1 A	TGGATCACCTGAGCTCAGTG	TTT	TTTAAAACGACGGCCAGTGAGCTT		TTT	CCGCATCAAATGCCATAAG	69
CP SHV A	TGTCGGGAGCAGTTAGAGT	TTT	TTTAAAACGACGGCCAGTGAGCTT		TTT	AGGGTGGCTAACAGAGGAGAT	70
CP TEM A	ATGCTGACTGATCTGCGTGT	TTT	TTTAAAACGACGGCCAGTGAGCTT		TTT	TGCTTAATCAGTGAGGCACCTA	72
CP13 c.pneu. P127	CAACGCCCGGAAGGA		GCGCGTGTATGCAGCTCCTCGAGTAGCCG			AGGGCAGATTATCTATGTA	63
CP13 c.pneu. P865	ATAGCGCGCCCCAAA		GCGCGTGTATGCAGCTCCTCGAGTAGCCG			CTATCCTCTAGAAAGATAG	63
CP13 c.pneu. P211	TTCGGCTTCGACCCG		GCGCGTGTATGCAGCTCCTCGAGTAGCCG			GATCCCCTTCTTTAATATAT	64
CP13 b.per. P721	CGGTCGTGTCGCAAC		GCGCGTGTATGCAGCTCCTCGAGTAGCCG			CGCTCATGCACGAAAG	60
CP13 b.per. P556	TGTTCCCGCCCTTCG		GCGCGTGTATGCAGCTCCTCGAGTAGCCG			TTCGGAAAGAAAGATGTG	62
CP13 b.per. P60	CCGCGCACAAAGACT		GCGCGTGTATGCAGCTCCTCGAGTAGCCG			AGGCCGAAGCCCGT	58

CP13 k.king. P96	CCTGCGGCGATCTCA		GCGCGTGTATGCAGCTCCTCGAGTAGCCG		GCCACCAAGTACAAGTA		61
CP13 k.king.P1025	GGGGGCAGAGAGTGC		GCGCGTGTATGCAGCTCCTCGAGTAGCCG		GGCACAAGAGTATCTCT		61
CP13 k.king. P475	AGATCCGCTGTCCGC		GCGCGTGTATGCAGCTCCTCGAGTAGCCG		CAGTTAATGATATTAGCATC		64
CP13 m.hom. P982	AGTGCTCGCAACCGT		GCGCGTGTATGCAGCTCCTCGAGTAGCCG		AGACATATAGTGGAGGTTA		63
CP13 m.hom. P154	CCCACTGGCTGGCAA		GCGCGTGTATGCAGCTCCTCGAGTAGCCG		CGGATACGCATGGAAC		60
CP13 m.hom. P186	TGCTTCCAGACGCCC		GCGCGTGTATGCAGCTCCTCGAGTAGCCG		CACAACGGAACCATGC		60
Capture probes B	**5' Target Region 1**	**Spacer intern**	**Detection oligo**	**Spacer intern**	**Lock Region 2**		
CP CTX-M-1 B	TAACAACAGCGTGACGGTTG	TTT	TTTAAAACGACGGCCAGTGAGCTT	TTT	CCAGACCACTGCAGAAAACA		70
CP CTX-M-9 B	ATAGGTCACCAGAACCAGCG	TTT	TTTAAAACGACGGCCAGTGAGCTT	TTT	GAGAAAGGTGTGGCTCTGGA		70
CP OXA-1 B	TGATAATGCGATCTTGAAAGTTG	TTT	TTTAAAACGACGGCCAGTGAGCTT	TTT	GAGACAGTTGGAGGAGGTGG		73
CP SHV B	AATACACAGGCGAATATAACGCA	TTT	TTTAAAACGACGGCCAGTGAGCTT	TTT	AAGGACAACAGCTCGACACC		73
CP TEM B	TCTCAGCGATCTGTCTATTTCG	TTT	TTTAAAACGACGGCCAGTGAGCTT	TTT	CCACCTCCTCCAACTGTCTC		72
CP52 c.pneu. P108	CTACTAACCCTTCCGC	TTTTTTTTT			TGTTCCCGCCCTTCG		41
CP52 c.pneu. P546	TTTTAAATGCTGACTTGGG	TTTTTTTTT			CGACCGCGCACAAAA		44
CP52 c.pneu. P191	ATTAGATGCCTAATTACACT	TTTTTTTTT			TCTCCGCGTTAGCGG		45
CP52 b.per. P737	TGTGGGAGCAAACAG	TTTTTTTTT			GAGTGCCGCCTTCCA		41
CP52 b.per. P574	AAATCCCAGGGCTTAAC	TTTTTTTTT			CCTTCAGCAGCCCGT		42
CP52 b.per. P46	GCTGCCGTCCGACT	TTTTTTTTT			TGAGATCGCCGCAGG		39
CP52 k.king. P79	CTCGTGGCTGCCGT	TTTTTTTTT			TTTTGTGCGCGGTCG		39
CP52 k.king.P1008	ACTCTCTTCCGTACATG	TTTTTTTTT			GAGTGCCGCCTTCCA		42
CP52 k.king. P455	AACCTTTTCTTCCCTAACA	TTTTTTTTT			GCCAGCAGACGGGAA		44
CP52 m.hom. P1001	TCGGAGTGACAGATGG	TTTTTTTTT			TTATCCGCGAGGGCG		41
CP52 m.hom. P170a	CGCATGGTTCCGTTGT	TTTTTTTTT			CAGCGCACGTAACGG		41
CP52 m.hom. P170b	GGTTCCATGCGTATCC	TTTTTTTTT			TGTCAGACCAGCCGC		41

Table 7. Oligonucleotides used for ligation based on-chip detection methods of β-lactamases presented in Figure 11 and Figure 12

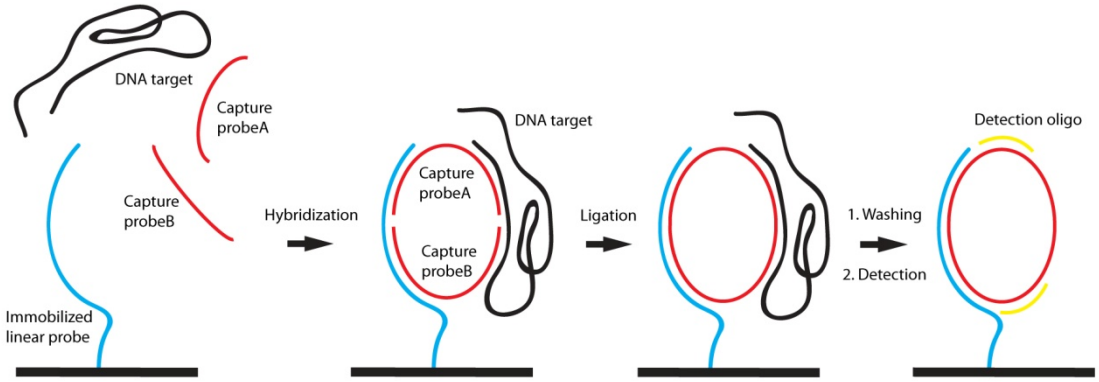
A new class of probes, looplock probes, was designed for the five clinically most frequent β-lactamase genes of CTX-M-1, CTX-M-9, OXA-1, TEM and SHV. The mode of action of these probes is shown in Figure 14.

Figure 14. Schematic overview of the ligation process using looplock probes. Yellow sequences indicate regions responsible for looplock and capture probe recognition, blue indicates the region on the capture probe detecting a specific DNA target, and red where detection oligonucleotides bind. After annealing of capture probes, looplock probe and the DNA target to each other, a ligase concatenates the ligated capture probes to the looplock probe. All unligated capture probes are removed during a stringent washing step using boiling water. Detection oligonucleotides are used to validate if the ligation reactions were successful.



These probes were compared with existing ligation dependent ligation methods using immobilized linear probes (Figure 15). The immobilized linear probes were ordered in two variants; one variant had dNTPs at its 3' end, the other had ddNTPs at the 3' end. The two variants were identical except this difference including their nucleotide sequences. Additionally, all immobilized probes had a sequence complementary to a capture probe A and a capture probe B. The immobilized linear probes had the same nucleotide sequence to bind capture probes as their analogous looplock probes.

Figure 15. Schematic overview of the ligation process using an immobilized linear probe to bind capture probes. The process is analogous to the one presented in Figure 14 except that no boiling water is used for washing but a less stringent washing buffer.



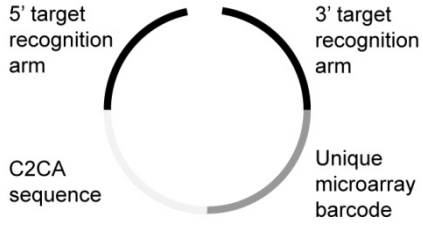
Capture probes are linear oligonucleotides that were used in the liquid phase of the detection reaction. Two capture probes (A and B) were necessary for the detection of a gene target. Every capture probe had two sequences at their ends to detect a gene target and their corresponding immobilized probe on the glass slide. The 3' end of capture probe A and the 5' end of capture probe B annealed to a gene target and got ligated. In parallel, the 5' end of capture probe A and the 3' end of capture probe B annealed to their common immobilized probe on the slide and were ligated. After the capture probes ligated at both ends, the resulting molecule was a circular DNA strand. Every capture probe had a universal sequence corresponding to the fluorescently labelled detection oligonucleotide BSrev (5’ Cy5-AAGCTCACTGGCCGTCGTTTTAAA). The mentioned oligonucleotides were ordered from Microsynth.

2.1.9 Padlock probes, synthetic templates and microarray probes

Padlock probes were designed for the 33 clinically most common β-lactamases (Table 8). The β-lactamase target gene sequences were cut *in silico* with the restriction enzymes *AluI* and *HpyF3I*. The obtained fragments were used as input sequence in the online software Primer3 plus (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) to calculate the 5’ and 3’ targeting arms of the padlock probes. Oligonucleotide sequences of the padlock probes designed in this study are shown in supplementary Table 1. Each gene-target was detected by two to four padlock probes resulting in 96 probes for the whole β-lactamase assay. Every padlock

probe contained a universal circle-to-circle amplification (C2CA) sequence and a unique sequence tag (80). A schematic overview of the designed padlock probes is shown in Figure 16. The polarity of oligonucleotides was referred to as "+", and of their complementary sequences as "-". Synthetic DNA templates were used to evaluate the ligation and subsequent amplification processes of every padlock probe (supplementary Table 2). Monomerization and ligation during C2CA was directed by the replication oligonucleotides $C2CA^{+}$ (GTGTATGCAGCTCCTCGAGTA) and $C2CA^{-}$ (TACTCGAGGAGCTGCATACAC) with an included restriction site for *AluI*. The above mentioned oligonucleotides were ordered from Integrated DNA Technologies (USA).

Figure 16. Schematic overview of the elements on the padlock probes. The 5' and 3' target recognition arms anneal specifically to a target DNA sequence and are subsequently ligated. The C2CA sequence contains a restriction site and is necessary for the amplification reactions. Every ligated padlock probe can be assigned with the unique microarray barcode element to a microarray spot in the final detection hybridization. The average nucleotide length of padlock probes in this study is 83 nt.



A microarray was used to detect ligated padlock probes. The array consisted of 274 oligonucleotides, only 96 were used for padlock probe detection (81). Every sequence-tagged padlock probe was complementary to only one microarray oligonucleotide which had 5' amino-modified-$(T)_{10}$-tails. Tag sequences were designed with ARB and had melting temperatures around 65°C. The fluorescently labeled detection oligonucleotide BSrev was used in every experiment as internal control to monitor the hybridization process. Microarray oligonucleotides are shown in supplementary Table 3 and were ordered from Microsynth.

Class A	Class B	Class C	Class D
CTX-M-1	IMP-1	ACT-1	OXA-1
CTX-M-2	IMP-24	CMY-1	OXA-2
CTX-M-8	VIM-1	CMY-2	OXA-23
CTX-M-9	VIM-2	DHA-1	OXA-24
CTX-M-25	NDM-1	FOX-1	OXA-48
GES-1		MIR-1	OXA-51
IMI-1			OXA-58
KPC-1			
PER-1			
RTG-4			
SFO-1			
SHV			
SME-1			
TEM			
VEB-1			

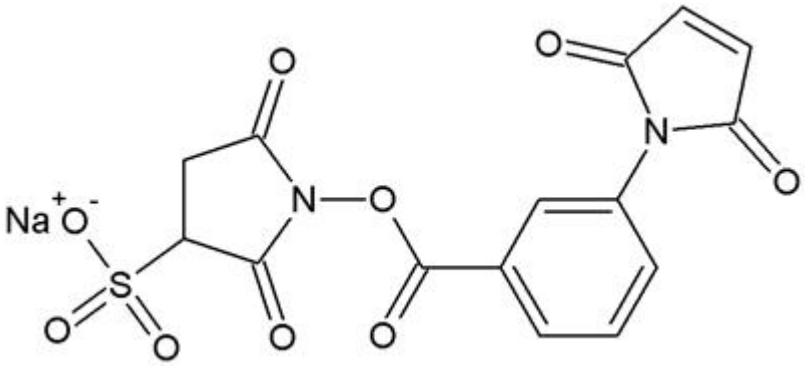
Table 8. Clinical important β-lactamases and their classification according sequence similarity. The padlock probe assay was designed to detect the genes of these enzymes

2.1.10 Glass slide preparation and spotting for on-chip PCR

First, glass slides were washed by sonication in acetone for ten minutes, followed by washing in ddH_2O for two minutes twice. Sonication was repeated in a 1 M NaOH solution for ten minutes. Slides were immersed overnight in 1 M HCl.

Washing was continued for four minutes in ddH_2O twice. Then, slides were rinsed in Ethanol and air-dried. The slides were subsequently immersed in dry acetone with 5% 3-aminopropyl-trimethoxysilane for one hour to coat the surface with aminosilane resulting in a nonpolar surface. Washing three times in acetone for five minutes each time was followed by rinsing the slides in ethanol. To fix the chemical surface on the glass slides, they were baked for 50 minutes at 90°C. Then, a freshly prepared cross-linker solution was applied on the top of the slides for five hours at room temperature.

Figure 17. Chemical structure of s-MBS. The sulfite modification makes the molecule water soluble. The molecule is reactive towards amino and thiol groups. The reactive sites are the sulfo-NHS ester and the maleimide.



The cross-linker solution consisted of 20 mM s-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfo-succinimide ester) in PBS (0.1 M NaH_2PO_4, 0.15 M NaCl, pH 7.2). The cross-linker provides chemical functional groups that can link the aminosilane-surface with a thiol-modified oligonucleotide. Furthermore, it enables a very thermostable, covalent binding of the oligonucleotides to the surface. Due to the thiol-modification at the 5' end of the oligonucleotides, they bind exclusively with the 5' end to the surface. Slides were immersed in PBS after the application of the cross-linker, then briefly in ddH_2O and finally in ethanol. Slides were air-dried and stored overnight.

On the third day of the procedure, oligonucleotides were spotted onto the slides. The thiol-modified oligos were prepared as 50 µM solution in NaPi (0.1 M NaH_2PO_4, 0.15 M NaCl, pH 6.5). The oligonucleotides (Table 2) were applied with the contact-spotter Genemachines OmniGrid 100 (Digilab Genomic Solutions, USA) onto the slides. Then, the slides were incubated in a hybridization chamber with humid atmosphere at room temperature for five hours to enable the covalent binding of the oligos to the slide surface. Afterwards, slides were washed in NaPi for five minutes twice. To deactivate the free reactive groups on the slide surface the slides were treated with 10 mM mercaptoethanol in NaPi for one hour at room temperature. Then, slides were again washed in NaPi. To remove unbound oligonucleotides, slides were immersed in a saline solution (1.5 M NaCl, 10m M NaH_2PO_4, pH 7) for ten minutes. The manufacturing of the slides was finalised with washing in 5xSSC buffer (0.75 M NaCl, 0.075 M Na

citrate, pH 7) with 0.1% Tween for ten minutes, rinsing in 5xSSC and storing in 5xSSC at 4°C.

2.1.11 Microarray spotting for padlock probe and solid support ligation assay

5' and 3' end amino-modified oligonucleotides (supplementary Table 4, Table 6, and Table 7) were printed using the contact arrayer Omnigrid at a concentration of 50 µM in 3×SSC and 1.5 M betaine monohydrate onto glass slide with an aldehyde surface (VSS-25F Silylated Slides, Cel Associates). Air humidity was between 55% and 60%. Spotting was carried out with SMP3 pins (TeleChem, Sunnyvale, California) leading to a spot size of 100 µm diameter. Four replicates of each probe were printed per microarray slide. After spotting, slides were stored at room temperature.

2.1.12 Spotting efficiency and thermal stability of on-chip PCR slides

Tests to evaluate the efficiency of spotting were performed. The blocked on-chip PCR slides were hybridized with fluorescently labelled adenine-decamers (Cy5-polyA-oligo). The spotted oligonucleotides had on their 5' end a nucleotide spacer consisting of ten thymines. The polyA-oligo could anneal to this spacer, and thus, reveal the immobilization of the thiol-modified oligonucleotides on the slides.

For this purpose, a mix consisting of 25 µl ddH_2O, 25 µl ExpressHyb Hybridization Solution (Clonetech, USA) and 1 µl polyA-oligo (50 µM) was pipetted onto on-chip PCR slides with a lifterslip. The slides were put in a hybridization chamber with humid atmosphere and incubated at room temperature for one hour. After hybridization, slides were washed in 2xSSC with 0.1% SDS for five minutes, followed by washing in 0.2xSSC for two minutes and a final washing step in ddH_2O for one minute. Slides were dried in a centrifuge and scanned in a Genepix 4000A microarray scanner (Axon Instruments, USA). Data was analysed with Excel 2007 (Microsoft, USA).

After determination of spotting quality, the thermal stability of the used oligonucleotide immobilization chemistry was evaluated. On-chip PCR slides were transferred into boiling water for two hours. The evaluation of the thermal stability was determined analogous to the spotting efficiency with Cy5-labelled polyA-oligos as described above. Slides were scanned in a Genepix 4000A microarray scanner.

2.1.13 On-chip PCR chamber and sealing

Surface temperatures of the on-chip PCR slides were measured during thermal cycling with external thermo-sensors. The program in the thermo cycler was as follows: 95°C at 30 seconds, 58°C at 30 seconds and 72°C for 30 seconds. These three temperature steps were repeated 30 times.

Different approaches to create a reaction chamber on an on-chip PCR slide were evaluated. All chambers were tested with different PCR-reagents in a thermal *In Situ* slide cycler (PTC-200; MJ research, USA). Thermal cycling was carried out as follows: 95°C for five minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for one minute; and a final cycle at 72°C for ten minutes. Commercially available and self-sticking reaction chambers with 18 µl, 30 µl 50 µl and 100 µl reaction volume (HybriWell; Grace-Biolab, USA) were tested with and without additional sealing. The chambers were sealed on their edges with nail polish, a paraffin pen (PAP Pen 520; Kisker, Germany), self-sealing solution (LTF Labortechnik, Germany) or mineral oil (Sigma-Aldrich, USA).

Liquid reaction chambers with a polar phase consisting of the PCR-mix and a nonpolar coating phase surrounding the PCR-mix were also tested.

Self-made chambers consisted of a cover slip or a lifter slip (Thermo Fisher Scientific, USA) on a microarray slide. The PCR-mix was pipetted under these glass slips and sealed with nail polish, self-sealing solution or mineral oil. In other approaches, reaction chambers were sealed with aluminium or polyolefin PCR foils (StarSeal sealing tape; Starlab, Germany) by wrapping the foil around the on-chip PCR slide with the lifter slip on top of it.

The chemical composition of different commercial PCR-mixes and its impact on evaporation was also tested. Therefore, following PCR-kits were tested: Phusion and Phusion Hotstart (New England Biolabs, USA), 16S Basic PCR kit (Molzym), TypeIt PCR kit and Multiplex PCR kit (Qiagen). Evaporation could not be measured absolutely due to the lack of appropriate measuring equipment. Thus, the amount and size of air bubbles formed under the lifter slips was compared when different kits were used.

2.1.14 Blocking of on-chip PCR slides

Before their use, on-chip PCR slides were blocked to reduce background fluorescence and the attachment of PCR reagents to the surface. Three approaches were compared. The first one used proteins to block the surface and was based on three steps: incubation in 5xSCC with 0.1% Tween and 0.1% BSA for ten minutes. Then, slides were washed in 5xSSC for two minutes, followed by washing in ddH_2O for one minute. Slides were always dried with a centrifuge after washing.

The second blocking method was based on urea. Slides were incubated in 3 M urea with 0.5% SDS for 30 minutes and then washed in ddH_2O for five minutes. The third approach tested if blocking was necessary at all and washed the slides in ddH_2O for two minutes twice.

2.1.15 Testing of PCR-kits

Various PCR kits were tested for their applicability in on-chip PCR. Reaction mixtures with Phusion and Phusion Hotstart polymerase were pipetted as follows: 36 µl ddH_2O, 10 µl Phusion HF Reaction Buffer (5x), 1 µl dNTPs (2.5 mM), 1.5 µl DMSO (100%), 1 µl BSA (2 µg/µl), 0.5µl polymerase, and 2 µl of genomic DNA.

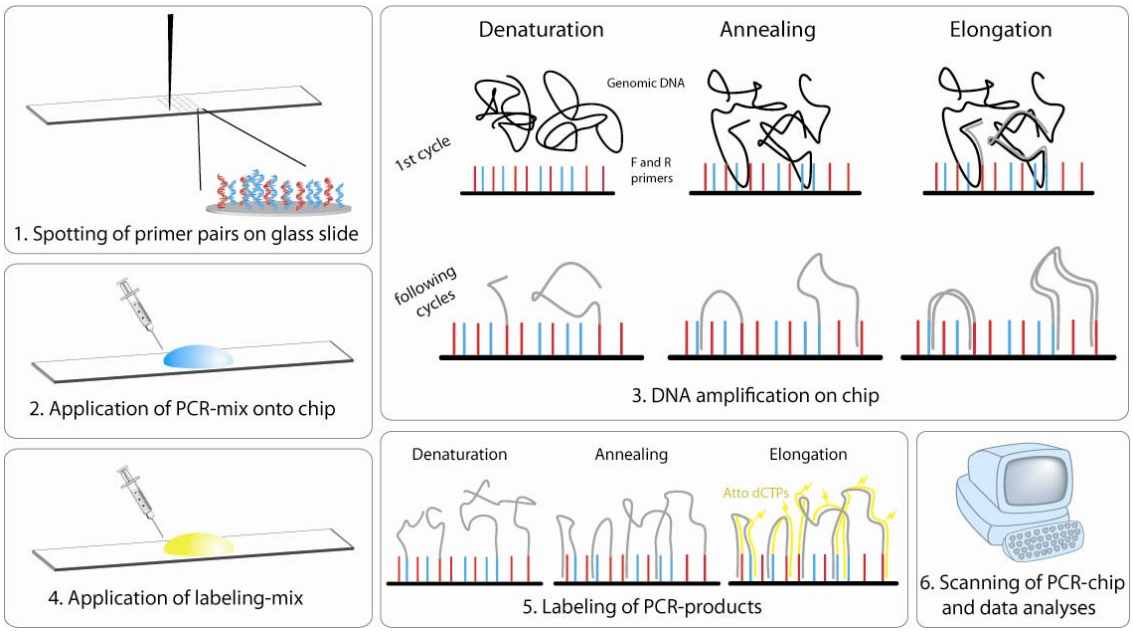
16S Basic Molzym kit was pipetted as follows: 19.4 µl ddH_2O, 20 µl Molzym Mastermix, 2 µl BSA (2 µg/µl), 1.6µl polymerase, and 2 µl genomic DNA.

The TypeIt and Multiplex PCR kit reaction mixtures consisted of: 11 µl ddH_2O, 25 µl Mastermix, 2 µl BSA (2 µg/µl), and 2 µl genomic DNA.

The ImmoMix PCR kit (Bioline, UK) was also tested: 23 µl ddH_2O, 25 µl Mastermix, and 2 µl genomic DNA.

PCR mixtures were pipetted onto on-chip PCR slides with lifterslips and wrapped up with polyolefin PCR foils. On-chip PCR was performed in the thermal cycler PTC-200. Cycling was carried out as follows: 95°C for five minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for one minute; and a final cycle at 72°C for ten minutes.

Figure 18. Schematic illustration of the experimental procedure of the on-chip PCR



2.1.16 Influence of concentrations of immobilized primers

The impact of different concentrations of immobilized oligonucleotides during on-chip PCR was tested. The primers TEM 20F, TEM 140F, TEM 204R, TEM 233R, TEM 789R, OXA 55F, OXA 472R, 45F++ and 1386R+ were spotted in the concentrations 20µM, 40µM, 60µM and 80µM according to the scheme in Table 9 . Every primer was spotted together with its corresponding primer.

forward primer 20µM reverse primer 20µM	forward primer 20µM reverse primer 40µM	forward primer 20µM reverse primer 60µM	forward primer 20µM reverse primer 80µM
forward primer 40µM reverse primer 20µM	forward primer 40µM reverse primer 40µM	forward primer 40µM reverse primer 60µM	forward primer 40µM reverse primer 80µM
forward primer 60µM reverse primer 20µM	forward primer 60µM reverse primer 40µM	forward primer 60µM reverse primer 60µM	forward primer 60µM reverse primer 80µM
forward primer 80µM reverse primer 20µM	forward primer 80µM reverse primer 40µM	forward primer 80µM reverse primer 60µM	forward primer 80µM reverse primer 80µM

Table 9. Spotting pattern of on-chip PCR primers

2.1.17 Labelling of on-chip PCR products

To visualize on-chip PCR products, fluorescent labels were used. The method of choice was based on the incorporation of Atto-532 labelled dCTPs (MoBiTec, Germany) with a thermostable polymerase lacking 3' end exonuclease activity. In the labelling mixture, the $Vent_R$(exo-) polymerase (New England Biolabs, UK) was used with the following reagents: 18.45 µl H_2O, 2.5 µl $Vent_R$(exo-) buffer, 0.25 µl $MgSO_4$ (100 mM), 0.5 µl dNTPs (2.5 mM), 0.5 µl Atto532-dCTP (100 nM), 1 µl BSA (2 µg/µl), 1 µl Tween (1%) and 0.8µl $Vent_R$(exo-) polymerase. The labelling mixtures were pipetted onto on-chip PCR slides that already had undergone on-chip PCR. Then, slides were wrapped up with polyolefin PCR foils. Labelling was performed in the thermal cycler PTC-200. Cycling was carried out as follows: 95°C for five minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for one minute; and a final cycle at 72°C for ten minutes.

After labelling, slides were washed in 2xSSC with 0.1% SDS for five minutes, followed by washing in 0.2xSSC for two minutes, and a final washing step in ddH_2O for one minute. Slides were dried in a centrifuge and scanned in the Genepix 4000A microarray scanner. Data was analysed with Excel 2007.

2.1.18 Liquid phase primers

To evaluate the specificity of the used on-chip PCR primers, a conventional PCR in tubes was performed. PCR primers with the same DNA sequences as the on-chip PCR primers but without polyT-spacer were used (Table 10). For the specificity tests, the 16S Basic Molzym kit was used and pipetted as follows: 12.2 µl H_2O, 10 µl Molzym Mastermix, 0.5 µl forward primer (10 µM), 0.5 µl reverse primer (10 µM), 0.8 µl polymerase, and 1 µl genomic DNA.

Primer name	Sequence 5' 3'	Length	Product size with TEM 20F	Product size with TEM 140F	Annealing temperature
TEM 20F	GTGTCGCCCTTATTCCCTTT	20			56°C
TEM 140 F	TGGATCTCAACAGCGGTAAGA	21			56°C
TEM 789R	TTCATCCATAGTTGCCTGACTC	22	769 bp	649 bp	56°C
TEM 233R	GGCGTCAACACGGGATAATA	20	213 bp	93 bp	56°C
TEM 204R	CCACATAGCAGAACTTTAAAAGTG	24	184 bp	64 bp	56°C

Table 10. Liquid phase primers used in on-chip PCR

2.1.19 Specificity and sensitivity of on-chip PCR

Specificity in PCR reactions can be achieved through the adjustment of parameters including primer sequence, annealing temperature and salt concentrations. These parameters were also varied in the on-chip PCR experiments in order to obtain high specificity. Tests with dilution series of genomic DNA and PCR products of the genes bla_{TEM}, bla_{SHV}, bla_{OXA-1} and $bla_{CTX-M-1}$ were performed to determine specificity and sensitivity of the assays. Probes were designed that annealed to the desired on-chip PCR products to evaluate the results of these experiments (Table 11). These probes specific for certain genes were detected with the fluorescently labelled detection oligonucleotide BSrev 2xATTO annealing to their 3' end.

For this purpose, a mixture consisting of 20 µl ddH_2O, 25 µl ExpressHyb Hybridization Solution, 1 µl of every on-chip PCR product specific probe (50 µM) and 1 µl BSrev 2xATTO (50 µM) was pipetted on a already cycled on-chip PCR slides. The slide was put in a hybridization chamber with humid atmosphere and incubated at 65°C for one hour. After hybridization, the slide was washed in 2xSSC with 0.1% SDS for five minutes, followed by washing in 0.2xSSC for two minutes and a final washing step in ddH_2O for one minute. The slide was dried in a centrifuge and scanned in a Genepix 4000A microarray scanner.

Probe name	Genes targeted	Sequence 5' 3'	Lenght
Sonde SHV 306	SHV variants	CTCGCCGGTCAGCGAAAATTTAAAACGACGGCCAGTGAGCTT	42
Sonde OXA 144	variants of OXA-1 group	CGATGCATCCACAAACGCTTTAAAACGACGGCCAGTGAGCTT	42
Sonde CTX-M 465	variants of CTX-M-1 group	CGCCCGACAGCTGGGAGATTTAAAACGACGGCCAGTGAGCTT	42
Sonde TEM 624	TEM variants	CTGGATGGAGGCGGATAATTTAAAACGACGGCCAGTGAGCTT	42
BSrev 2xATTO		5' Atto-620-AAGCTCACTGGCCGTCGTTTTAAA-Atto-620-3'	24

Table 11. Probes to detect on-chip PCR products. The last row lists the detection oligonucleotide BSrev 2xATTO

2.1.20 Multiplex solid support ligation assay

The solid support ligation assays detecting β-lactamase genes were tested with sequenced PCR products (bla_{TEM}, bla_{SHV}, bla_{OXA-1}, $bla_{CTX-M-1}$ and $bla_{CTX-M-9}$), genomic DNA of *E. coli* and *K. pneumoniae* and synthetic DNA templates (Supplementary Table 3). In the first step, PCR products and genomic DNA had to be digested with *AluI* and

HpyF3I in the recommended buffers (Fermentas) for one hour at 37°C, following deactivation at 65°C for ten minutes. This step was obsolete if synthetic DNA templates were used. The ligation reaction mixture using immobilized linear probes and specificity probes located next to the immobilized probes, was as follows (Figure 12): Twenty-five microlitre of ampligase buffer containing amounts of each specificity probe ranging from 1 pM to 10 µM, 5 µg BSA, and five units ampligase (Epicenter, USA) were mixed and pipetted onto the microarray slide. To the ligation reaction mixture using specificity probes, which are several nucleotides apart from the immobilized probes, three units of $Vent_R$(exo-) polymerase and 125 µM dNTPs were additionally added (Figure 13). For the reactions described in Figure 14 and Figure 15 ligation mixtures were as follows: Twenty-five microlitre of ampligase buffer containing variable amounts of capture probes ranging from 1 pM to 1 µM, 5 µg BSA, and five units ampligase were mixed and pipetted onto the microarray slide. All ligation mixtures included 1 µl of the DNA digestion or 100 nM synthetic DNA as templates, respectively. The slide was sealed and placed in a thermal cycler after the application of the ligation mixture onto the slide surface. Thermal cycling was performed at 95°C for five minutes, following annealing at varying temperatures and incubation times starting from 30 minutes to 24 hours, and a final step of 95°C for two minutes. If looplock probes were analysed, slides were put in boiling ddH_2O for ten minutes. Subsequently, labelling with the fluorescently labelled detection oligonucleotides BSrev 2xAtto, C2CA1 Atto532 (5' Atto532-GTGTATGCAGCTCCTCGAGTA-3'), or C2CA2 Atto532 (5' Atto532-TACTCGAGGAGCTGCATACAC-3') was performed.

2.1.21 Fluorescence labelling of immobilized ligation products

A mixture consisting of 25 µl of ddH_2O, 25 µl ExpressHyb Hybridization Solution (Clonetech, USA) and 1 µl of the fluorescently labelled detection oligonucleotide (50µM; BSrev 2xAtoo, C2CA1 Atto532, C2CA2 Atto532) was pipetted on the microarray slide containing the ligation products. The slide was sealed with a lifterslip and incubated in a hybridization chamber with a humid atmosphere at 65°C for one hour.

Then, the fluorescently labelled solid support ligation slides were prepared for analysis. First, slides were washed in 2xSSC with 0.1% SDS for five minutes, followed by

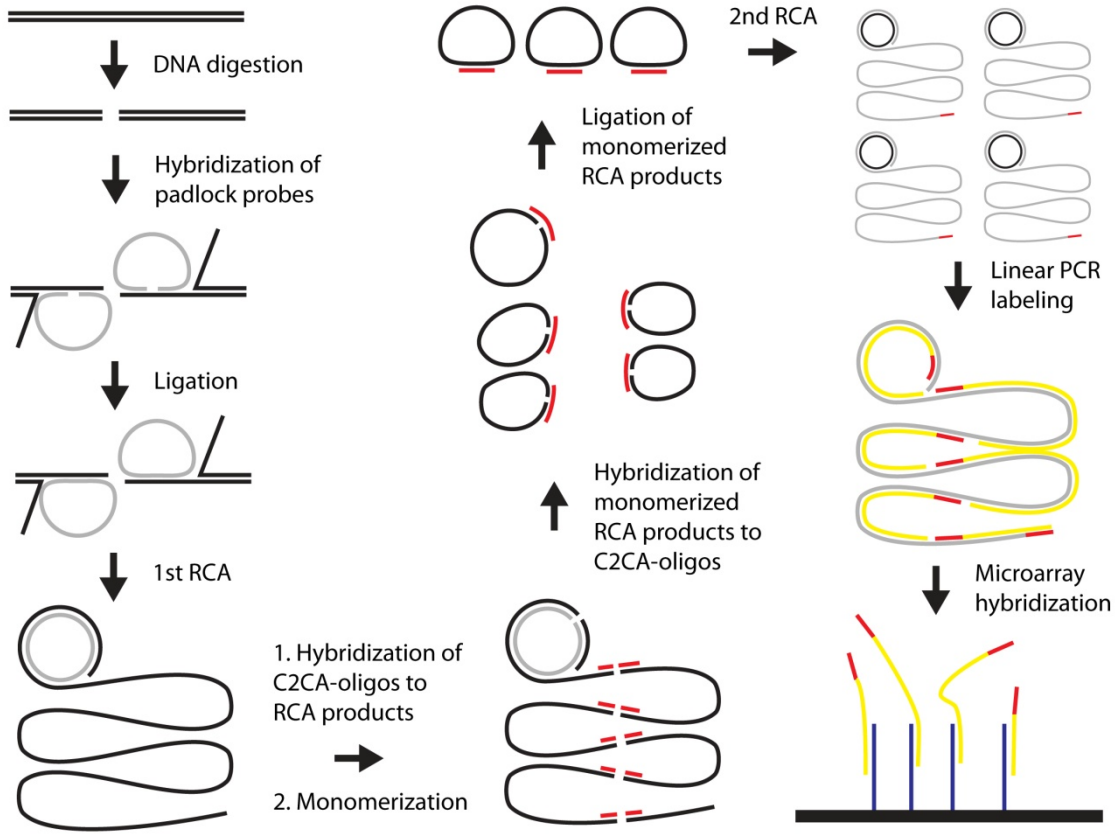
washing in 0.2xSSC for two minutes, and washing in ddH_2O for one minute. Subsequently, slides were dried in a centrifuge and scanned in the Genepix 4000A microarray scanner. The resulting data was analysed using Microsoft Excel 2007.

2.1.22 Circle-to-circle amplification (C2CA)

The method of choice for DNA amplification is based on RCA. The advantages of this method are the avoidance of thermal cycling and the amplification of only circular DNA molecules in high numbers, effects that are possible due to the strand displacement capability of the φ29 polymerase. The C2CA procedure consists of two single RCA reactions separated by an enzymatic restriction step (Figure 19).

Ligated and, thus, circularised padlock probes were replicated in φ29 polymerase buffer (33 mM Tris-acetate (pH 7.9 at 37°C), 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20 and 1 mM DTT) containing 125 µM dNTPs, 4 µg BSA, 250 pM $C2CA^-$, four units of φ29 DNA polymerase (Fermentas, USA) and 10 µl ligation products in a total volume of 20 µl at 37°C for 20 minutes. The two RCAs were primed with $C2CA^-$ or $C2CA^+$; however, in the padlock probe evaluation experiments using synthetic DNA templates, the first RCA was primed with the synthetic template itself. The first RCA was terminated by a two-minute incubation at 65°C. Monomerization of the RCA products was performed in φ29 polymerase buffer containing the C2CA+ oligonucleotide complementary to the amplification sequence in the RCA products. A 5-µl aliquot of φ29 polymerase buffer containing 1 µg BSA, 0.8 µM C2CA+ and five units of the restriction enzyme AluI was added to the first RCA, followed by incubation at 37°C for 5 minutes. After monomerization, the enzyme was inactivated at 65°C for five minutes. To circularise the monomerized RCA products, 10 µl of φ29 polymerase buffer containing 2 µg BSA, 0.25 mM dNTPs, 3 mM ATP, 0.5 units T4 DNA ligase (Thermo Scientific) and three units φ29 DNA polymerase were added to the previous restriction reaction and incubated for 20 minutes at 37°C. Because the reaction mixture also contained φ29 DNA polymerase, an immediate RCA occurred in the same reaction mixture after circularization of the monomerized RCA products. The second RCA and ligation were terminated by a two-minute incubation at 65°C.

Figure 19. Schematic overview of the padlock probe-based multiplex detection assay. Padlock probes have specific 5' and 3' ends for gene-target detection, a universal C2CA-sequence for amplification and a unique microarray sequence tag. The first step is the digestion of sample DNA with restriction enzymes, followed by annealing of padlock probes to their gene targets and subsequent ligation. Circularized padlock probes are amplified via RCA. Single-stranded RCA-products are incubated with C2CA-oligonucleotides which have an internal restriction site allowing monomerization of RCA-products with a restriction enzyme. Then, monomerized RCA-products circularize and anneal to excess C2CA-oligos that were not cut in the previous reaction. RCA-products are ligated and amplified in a second RCA. Those products are used as templates in a linear PCR with a C2CA-oligonucleotide as only primer resulting in single-stranded and fluorescently labelled DNA which is hybridized to a microarray.



2.1.23 Fluorescence labelling of C2CA products

To visualize C2CA products, fluorescent labelling was used (Figure 19). A linear PCR using only one primer instead of a primer pair was established for the incorporation of Atto-532 labelled dCTPs (MoBiTec, Germany) with a thermostable DNA polymerase lacking exonuclease activity. A 25 µl reaction mixture in $Vent_R$ (exo-) buffer (20 mM Tris-HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1% Triton X-100)

containing additionally 1.6 units $Vent_R$ (exo-)-polymerase (New England Biolabs), 5 µg BSA, 1 mM $MgSO_4$, 2 nM Atto532-dCTP, 1 µM $C2CA^-$, 50 µM dNTPs, and 6 µl C2CA products was used. Thermal cycling was carried out as follows: 95°C for five minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds; and a final cycle at 72°C for ten minutes.

2.1.24 Evaluation of padlock probes

The purchased padlock probes were tested with synthetic target oligonucleotides in a ligation reaction to determine their functionality and specificity. Synthetic DNA templates that represented the same gene were pooled and used in a single ligation reaction. A ligation mixture including all 96 padlock probes was incubated with the synthetic DNA template mix. DNA circles were prepared by the ligation of a 1-pM mixture of each padlock probe in ampligase buffer (20 mM Tris-HCl (pH 8.3), 25 mM KCl, 10 mM $MgCl_2$, 0.5 mM NAD, and 0.01% Triton® X-100), five units ampligase (Epicentre), 4 µg BSA, and 8.3 pM synthetic template in 20 µl at 60°C for one hour. The ligated probes were amplified using C2CA, and the generated C2CA products served as templates for labelling with fluorescent dCTPs in a linear PCR. These PCR products were hybridised to a DNA microarray slide. The padlock probes harboured a unique sequence tag complementary to a microarray probe. The microarray slides were scanned and analysed after washing. The cut-off for positive microarray signals was twice the mean fluorescence intensity of all the microarray probes used for padlock detection, and a heatmap was created for better visual presentation of the data. The microarray results from 35 experiments (one per gene plus two negative controls) were normalised, and the fluorescence intensity values were transformed into log(2) values. The resulting matrix was visualised in Microsoft Excel 2007.

2.1.25 Padlock probe assay testing using PCR products

The β-lactamase detection assay was also evaluated using the sequenced PCR products of five β-lactamase genes (*bla*$_{CTX\text{-}M\text{-}1}$, *bla*$_{CTX\text{-}M\text{-}9}$, *bla*$_{OXA\text{-}1}$, *bla*$_{SHV}$ and *bla*$_{TEM}$). Aliquots of 2 µl of 1:10 dilutions (10^0 - 10^9 PCR copies/µl) of these PCR products were added to a DNA digestion reaction (a total volume of 10 µl) containing *AluI* and *HpyF3I* (Fermentas) for one hour at 37°C according the manufacturer's recommendations, with

a subsequent inhibition step at 65°C for ten minutes. Ten microliters of ampligase buffer containing 2 pM of each padlock probe, 4 µg BSA, and five units ampligase were added to the 10 µl of the PCR digestion mixture. The padlock probe assay was placed in a thermal cycler at 95°C for five minutes and then at 60°C for various incubation times from 30 minutes to eight hours, followed by a final step of 95°C for two minutes. The different ligation times were tested to analyse the impact of ligation time on the sensitivity of the assay. After ligation, C2CA was performed using 10 µl of the ligation reaction, followed by fluorescence labelling with $Vent_R$(-exo) DNA polymerase and analysis using the barcode sequence microarray.

2.1.26 Pre-amplification of genomic DNA

The sensitivity of the β-lactamase detection assay using genomic DNA as template could be increased if genomic DNA was preamplified in a 25-multiplex PCR. The Mastermix 16S Basic PCR Kit (Molzym, Germany) was used according to the manufacturer's instructions. The total volume of the PCR was 10 µl containing 1 pM of 25 different primer pairs and 1 µl of the genomic DNA extract. Primers used for multiplex PCR are listed in Table 4. Thermal cycling was carried out as follows: 95°C for five minutes; 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 90 seconds; and a final cycle at 72°C for ten minutes.

2.1.27 Padlock probe based multiplex genotyping

The padlock probe assay detecting β-lactamase genes was tested with 70 clinical bacterial strains. A schematic overview of the whole experimental procedure, starting from DNA digestion until microarray detection, is illustrated in Figure 19. Prior to the ligation step of padlock probes, 10 µl of the genomic DNA extracts was digested with *AluI* and *HpyF3I* in the recommended buffers (Fermentas) for one hour at 37°C according the manufacturer's recommendations, followed by enzyme inhibition at 65°C for ten minutes. If genomic DNA was pre-amplified in the multiplex PCR, it was also digested according the manufacturer's recommendations. Ten microliters of ampligase buffer containing 2 pM of each padlock probe, 4 µg BSA, and five units ampligase were mixed with 10 µl of the digestion mix. The padlock probe assay was placed in a thermal cycler at 95°C for five minutes, and then 60°C for various incubation times from 30

minutes to 24 hours, followed by a final step of 95°C for two minutes. For the final analysis of the 70 clinical isolates, the genomic DNA was not pre-amplified; thus, a ligation time of 23 hours was chosen. After ligation, C2CA was performed using 10 µl of the ligation reaction, followed by fluorescence labelling with $Vent_R$(-exo) DNA polymerase and analysis using the barcode sequence microarray. The cut-off for a positive microarray signal was the mean fluorescence intensity of all microarray probes used for padlock detection. Two criteria had to be fulfilled for the detection of a gene to be considered positive. The first criterion was a positive signal for at least two corresponding microarray probes. For the second criterion, the mean gene value of the microarray probes corresponding to the gene was calculated; the second criterion was fulfilled if this value was above twice the mean fluorescence intensity of all the mean gene values.

2.1.28 Microarray analysis of padlock probe assay

A mixture consisting of 25µl of labelled C2CA products, 25µl ExpressHyb Hybridization Solution (Clonetech) and 1µl BSrev (50µM) was pipetted on a microarray slide and using a lifterslip for coverage. The slides were put in a hybridization chamber with humid atmosphere and incubated at 65°C for one hour. Subsequently, slides were washed in 2xSSC and 0.1% SDS for five minutes, followed by washing in 0.2x SSC for two minutes and a final washing step in ddH_2O for one minute. Slides were dried in a centrifuge and scanned in the Genepix 4000A microarray scanner. Data was analysed using GenePix Pro 6.0 and Microsoft Excel 2007.

2.1.29 Diversity analyses

Based on the sequencing results, a DNA sequence database alignment was created (75;82). The obtained β-lactamase sequences were imported into ARB and their deduced amino acid sequences were manually aligned. Subsequently, the nucleotide sequences were aligned according to the amino acid alignment. Nucleotide sequences were grouped in ARB according to their sequence similarity. BLAST analyses of nucleotide and amino acid sequences were performed to classify the identified sequences.

Phylogenetic trees were calculated with distance-matrix (PHYLIP distance matrix with FITCH, global rearrangements and randomized input order; Neighbour Joining with Jukes-Cantor correction), maximum-likelihood (PHYLIP ProML with JTT) and maximum-parsimony (PHYLIP protein parsimony with bootstraps, 1000 resamplings) methods. Consensus trees were drawn based on the trees obtained with the above mentioned methods proposed by Ludwig et al. (82).

The biostatistical software EstimateS was used to calculate rarefaction curve, Chao1 and ACE values (83). These values are used in general ecology but also in microbial ecology to estimate the total number of species in an investigated habitat since it is hardly possible to determine the total number of species experimentally. Especially in species-rich communities, such as those of tropical invertebrates, microbial or plant communities, it is impracticable to sample all species.

These species richness estimators use the observed abundance of species to perform their calculations and can be equally used to calculate the incidence of genes. When starting analyses of very species-rich communities, it is likely that with every individual sampled a different species is identified. After intensive sampling, it becomes unlikely to discover not so far observed species because most individuals have been recorded. Theoretically, in an extensively sampled habitat, all present species have been documented and sampling of new individuals would not lead to the discovery of new species. This perfectly documented habitat plotted as a graph with the number of observed species on the y-axis and the sampled individuals on the x-axis would result in a jagged curve, also referred to as accumulation curve, with a horizontal asymptote. Rarefaction curves are the result of repeated randomizing of the sample order, and thus are smooth curves corresponding to an accumulation curve. In this study, rarefaction curves using the Coleman method and 1000 randomizations were calculated. Rarefaction curves are used to compare species diversity of different samples or communities. Using this method, also communities with varying sample numbers can be compared.

Species richness estimators ACE and Chao1 estimate the point when further sampling effort would not result in the observation of new species. These asymptotic estimators use the information on the distribution of rare species for their calculations.

In the General Hospital of Vienna study, I used these species richness estimators to calculate the number of species with different β-lactamase genotypes, the number of β-lactamase genes and their subtypes in the General Hospital of Vienna. The genetic diversity within *Enterobacteriaceae*, tested phenotypically ESBL-positive, AmpC-positive or generally β-lactamase-positive also including carbapenemases, was compared. Input parameters for the estimators used in this study were as follows (Figure 20): a gene-group was defined as the set of genes that was detected by a single primer pair. DNA sequences coding for different amino acid sequences were classified in this study as different genes. A single gene could be subcategorized due to silent mutations into several variants. These variants were assigned in this study as unique β-lactamase DNA sequences. Unique genotypes harboured different combinations of unique β-lactamase DNA sequences. The workflow of the statistical analyses is illustrated in Figure 21.

Figure 20. Illustration of the classification system used in the statistical analysis. TEM-1 var1 and TEM-1 var2 have the same amino acid sequence but distinct nucleotide sequences due to silent mutations. A unique genotype consists of combinations of unique β-lactamase sequences.

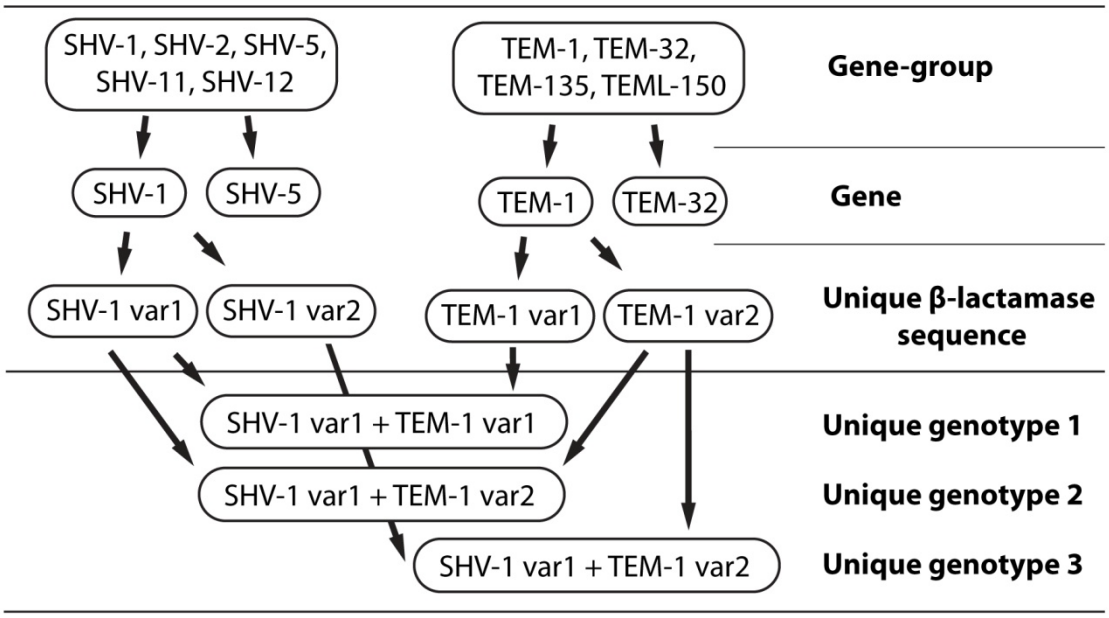
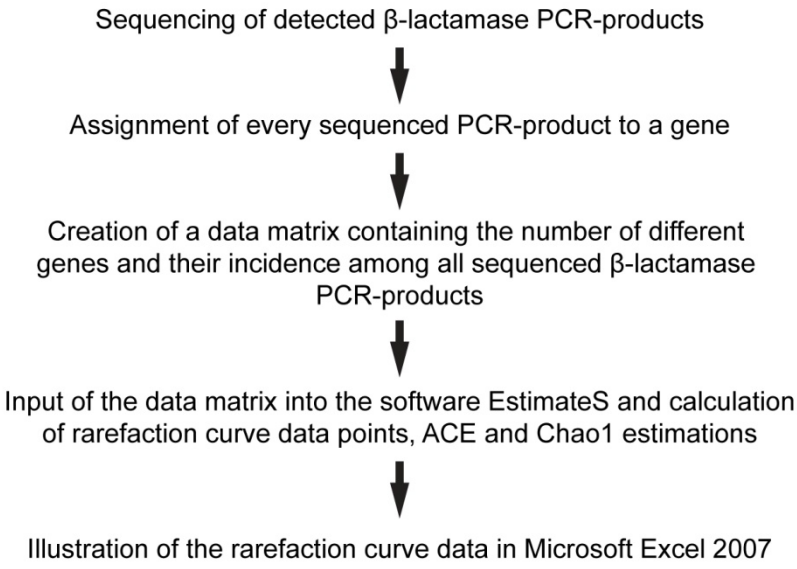


Figure 21. Schematic workflow of the statistical analysis estimating the genetic diversity of β-lactamase genes in the hospitals. The workflow for the analyses of the gene-groups and unique β-lactamase DNA sequences was identical. For the analyses of the diversity of unique resistance genotypes, the number of different genotypes and their incidence in the characterized clinical isolates was used for the data matrix.



In the study analysing the clinical samples from Graz, rarefaction curves and the Chao1 and ACE models were used to estimate the number of isolates with a unique β-lactamase genotype and the number of different β-lactamase genes among the 1101 ESBL-positive isolates of *Enterobacteriaceae*.

2.1.30 Nucleotide sequence accession numbers

The nucleotide sequences determined in the study analysing ESBL-positive bacterial isolates from the Medical University of Graz will appear in GenBank under the accession numbers JN676824 to JN676893. The DNA sequences obtained from the clinical isolates harbouring β-lactamases from the General Hospital of Vienna will appear in GenBank under the accession numbers JX268601 to JX268788.

3 Results

3.1 Emergence of several β-lactamase genes in Austria

In the present study, I screened clinical isolates of *Enterobacteriaceae* from the General Hospital of Vienna for the presence of a wide range of β-lactamase genes to provide clinicians with information on the disseminated resistance mechanisms in the hospital. This genetic information enables physicians to select more specific antibiotic treatments and helps to avoid the application of some antibiotics stimulating new point mutations, and thus new resistance mechanisms. Isolates phenotypically tested positive for ESBL, AmpC or carbapenemases were analysed by PCR. Because even sophisticated molecular analyses detecting many genes in hundreds of isolates face the problem how to evaluate the significance of the obtained data, phylogenetic and statistical calculations were performed to estimate the genetic diversity. Ecologists have a similar problem when they analyse an environment with the need to estimate the number of different species in the sampled area. Thus, statistical models such as the species richness estimators ACE and Chao1 were introduced in order to estimate the number of different species but also genes in the investigated environment. Rarefaction curve analyses provide information about the significance of these estimations (84-86). This study gives an extremely detailed picture about the dissemination of β-lactamase genes in the General Hospital of Vienna.

3.1.1 Emergence of known and new β-lactamase genes in Austria

Six new β-lactamases that had not been described before were identified. Five out of the six differed only in a single amino acid from known enzymes. The probability that these single point mutations are the result of sequencing errors is low, due to the fact that all genes were sequenced with a forward and reverse primer. Thus, overlapping sequence information was available on both DNA strands. $bla_{CTX\text{-}M\text{-}new1}$, $bla_{CTX\text{-}M\text{-}new2}$, and $bla_{CTX\text{-}M\text{-}new3}$ were most closely related to $bla_{CTX\text{-}M\text{-}15}$. $bla_{CTX\text{-}M\text{-}new1}$ had an arginine

instead of a glycine at amino acid (aa) position 22. *bla*$_{CTX\text{-}M\text{-}new2}$ had glutamic acid instead of a glycine at aa-position 45. *bla*$_{CTX\text{-}M\text{-}new3}$ had a serine instead of a glycine at aa-position 46. *bla*$_{CTX\text{-}M\text{-}new4}$ was most closely related to *bla*$_{CTX\text{-}M\text{-}1}$ but differed at aa-position 77 because glutamic acid was incorporated instead of valine. *bla*$_{CTX\text{-}M\text{-}new5}$ was similar to *bla*$_{CTX\text{-}M\text{-}14}$, but an isoleucine was incorporated instead of methionine at aa-position 138.

bla$_{CMY\text{-}new}$ had four substitutions in comparison to *bla*$_{CMY\text{-}2}$: Asparagine 214 to serine, aspartic acid 218 to asparagine, arginine 252 to histidine, and alanine 273 to glutamic acid. The newly identified CMY β-lactamase gene was most related to *bla*$_{CMY\text{-}41}$.

In this study, we also report the emergence of the β-lactamase genes *bla*$_{CMY\text{-}2,\ -4,\ -42}$, *bla*$_{CTX\text{-}M\text{-}27,\ -38}$, *bla*$_{DHA\text{-}1}$, *bla*$_{OXA\text{-}72}$, *bla*$_{SHV\text{-}26,\ -33}$, *bla*$_{TEM\text{-}32,\ -135}$, *bla*$_{TEML\text{-}150}$, *and bla*$_{VIM\text{-}4}$ in Austria. *bla*$_{TEML\text{-}150}$ is an unassigned TEM gene present in the β-lactamase engineering database of the University of Stuttgart (http://www.laced.uni-stuttgart.de).

3.1.2 Distribution of β-lactamase genes in *Enterobacteriaceae*

The routine diagnostic laboratory in the General Hospital of Vienna identified in 657 isolates of β-lactamase-positive *Enterobacteriaceae* AmpC, ESBL, and carbapenemase resistance mechanisms by phenotypic tests in the year 2011. In the present study, out of these 657 isolates 108 consecutive were chosen from different patients for a detailed genetic analysis. In 89 of the genetically investigated clinical strains, genes encoding β-lactamases could be detected. In total, 188 β-lactamase DNA sequences were identified in these 89 isolates by PCR analysis and further sequenced. Sequence analyses revealed the presence of ten gene-groups comprising 30 different genes (Table 12). Forty different unique β-lactamase DNA sequences (e.g SHV-1 var1, SHV-1 var2) represented due to silent mutations only 30 different genes (e.g. SHV-1).

Exclusively in phenotypically AmpC-positive isolates, AmpC-genes of the CMY-2 group were present with a frequency of 38%. In 59% of phenotypically AmpC-positive isolates, *bla*$_{DHA\text{-}1}$ was detected and 56% harboured additional *bla*$_{SHV\text{-}11}$. Surprisingly, other TEM variants than *bla*$_{TEM\text{-}1}$ were only present in AmpC-positive isolates.

Gene-groups / Genes	Number of observed β-lactamase sequences in 89 β-lactamase-positive isolates	32 AmpC-positive isolates	53 ESBL-positive isolates	4 carbapenemase-positive isolates
1. CMY-2 group	**12**	**12**		
CMY-2	8	8		
CMY-4	2	2		
CMY-42	1	1		
CMY-new	1	1		
2. CTX-M-1 group	**25**		**24**	**1**
CTX-M-1	3		2	1
CTX-M-15	18		18	
CTX-M-new1	1		1	
CTX-M-new2	1		1	
CTX-M-new3	1		1	
CTX-M-new4	1		1	
3. CTX-M-9 group	**18**		**18**	
CTX-M-14 var1	3		3	
CTX-M-14 var2	4		4	
CTX-M-27 var1	1		1	
CTX-M-27 var2	3		3	
CTX-M-38	6		6	
CTX-M-new5	1		1	
4. DHA	**21**	**19**	**2**	
DHA-1	21	19	2	
5. KPC	**2**			**2**
KPC-2	2			2
6. OXA-1 group	**32**	**18**	**14**	
OXA-1	32	18	14	
7. OXA-24 group	**1**			
OXA-72	1			
8. SHV	**38**	**18**	**17**	**3**
SHV-1 var1	2		2	
SHV-1 var2	1		1	
SHV-2 var1	1		1	
SHV-2 var2	3		3	
SHV-5	1		1	
SHV-11 var1	21	17	3	1
SHV-11 var2	3		3	
SHV-11 var3	1		1	
SHV-11 var4	1	1		
SHV-12	2		1	1
SHV-26	1			1
SHV-33	1		1	
9. TEM	**38**	**9**	**27**	**2**
TEM-1 var1	31	6	24	1
TEM-1 var2	2		2	
TEM-1 var3	1		1	
TEM-1 var4	1			1
TEM-32	1	1		
TEM-135	1	1		
TEML-150	1	1		
10. VIM	**1**			**1**
VIM-4	1			1

Table 12. Detected β-lactamase genes and their observed frequency in the characterized 89 clinical isolates. β-Lactamase-positive isolates include AmpC-, ESBL-, and carbapenemase-positive isolates. One hundred eighty-eight β-lactamase DNA sequences were detected in 89 isolates.

In 78% of ESBL-positive isolates, CTX-M genes were encountered, and in 52% TEM-1. *K. pneumoniae* isolates harboured 92% of the detected SHV genes. In ESBL-positive *E. coli*, bla_{SHV-5} and bla_{SHV-2} were detected, and in an ESBL-positive isolate of *Serratia marcescens*, bla_{SHV-12}. $bla_{SHV-1,\ -2,\ -5,\ -33}$ were found exclusively in ESBL-positive isolates.

Carbapenem resistance was in two cases mediated by bla_{KPC-2} and in one case by bla_{VIM-4}. In two phenotypically carbapenem-resistant isolates, no carbapenemase could be identified. One of these isolates was an *Enterobacter cloacae* strain (C8) with no β-lactamase detected by PCR, the other was a *Klebsiella pneumoniae* isolate (C21) with $bla_{CTX-M-1}$ and bla_{SHV-26} present.

In total, 51 different β-lactamase genotypes were detected. The most frequently detected β-lactamase genotype was observed 16 times in AmpC-positive *Klebsiella pneumoniae* isolates consisting of bla_{DHA-1}, bla_{OXA-1}, and bla_{SHV-11}. The second most common genotype contained $bla_{CTX-M-15}$ and bla_{OXA-1} in ESBL-positive *E. coli* and was observed eight times. Other genotypes were less dominant and identified only three times or less (Table 13).

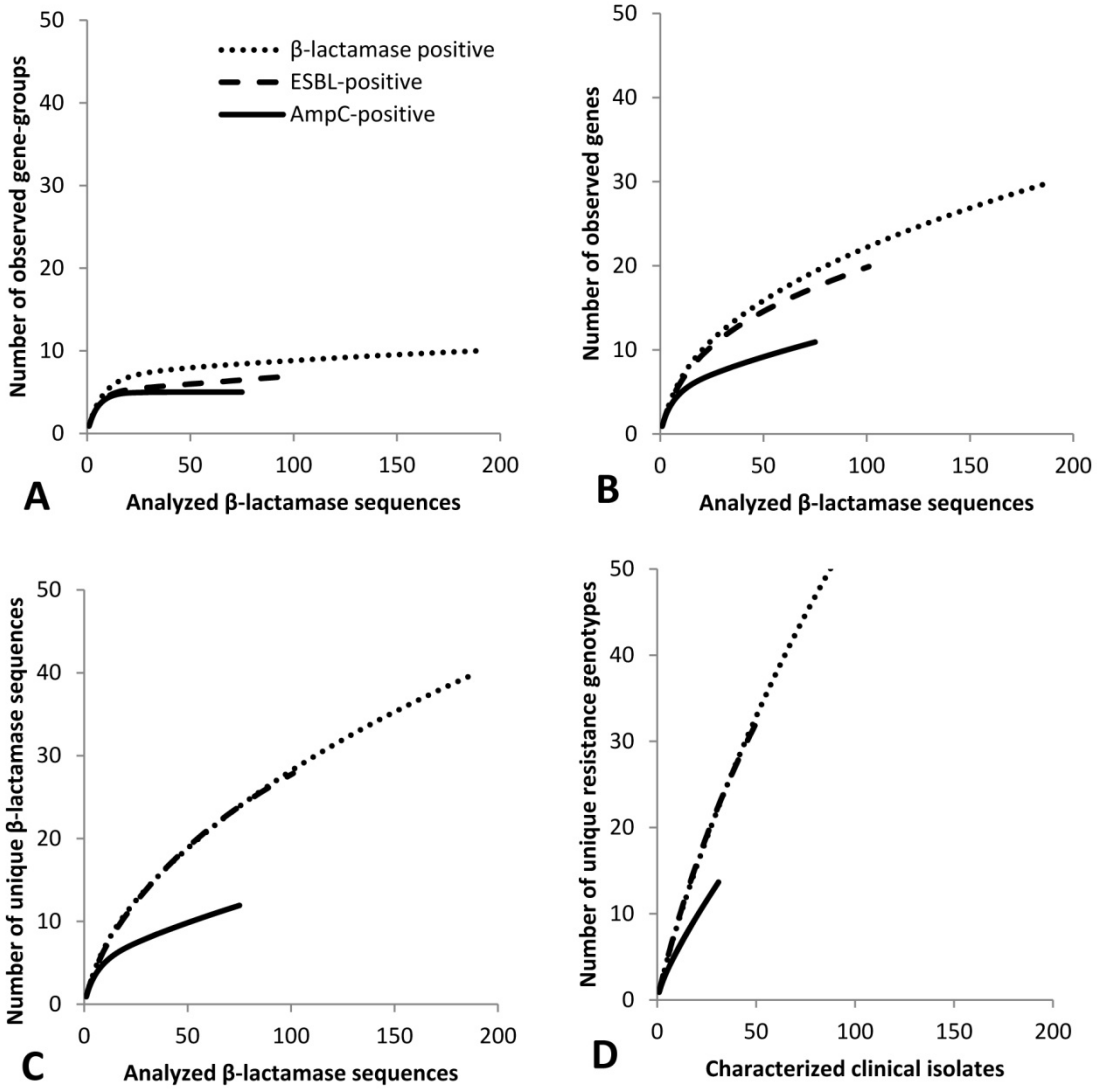
Isolate	Species	Isolation site	Resistance phenotype	β-lactamase genes	Genotype
C11	E. coli	Stool	AmpC-BL	CMY-new	1
C14	E. coli	Urine	AmpC-BL	CMY-2	2
C29	E. coli	Urine	AmpC-BL	CMY-2	2
C16	E. coli	Swab	AmpC-BL	CMY-2, TEM-1 var1	3
C18	E. coli	Urine	AmpC-BL	CMY-2, TEM-1 var1	3
C37	E. coli	Urine	AmpC-BL	CMY-2, TEM-1 var1	3
C47	E. coli	Stool	AmpC-BL	CMY-2, TEM-135	4
C36	E. coli	Stool	AmpC-BL	CMY-2, TEM32	5
C7	E. coli	Urine	AmpC-BL	CMY-2, TEML-150	6
C5	E. coli	Urine	AmpC-BL	CMY-42, TEM-1 var1	7
C28	P. mirabilis	Urine	AmpC-BL	CMY-4	8
C48	P. mirabilis	Skin	AmpC-BL	CMY-4, TEM-1 var1	9
6	E. coli	Urine	ESBL	CTX-M-27 var1, TEM-1 var1	10
15	M. morganii	Stool	ESBL	CTX-M-new1, DHA-1, OXA-1, TEM-1 var1	11
5	E. coli	Urine	ESBL	CTX-M-new2, TEM-1 var1	12
10	K. pneumoniae	Bronchial secretion	ESBL	CTX-M-new3, OXA-1, SHV-11 var1, TEM-1 var1	13
9	E. coli	Urine	ESBL	CTX-M-new4	14
1	E. coli	Urine	ESBL	CTX-M-new5, TEM-1 var1	15
24	E. coli	Stool	ESBL	CTX-M-1	16
42	E. coli	Swab	ESBL	CTX-M-1	16
C21	K. pneumoniae	Dialysate	Carbapenemase	CTX-M-1, SHV-26	17
C45	K. oxytoca	Swab	ESBL	CTX-M-14 var1	18
45	E. coli	Urine	ESBL	CTX-M-14 var1, CTX-M-15 var1	19

47	K. oxytoca	Urine	ESBL	CTX-M-14 var1, TEM-1 var1	20
17	E. coli	Stool	ESBL	CTX-M-14 var2	21
40	E. coli	Swab	ESBL	CTX-M-14 var2	21
18	E. coli	Swab	ESBL	CTX-M-14 var2, TEM-1 var1	22
23	E. coli	Swab	ESBL	CTX-M-14 var2, TEM-1 var1	22
30	E. coli	Drain fluid	ESBL	CTX-M-15 var1	23
34	E. coli	Blood	ESBL	CTX-M-15 var1	23
52	E. coli	Urine	ESBL	CTX-M-15 var1	23
33	K. pneumoniae	Bronchial lavage	ESBL	CTX-M-15 var1, DHA-1, SHV-11 var1, TEM-1 var3	24
3	E. coli	Urine	ESBL	CTX-M-15 var1, OXA-1	25
12	E. coli	Swab	ESBL	CTX-M-15 var1, OXA-1	25
13	E. coli	Urine	ESBL	CTX-M-15 var1, OXA-1	25
31	E. coli	Urine	ESBL	CTX-M-15 var1, OXA-1	25
32	E. coli	Urine	ESBL	CTX-M-15 var1, OXA-1	25
49	E. coli	Urine	ESBL	CTX-M-15 var1, OXA-1	25
51	E. coli	Swab	ESBL	CTX-M-15 var1, OXA-1	25
55	E. coli	Sinus maxillaris	ESBL	CTX-M-15 var1, OXA-1	25
43	K. pneumoniae	Swab	ESBL	CTX-M-15 var1, OXA-1, SHV-11 var1, TEM-1 var1	26
19	E. coli	Stool	ESBL	CTX-M-15 var1, OXA-1, TEM-1 var1,	27
4	K. pneumoniae	Urine	ESBL	CTX-M-15 var1, OXA-1, TEM-1 var2,	28
44	K. pneumoniae	Stool	ESBL	CTX-M-15 var1, SHV-11 var3	29
28	E. coli	Urine	ESBL	CTX-M-15 var1, TEM-1 var1	30
14	E. coli	Stool	ESBL	CTX-M-27 var2	31
37	E. coli	Swab	ESBL	CTX-M-27 var2	31
54	E. coli	Urine	ESBL	CTX-M-27 var2	31
7	K. pneumoniae	Urine	ESBL	CTX-M-38, SHV-11 var2, TEM-1 var1,	32
48	K. pneumoniae	Stool	ESBL	CTX-M-38, SHV-11 var2, TEM-1 var1,	32
50	K. pneumoniae	Stool	ESBL	CTX-M-38, SHV-11 var2, TEM-1 var1,	32
20	K. pneumoniae	Swab	ESBL	CTX-M-38, TEM-1 var1	33
26	K. oxytoca	Stool	ESBL	CTX-M-38, TEM-1 var1	33
27	E. coli	Stool	ESBL	CTX-M-38, TEM-1 var2	34
C53	K. pneumoniae	Urine	AmpC-BL	DHA-1, OXA-1	35
C1	K. pneumoniae	Swab	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C2	K. pneumoniae	Swab	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C6	K. pneumoniae	Swab	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C12	K. pneumoniae	Swab	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C25	K. pneumoniae	Tracheal secretion	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C26	K. pneumoniae	Urine	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C30	K. pneumoniae	Blood	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C31	K. pneumoniae	Bronchial secretion	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C33	K. pneumoniae	Swab	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C34	K. pneumoniae	Drain fluid	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C38	K. pneumoniae	Swab	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C43	K. pneumoniae	Swab	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C44	K. pneumoniae	Urine	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C49	K. pneumoniae	Sputum	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C50	K. pneumoniae	Sputum	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C52	K. pneumoniae	Blood	AmpC-BL	DHA-1, OXA-1, SHV-11 var1,	36
C20	K. pneumoniae	Stool	AmpC-BL	DHA-1, OXA-1, SHV-11 var4,	37
C9	K. pneumoniae	Bronchial lavage	AmpC-BL	DHA-1, SHV-11 var1	38
C19	K. pneumoniae	Swab	Carbapenemase	KPC-2, SHV-11 var1	39
C39	K. pneumoniae	Swab	Carbapenemase	KPC-2, SHV-12, TEM-1 var4,	40
29	E. coli	Anal swab	ESBL	OXA-1	41

22	K. pneumoniae	Stool	ESBL	OXA-72, SHV-33, TEM-1 var1,	42
39	K. pneumoniae	Anal swab	ESBL	SHV-1 var1, TEM-1 var1	43
46	K. pneumoniae	Stool	ESBL	SHV-1 var1, TEM-1 var1	43
53	K. pneumoniae	Urine	ESBL	SHV-1 var2, TEM-1 var1	44
8	S. marcescens	Urine	ESBL	SHV-12, TEM-1 var1	45
16	K. pneumoniae	Urine	ESBL	SHV-2 var1	46
11	K. pneumoniae	Urethra	ESBL	SHV-2 var2	47
41	K. pneumoniae	Stool	ESBL	SHV-2 var2	47
21	E. coli	Anal swab	ESBL	SHV-2 var2, TEM-1 var1	48
38	E. coli	Anal swab	ESBL	SHV-5, TEM-1 var1	49
C41	E. coli	Urine	AmpC-BL	TEM-1 var1	50
35	E. coli	Swab	ESBL	TEM-1 var1	50
C32	E. cloacae	Swab	Carbapenemase	TEM-1 var1, VIM-4	51

Table 13. Results obtained from the genetic analysis of β-lactamase-positive isolates of *Enterobacteriaceae* from the General Hospital Vienna.

Figure 22. Rarefaction curve analyses of the detected β-lactamase genes in the General Hospital of Vienna. (A-C) The observed number of gene-groups, genes and unique β-lactamase sequences was plotted versus the number of observed β-lactamase nucleotide sequences. (D) The number of observed β-lactamase genotypes was plotted versus the number of tested clinical isolates harbouring β-lactamases.



3.1.3 β-Lactamase gene diversity in General Hospital of Vienna

To estimate the total diversity of β-lactamase genes in the General Hospital of Vienna, statistical algorithms were used. Rarefaction curves were calculated to compare the genetic diversity of different β-lactamase phenotypes. In general, AmpC-positive isolates showed less genetic diversity of β-lactamases than ESBL-positive ones as shown in all graphs in Figure 22. The rarefaction curves in Figure 22A and Figure 22B resulting from all β-lactamase-positive isolates (including AmpC-positive, ESBL-

positive and carbapenemase-positive) are clearly above the ones of solely ESBL-positive. This is logical because the genes of the carbapenemase-positive isolates contribute to this higher diversity. Interestingly, the corresponding rarefaction curves in Figure 22C are almost identical, which is indicating a higher number of silent mutations in ESBL-positive isolates in comparison to other β-lactamase-positive isolates.

Only the rarefaction curves in Figure 22A approach the horizontal asymptote. This shows that the majority of gene-groups in β-lactamase positive isolates were detected and that extensive analyses of resistant strains in the General Hospital of Vienna would reveal only a few further new gene-groups. Chao1 and ACE models estimated that only two or three more gene-groups, respectively are present in this clinical environment (Table 14).

	β-lactamase-positive		ESBL-positive		AmpC-positive	
	Chao1	ACE	Chao1	ACE	Chao1	ACE
Gene-groups	12 ±SD 4	13	8 ±SD 2	8	5 ±SD 0	5
Genes	55 ±SD 19	52	40 ±SD 20	32	24 ±SD 17	21
Unique β-lactamase sequences	77 ±SD 23	77	48 ±SD 14	45	30 ±SD 24	27
β-Lactamase genotypes	123 ±SD 35	152	77 ±SD 27	97	75 ±SD 71	56

Table 14. Results of the statistical calculations using the species richness estimators Chao1 and ACE to estimate the number of gene-groups, genes, unique β-lactamase sequences and β-lactamase genotypes in the General Hospital of Vienna.

In Figure 22B and Figure 22C, the flattening character of the slopes is visible but the rarefaction curves are clearly not approximating the horizontal asymptote. Chao1 and ACE values indicate that approximately 55% of β-lactamase genes present in the hospital were detected in this study.

Not only the presence of individual β-lactamases is important but also how they cluster together in clinical isolates as their composite genotype is responsible for phenotypic β-lactam resistance. Therefore, the diversity of β-lactamase genotypes was also analysed with rarefaction curve analysis. The slopes of the rarefaction curves in Figure 22D are nearly linear indicating that the sampling effort was not sufficient to estimate the total number of β-lactamase genotypes in the General Hospital of Vienna. Thus, the genotype numbers calculated with the asymptotic estimators have to be considered cautiously,

which is also reflected in the high standard deviation values and varying values calculated by the two methods (Table 14).

3.2 High genetic diversity of ESBL in south eastern Austria

In the present study, 33 ESBL-positive bacterial isolates collected from patients of the south-eastern Austrian city of Graz and its neighbourhood were studied for the presence of β-lactamase genes. The phenotypic characterizations were conducted in the central microbiological laboratory of the Medical University of Graz. Nucleotide sequence diversities were determined using phylogenetic analysis. The total number of β-lactamase genes and different resistance genotypes were calculated using richness estimators.

The investigation is technically almost identical with the one analysing clinical isolates from the General Hospital of Vienna. The sample number was significantly less, as only bacterial strains which were tested phenotypically as ESBL-positive, were genetically analysed. Additionally, a phylogenetic consensus tree was created, illustrating the diversity of the detected serine-β-lactamase genes. ESBL-positive isolates from the current biobank of the Medical University of Graz were already analysed in a previous study by Eisner *et al.* (87). The mentioned study investigated the emergence of ESBL-positive pathogens and the presence of CTX-M genes between 1998 and 2004. My study gives insight in the further development of the antibiotic resistance situation in this area.

3.2.1 Emergence of CTX-M-2 and DHA-1 genes

In the years 2009 and 2010, the Medical University of Graz received and analysed 180,604 patient samples from Graz and south-eastern Austria. 230,188 isolates could be identified. If clinical relevance was suspected, they were further tested for antibiotic susceptibility with cultivation based methods. The phenotypical characterization revealed 2177 ESBL-positive *Enterobacteriaceae*, mainly *E.coli* and *Klebsiella* spp., which represented 0.95% of all isolates in 2009 and 2010 (Table 15).

Antibiotic	*E.Coli*						*Klebsiella spp.*					
	2000		2009		2010		2000		2009		2010	
	%I	%R	%I	%R	%I	%R	%I	%R	%I	%R	%I	%R
Amoxicillin	0.9	**23.7**	1.6	**35**	1.3	**36**	-	-	-	-	-	-
Amoxi/Clav	4.4	1.8	8.2	3.8	7.5	3.2	2.8	1.7	5.4	9.8	3.5	7.8
Pip/Taz	0.4	0.3	0.7	0.7	0.7	0.8	1.3	2.7	1.4	3	1.2	5
Cefalotin	9.1	4.7	**19.1**	6.7	**19.3**	7.8	4	6.8	2.7	**14.4**	3.9	**10.1**
Cefuro/Axetil	1.6	0.5	3.1	5.6	3.2	5.9	2.3	4	3.3	7.8	3.5	8.9
Cefoxitin	0.6	0.5	1.5	0.7	0.9	0.5	0.9	2.1	1.5	2.1	2.5	3.2
Cefotaxime	0	0.1	0	5.2	0.1	5.4	0.1	1.7	0.2	4.6	0.6	4.9
Ceftazidime	0	0.1	0.1	5.2	0.1	5.4	0.2	2.7	0.1	4.8	0.9	5.1
Cefepime	0	0.2	0	**10.2**	0	6.2	0	2.9	0	7	0.3	5.8
Imipenem	0	0	0	0	0	0	0	0	0.2	0.2	0	2.6
Meropenem	-	-	0	0	0	0	-	-	0	0.2	0.3	0.5
Ertapenem	-	-	-	-	0	0.2	-	-	0	0	0	1.7
Gentamicin	0	1.1	0.1	4.6	0.1	4.3	0.9	0.8	0.9	**11.4**	0.4	6.4
Amikacin	0	0	0.1	0.6	0.2	0.1	0.2	0	0.3	0.3	0	1
Tetracyclin	-	-	0	**22.9**	0.1	**22.5**	-	-	0.5	**24.2**	1	**14.9**
Tigecycline	-	-	-	-	-	-	-	-	1.6	**11.1**	**12.9**	0
Trimethoprim	0.1	**16**	0	**25.5**	0	**27.6**	1	7.1	0.1	**15.6**	0.2	**14.7**
Trim/Sulfa	0.1	**13.3**	0	**22.8**	0	**23.3**	0.3	3.8	0.1	10	0.4	**12.6**
Fosfomycin	1.6	3.9	0	1.4	0	1	-	-	0	**39.4**	0	**30.2**
Ciprofloxacin	0.1	5	0.3	**14.7**	0.2	**15.1**	0.7	0.7	0.6	5.9	1.2	7.5
Norfloxacin	0	6.3	0.4	**16.3**	0.2	**18.1**	0	1.5	1.3	8.1	1.3	9.3
Nitrofurantoin	0.3	0.6	1.2	0.5	0.6	0.5	6.4	**10.7**	**14.4**	**15.3**	**12.1**	**14.4**

R resistant; I intermediate; - not tested. Values higher than ten are indicated with boldface digits.

Table 15. Results of phenotypical testing of the susceptibility against antibiotics from the Medical University of Graz

In all 33 isolates investigated, at least one β-lactamase gene could be identified. Genotypes were characterized by sequencing the PCR products and analyzing the DNA sequences with NCBI BLAST. In the 33 isolates investigated, 70 β-lactamase DNA sequences were found comprising 13 unique genes with the TEM-1 gene being the most common (in 58% of the isolates), followed by the genes coding for CTX-M-15 (55%), OXA-1 (46%), CTX-M-1 (9%), CTX-M-9 (9%), CTX-M-14 (9%), CTX-M-22 (6%), SHV-11 (6%), SHV-1 (3%), SHV-12 (3%), CTX-M-2 (3%), CTX-M-38 (3%), and DHA-1 (3%). CTX-M genes were found in 30 of 33 isolates investigated (Table 16).

Isolate	Species	Isolation site	β-lactamase(s)	AMX	AMC	MEC	PIP	CFZ	CEF	CXM	CPD	FOX	CTX	CAZ	FEP	ATM	IMI	MEM	ETP
09/120	*E. coli*	Urine	CTX-M-15, OXA-1, TEM-1	R	R	S	S	-	R	R	R	S	R	R	R	-	S	S	S
10/142	*E. coli*	Urine	CTX-M-15, OXA-1, TEM-1	R	R	S	R	R	R	R	-	S	R	R	-	R	S	S	S
10/143	*E. coli*	Urine	CTX-M-15, OXA-1, TEM-1	R	/	S	S	-	R	R	-	S	R	R	-	-	-	-	S
10/147	*E. coli*	Anal swab	CTX-M-15, OXA-1, TEM-1	R	S	-	S	-	R	R	-	S	R	R	R	R	S	S	S
10/152	*E. coli*	Urine	CTX-M-15, OXA-1, TEM-1	R	R	S	S	R	R	R	-	S	R	R	-	R	S	S	S
10/153	*E. coli*	Urine	CTX-M-15, OXA-1, TEM-1	R	R	-	S	-	R	R	-	S	R	R	R	-	-	S	S
10/141	*E. coli*	Sore	CTX-M-15, OXA-1, TEM-1	R	R	-	S	-	R	R	-	S	R	R	R	-	-	S	S
10/145	*E. coli*	Sore	CTX-M-15, OXA-1, TEM-1	R	R	-	S	-	R	R	-	S	R	R	R	S	S	S	S
09/133	*E. coli*	Groin	CTX-M-15, OXA-1	R	R	-	S	-	R	R	R	S	R	R	R	-	S	S	-
09/137	*E. coli*	Urine	CTX-M-15, OXA-1	R	/	S	R	-	R	R	R	S	R	R	R	-	S	S	S
10/135	*E. coli*	Urine	CTX-M-15, OXA-1	R	S	S	S	-	R	R	-	S	R	R	-	-	-	-	S
10/140	*E. coli*	Urine	CTX-M-15, OXA-1	R	S	S	S	-	R	R	-	S	R	R	-	-	-	-	S
10/146	*E. coli*	Stool	CTX-M-15, OXA-1	R	R	-	S	R	R	R	-	/	R	R	R	R	S	S	S
10/154	*E. coli*	Urine	CTX-M-15, OXA-1	R	/	S	S	-	R	R	-	S	R	R	-	-	-	-	S
09/134	*E. coli*	Ulcer natal cleft	CTX-M-9, TEM-1	R	S	-	S	-	R	R	R	S	R	R	R	-	S	S	-
09/140	*E. coli*	Urine	CTX-M-9, TEM-1	R	/	S	S	-	R	R	R	S	R	R	R	-	S	S	S
10/137	*E. coli*	Urine	CTX-M-9, TEM-1	R	/	S	S	-	R	R	-	S	R	R	-	-	-	-	S
09/136	*E. coli*	Urine	CTX-M-14, TEM-1	R	/	S	S	-	R	R	R	S	R	R	R	-	S	S	S
10/150	*E. coli*	Urine	CTX-M-14, TEM-1	R	/	S	S	R	R	R	-	S	R	R	-	R	S	S	S
09/116	*E. coli*	Vaginal swab	CTX-M-15	R	/	-	S	-	R	R	R	R	R	R	R	-	S	S	-
10/133	*E. coli*	Sore	CTX-M-15	R	S	-	S	-	R	R	-	S	R	R	R	R	S	S	S
10/148	*E. coli*	Anal swab	CTX-M-15, TEM-1	R	S	-	S	-	R	R	-	S	R	R	R	R	S	S	S
09/139	*K. pneumoniae*	Urine	CTX-M-1, SHV-11	R	/	-	S	-	R	R	R	S	R	R	R	-	S	S	S
10/136a	*E. coli*	Urine	CTX-M-1, TEM-1	R	R	R	/	-	R	R	-	S	R	R	-	-	-	-	S
10/139	*E. coli*	Anal swab	CTX-M-1	R	S	-	S	-	R	R	-	S	R	R	R	-	-	S	-
09/127	*K. pneumoniae*	Stoma	CTX-M-22, SHV-1, TEM-1	R	R	-	R	-	R	R	R	S	R	R	R	-	-	S	-
10/136b	*E. coli*	Urine	CTX-M-22	R	S	S	S	-	R	R	-	S	R	R	-	-	-	-	S
10/144	*E. coli*	Urine	CTX-M-14	R	R	S	S	-	R	R	-	S	R	R	-	-	-	-	S
10/149	*K. pneumoniae*	Urine	CTX-M-38, TEM-1	R	/	S	S	-	R	R	-	S	R	R	-	R	S	S	S
10/151	*K. pneumoniae*	Urine	CTX-M-2, SHV-12, TEM-1	R	/	-	S	R	R	R	-	S	R	R	-	R	S	S	S
10/134	*K. pneumoniae*	Urine	DHA-1, OXA-1, SHV-11	R	R	-	R	R	-	R	-	R	R	R	R	R	S	S	S
09/121	*K. pneumoniae*	Stool	TEM-1	R	R	-	R	-	R	R	R	R	R	R	R	-	S	S	-
09/123	*E. coli*	Urine	OXA-1	R	R	S	R	-	R	R	R	/	R	R	R	-	S	S	S

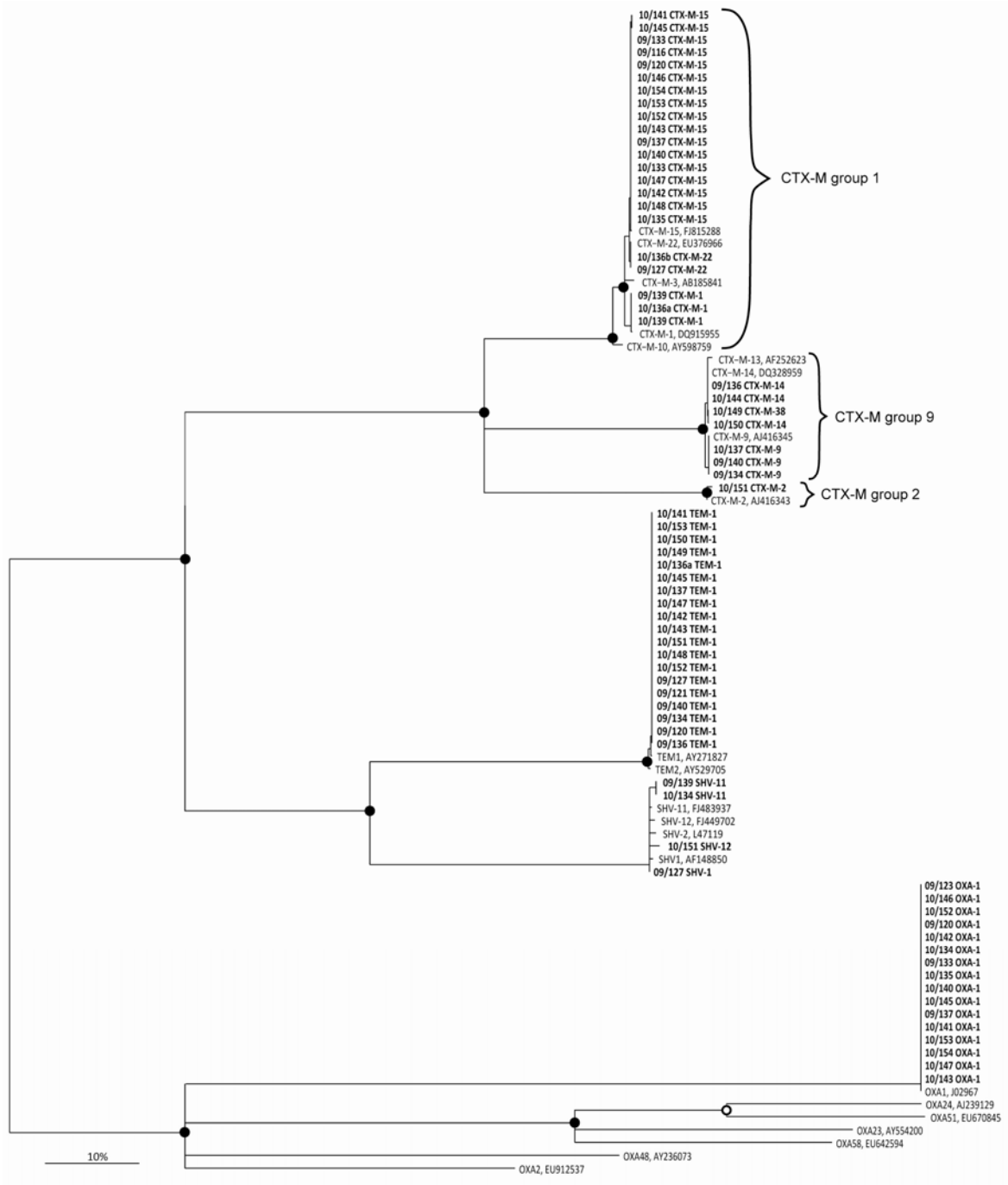
R resistant; / intermediate; S sensitive; - not tested.

Amoxicillin, AMX; Amoxicillin/Clavulanic acid, AMC; Mecillinam, MEC ; Piperacillin/Tazobactam, PIP ; Cefazolin, CFZ; Cefalotin, CEF ; Cefuroxime-Axetil, CXM; Cefpodoxime, CPD; Cefoxitin, FOX;

Cefotaxime, CTX; Ceftazidime, CAZ; Cefepime, FEP; Aztreonam, ATM; Imipenem, IMI ; Meropenem, MEM; Ertapenem, ETP

Table 16. Results obtained from the 33 isolates of *E.coli* and *K. pneumoniae* tested ESBL-positive at the Medical University of Graz

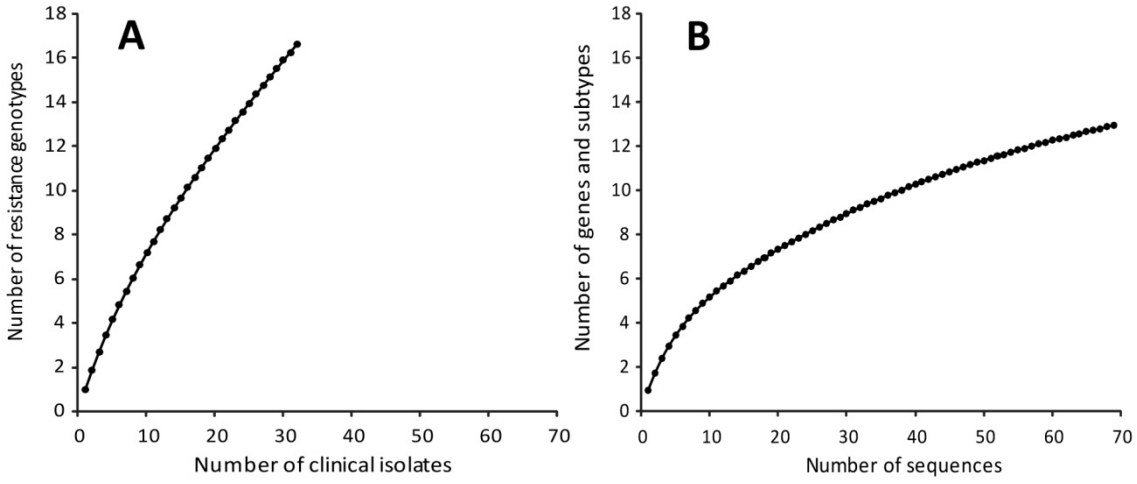
Figure 23. Phylogenetic consensus tree illustrating the members of Class A and Class D serine β-lactamase genes. The tree was calculated using serine β-lactamase DNA sequences obtained from this study (bold) and the most closely related β-lactamases. The open circles indicate lineages with 80-90% parsimony bootstrap score, the filled circles >90%. The scale bar indicates 10% of sequence divergence as estimated from distance-matrix analysis.



To investigate the relation between the bacteria and genes in more detail, phylogenetic trees of the serine β-lactamase genes sequenced were calculated and a consensus tree

established (Figure 23). Fifty-three sequences were found to be members of Class A, 16 of Class D, and one of Class C serine β-lactamases. Sequences belonging to the three phylogenetically different CTX-M groups 1, 2, and 9 could be identified. The CTX-M-15 genes of isolates 10/141 and 10/145 showed only a single mutation when compared to the 15 CTX-M-15 genes of the other isolates. This synonymous mutation affected nucleotide position 528 with adenosine instead of guanine. The only sequence (bla_{DHA-1}) belonging to Class C β-lactamases was not included in the phylogenetic tree as it showed very little sequence similarity, and thus cannot be implemented correctly in the used alignment.

Figure 24. Rarefaction curve analyses. (A) The number of β-lactamase observed genotypes plotted versus the number of clinical isolates characterized. (B) The number of different β-lactamases and subtypes plotted versus the number of β-lactamase sequences obtained during the characterization of bacterial isolates.



The diversity analyses revealed a high diversity of β-lactamase antibiotic resistance genotypes among the clinical isolates investigated. From 17 different resistance genotypes, only five could be observed in more than one isolate. The genotype comprising $bla_{CTX-M-15}$, bla_{OXA-1}, and bla_{TEM-1} was dominant (Table 16). The slope of the rarefaction curve is almost linear indicating a high diversity of antibiotic resistance genotypes (Figure 24A). Among ESBL-positive *E.coli* and *K. pneumoniae* isolates, the number of antibiotic resistance genotypes was found to be 54 using the ACE model and 53 using the Chao1 models. Rarefaction curve analysis revealed a flattening slope suggesting that the majority of β-lactamase genes in ESBL-positive *E.coli* and *K.*

pneumoniae characterized in this laboratory was identified (Figure 24B). The ACE model estimated 17 to be the total number of β-lactamase genes. The Chao1 model estimated the presence of 19 β-lactamase genes in the investigated region of south eastern Austria.

3.3 On-chip PCR

The characterization of clinical isolates presented in the previous two sections was performed using PCR based techniques. In the majority of clinical laboratories, PCR is the method of choice to gain more detailed information on pathogens because it comprises several advantages. It is cheap, robust, simple, fast, and has a high sensitivity. The most important limitation concerns its poor multiplexing capability which is highly necessary in order to examine the thousands of genetic elements altering various pathogens. Beside species and strain information, also the presence of antibiotic resistance mechanisms is of special importance during pathogen characterization.

The immobilization of primers to a surface seems to be an elegant solution to the problem of PCR multiplexing. The spatially separated primers cannot hybridize to the same target and also the immobilized PCR products cannot interact with other primer pairs. Furthermore, the formation of artificial DNA chimera molecules is prevented, because different, partially elongated PCR products cannot hybridize unspecifically and get elongated using the unspecific PCR fragment as template. Unfortunately, several studies did not succeed to establish an on-chip PCR protocol due to missing specificity of the reaction. Thus, the goal of my study was to identify reaction parameters that are responsible for the specificity of the on-chip PCR. The reaction parameters should be optimized and implemented in an assay detecting β-lactamase genes.

3.3.1 Spotting efficiency and thermal stability

The spotting of primers is a critical step because the used binding chemistry influences the properties of the primers. A low thermal stability of the primer-surface binding can result in detachment from the surface. In this particular case, the on-chip primers with their nucleotide spacers can easily bind unspecifically to genomic DNA and outcompete the PCR on the surface. Thus, and due to quality control, every batch of on-chip PCR slides was tested with PolyA-probes before use to evaluate spotting efficiency. The used surface chemistry in combination with thiol-modified primers produced consistent spots. The exposure of on-chip PCR slides to boiling water did not have any significant impact on signal intensities if polyA probes were hybridized to the slides subsequently.

3.3.2 Reaction chamber

An own on-chip PCR chamber had to be developed, as no commercial one is available. During on-chip PCR, reaction mixtures were heated up to 95°C. Evaporation of the reaction mixtures had to be prevented in order to maintain the correct concentrations of the reagents. In this work, various reaction chambers and their sealing were tested. The on-chip PCR had to be performed on a glass slide that can be analysed in a microarray scanner. A further requirement was thermostability of the reaction chamber. Sealing reagents were not allowed to inhibit the enzymatic reactions. Due to subsequent reactions after on-chip PCR, the exchange of enzymatic mixtures had also to be possible.

Measuring of temperatures on the slide surface revealed that the glass slides reacted in an acceptable manner to thermal cycling. The surface temperature on the slides was 95°C for 32 seconds, 58°C for 22 seconds and 72°C for 34 seconds. If temperature was increased, the step with the higher temperature was for 2-4 seconds longer than programmed. The annealing step following temperature decrease was eight seconds shorter than programmed, which is approximately one third of the programmed time.

Thermostable sealing of reaction chambers with nail polish, paraffin, self-sealing solution and mineral oil could not be achieved. Reaction mixtures in Hybriwells also evaporated. On-chip PCR slides with lifter slips on top of them could not be sealed with aluminium PCR foils. A positive result was obtained with lifter slips and polyolefin PCR foils wrapped around on-chip PCR slides.

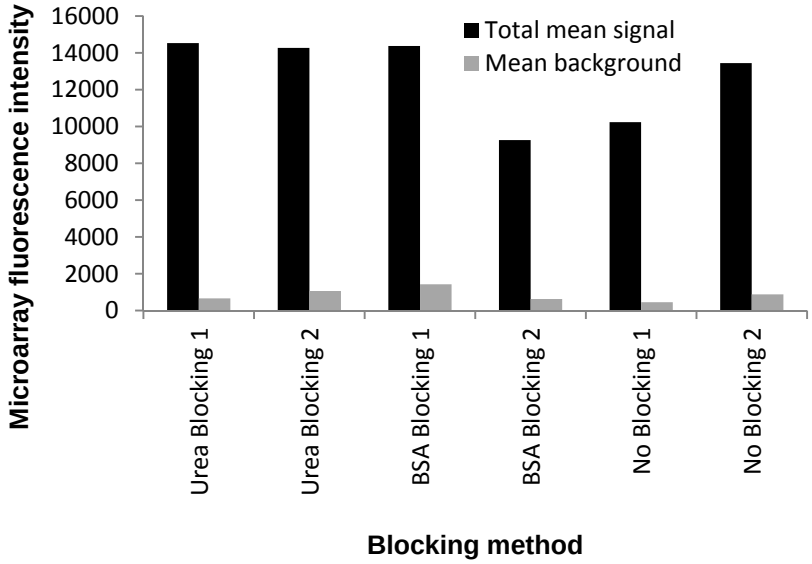
Tests with the PCR-kits Phusion polymerase, Phusion Hotstart polymerase, 16S Basic PCR kit, TypeIt PCR kit and Multiplex PCR kit were performed to investigate their properties regarding evaporation. Only the TypeIt PCR kit and the Multiplex PCR kit showed little volume change after thermal cycling. Solution Q, which is part of the TypeIt and the Multiplex PCR kit and not described in detail by the manufacturer, was present in those reaction mixtures and responsible for the low evaporation. If Solution Q was not used in reaction mixtures, evaporation was higher.

3.3.3 Blocking of slides

The tested blocking procedures with BSA and urea had no significant impact on fluorescence signals (Figure 25). Background fluorescence was also unaffected by

blocking. Tests with no blocking of slides produced similar signal intensities and background fluorescence values when compared to BSA blocked slides. Thus, slides were only washed twice with ddH_2O for two minutes before use in on-chip PCR.

Figure 25. On-chip PCR results showing the mean signal intensity of all primers on the chip and their mean background fluorescence using different blocking procedures.

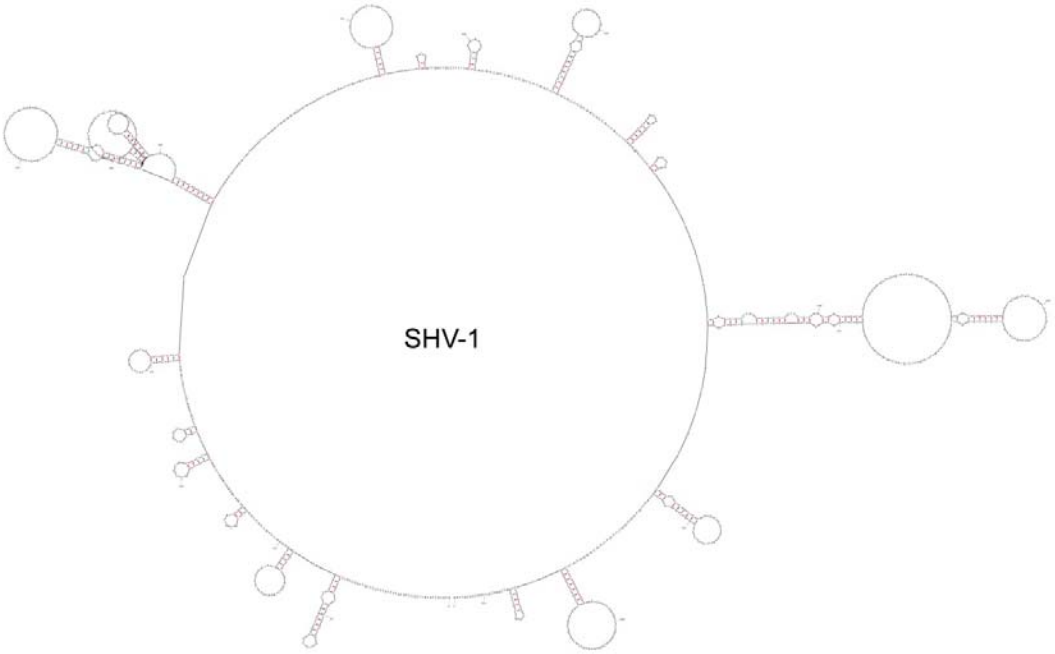


3.3.4 Properties of gene targets

The β-lactamase genes that were used as model for the development of the on-chip PCR were analysed regarding possible secondary structures and the varying GC content of their nucleotide sequence. The software tools Mfold and Freak were used for these purposes. PCR conditions occurring during the annealing step were simulated. The GC content varied between the investigated genes significantly. In some regions of the *bla*$_{OXA-1}$ gene, the GC content was close to 10%, whereas in the *bla*$_{SHV-1}$ gene a GC-content of almost 90% was observed.

Probable DNA secondary structures were found on all investigated genes. The higher the GC content of the investigated genes, the higher was the number of possible secondary structures (Figure 26 and Figure 27). For the β-lactamase gene *bla*$_{SHV-1}$, more than one structure was calculated. Two of the three possibilities are shown in Figure 26.

Figure 26. Schematic overview of the possible DNA secondary structures of *bla*$_{SHV-1}$ calculated with Mfold.



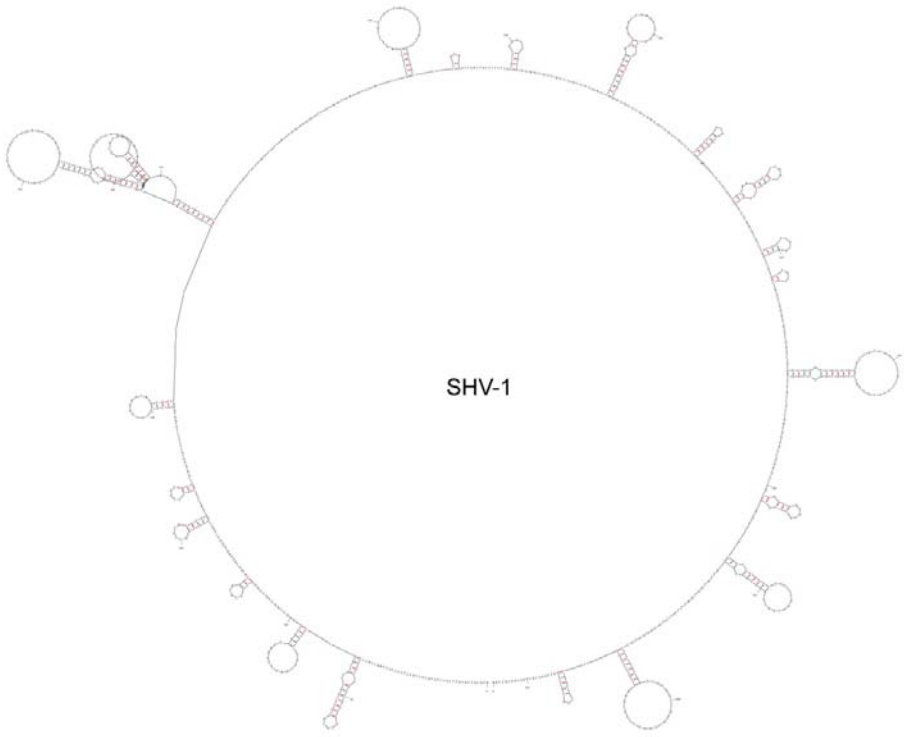
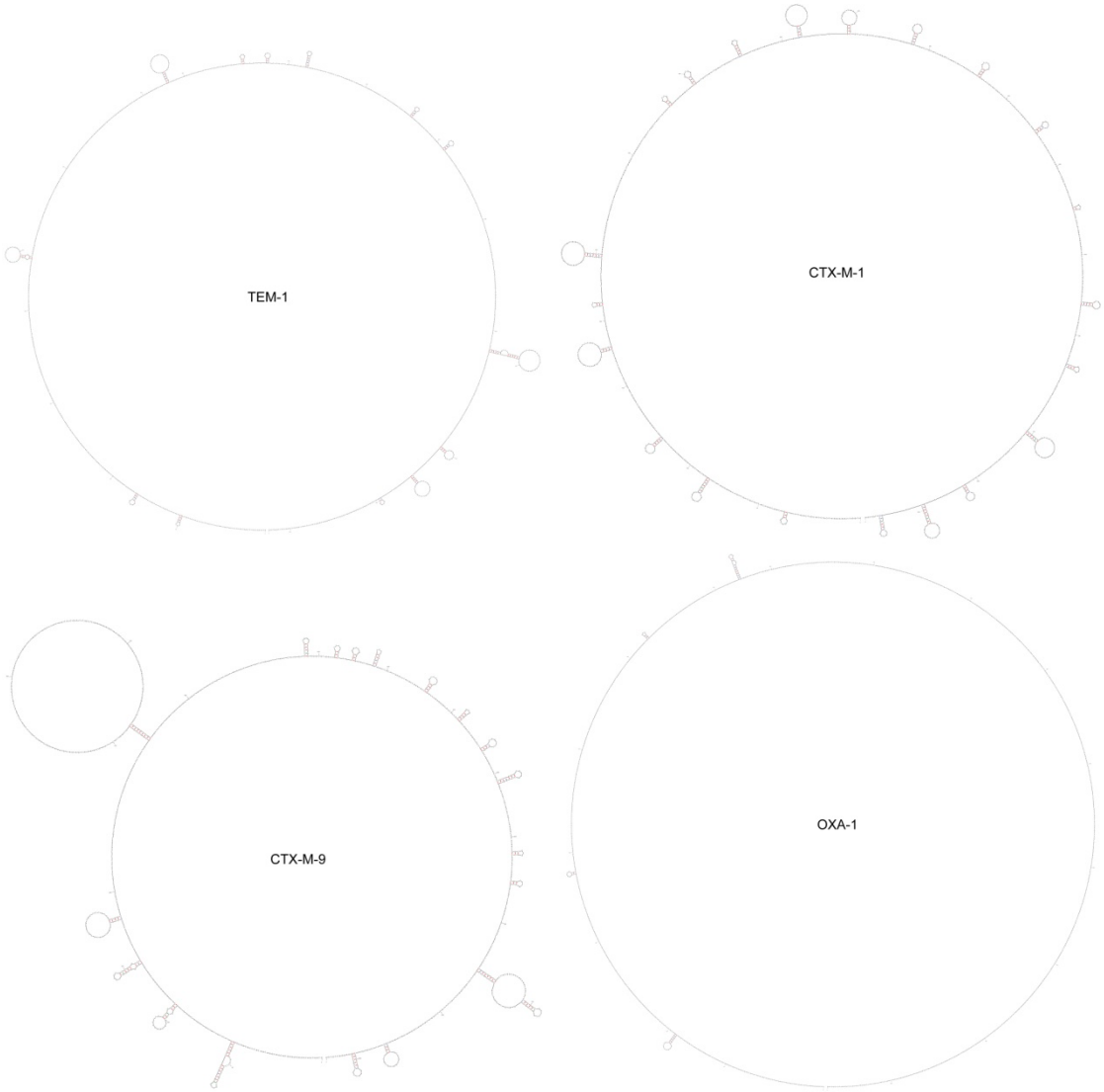


Figure 27. Schematic overview of the possible DNA secondary structures of the β-lactamase genes *bla*$_{TEM-1}$, *bla*$_{CTX-M-1}$, *bla*$_{CTX-M-9}$ and *bla*$_{OXA-1}$ calculated with Mfold.



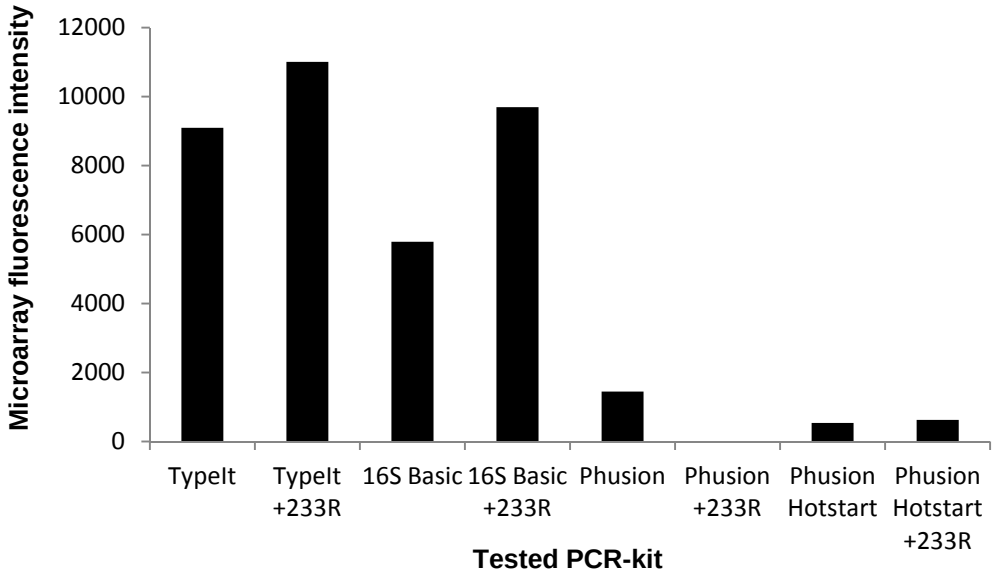
Interestingly, the first batch of primers, which was designed before the secondary structure analyses were calculated, anneals largely to the regions on the genes where secondary structure are formed. The focus on the design of this first batch of primers was on conserved regions because they should amplify as many as possible variants of their corresponding gene.

3.3.5 Testing of PCR-kits

Six different PCR-kits were tested for their use in on-chip PCR. In a parallel experimental setup, the liquid phase primer TEM 233R was added to the on-chip PCR

mixture. The goal was to observe the impact of additional liquid primers in the reaction mixture. Highest signal intensities were obtained with the TypeIt PCR-kit (Figure 28). No differences could be observed between the Multiplex PCR and the TypeIt PCR kit (data not shown). With the ImmoMix PCR kit, no fluorescence signal could be seen. The used liquid phase primer employed increased signal intensities of on-chip PCR. In subsequent experiments, the TypeIt PCR-kit was used.

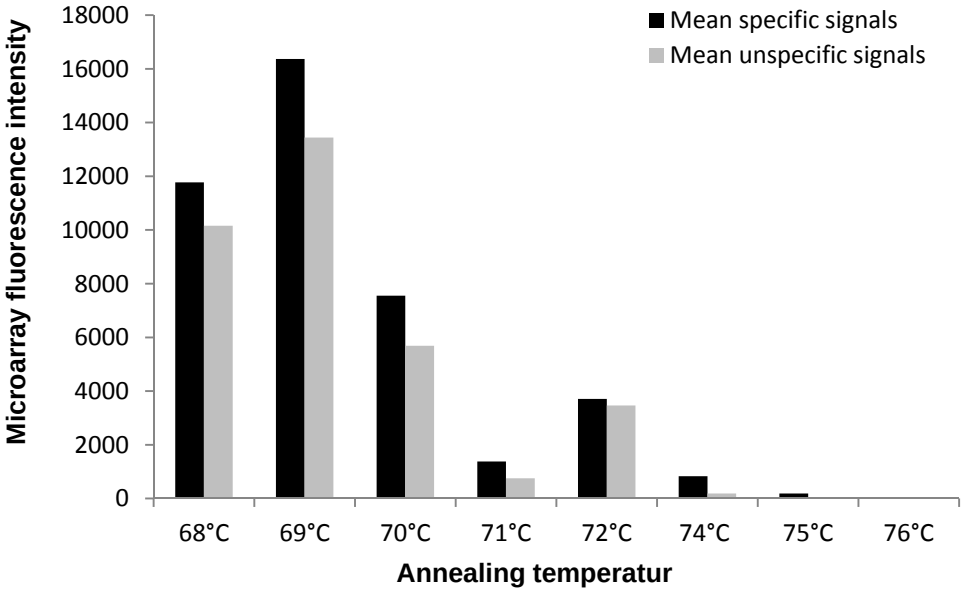
Figure 28. On-chip PCR results showing the mean signal intensities of all microarray spots with immobilized primers targeting the β-lactamase genes of TEM-1 and the impact of different PCR-kits on signal intensity. PCR products of *bla*$_{TEM-1}$ were used as template DNA in the on-chip reaction. The four best performing tested kits are illustrated. Columns labelled with +233R indicate experiments were the liquid phase primer TEM 233R was additionally added to the on-chip PCR reaction mixture.



3.3.6 Annealing temperature

The impact of different annealing temperatures during on-chip PCR was investigated in twelve separate experiments. In every experiment, two on-chip PCR slides per annealing temperature were tested. The number of different annealing temperatures per experiment varied between two and five. The mean signal intensities of all on-chip PCR experiments testing different annealing temperatures were used for the analysis (Figure 29). Highest signal intensities were observed at 69°C annealing temperature.

Figure 29. On-chip PCR results showing the impact of annealing temperature on signal intensity. The mean signal intensities were calculated from 20 different on-chip PCR slides using PCR products of the β-lactamase gene TEM-1 as template. The average fluorescence intensities of all microarray spots with intended on-chip PCR products were named as "mean specific signals". "Mean unspecific signals" comprised the mean fluorescence intensity of the microarray spots targeting the β-lactamase genes of SHV-1, OXA-1, and CTX-M-1, and the ribosomal 16S gene.



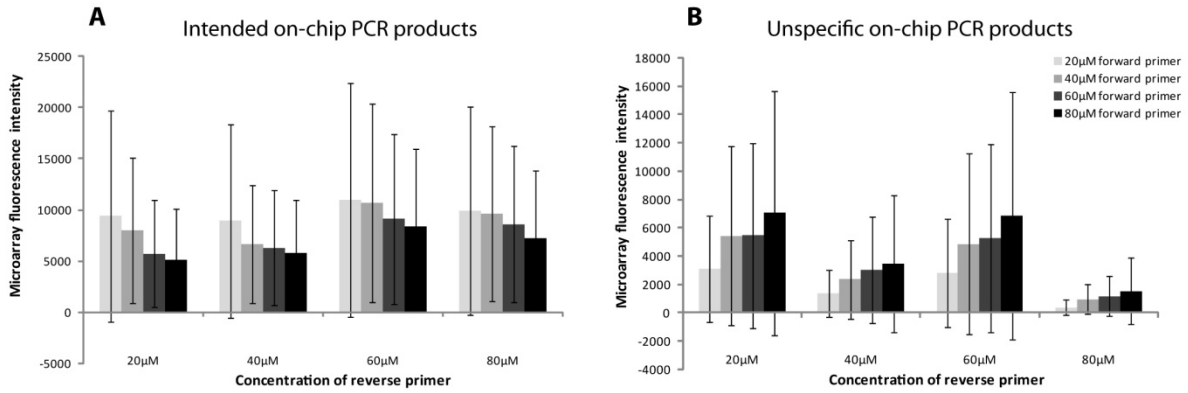
3.3.7 Immobilized primer concentrations

The amount of immobilized primers per surface area is an important parameter during on-chip PCR. It is influenced by the concentration of the primer solution before it is spotted. Too low primer concentrations will result in primers too far apart to anneal to immobilized on-chip PCR amplificates. Too high primer concentrations on the surface will provoke steric hindrance. Both above mentioned scenarios have an impact on the reaction mechanisms of the on-chip PCR and reduce its efficiency. Thus, experiments were designed to determine the optimal primer concentration that should be used for spotting. Concentrations started from 20 µM to 80 µM.

Eleven experiments were performed to determine the optimal primer concentration. The mean signal intensities of all spots with different primer concentrations that should give a specific signal were analysed and their standard deviation calculated (Figure 30A). The analysis showed no trend which favoured one of the tested concentrations. The high standard deviations illustrated as error bars show the high variance in signal intensity and the random impact of immobilized primer concentration. Primer sequence was a

more dominant factor as surprisingly some primers gave higher signals at lower spotting concentrations while others at higher concentrations. This phenomenon is also visible if the unspecific signals that were obtained during the on-chip PCR experiments were compared to the specific ones (Figure 30). The mean signal intensities of those primers showed different patterns using the same concentrations. Also the error bars of the mean unspecific signals in Figure 30B are higher than the signals themselves indicating the minor importance of immobilized primer concentration along the tested gradient.

Figure 30. On-chip PCR results illustrating the mean signal intensities of all on-chip PCR products collected from eleven experiments using the PCR products of *bla*$_{TEM-1}$ as DNA template. Signals were ordered according the concentration at which the primers were spotted to the slide. Error bars indicate the standard deviation. (A) Mean signal intensities of all intended on-chip PCR products. (B) Mean signal intensities of unspecific microarray spots targeting the β-lactamase genes of SHV-1, OXA-1, and CTX-M-1, and the ribosomal 16S gene.

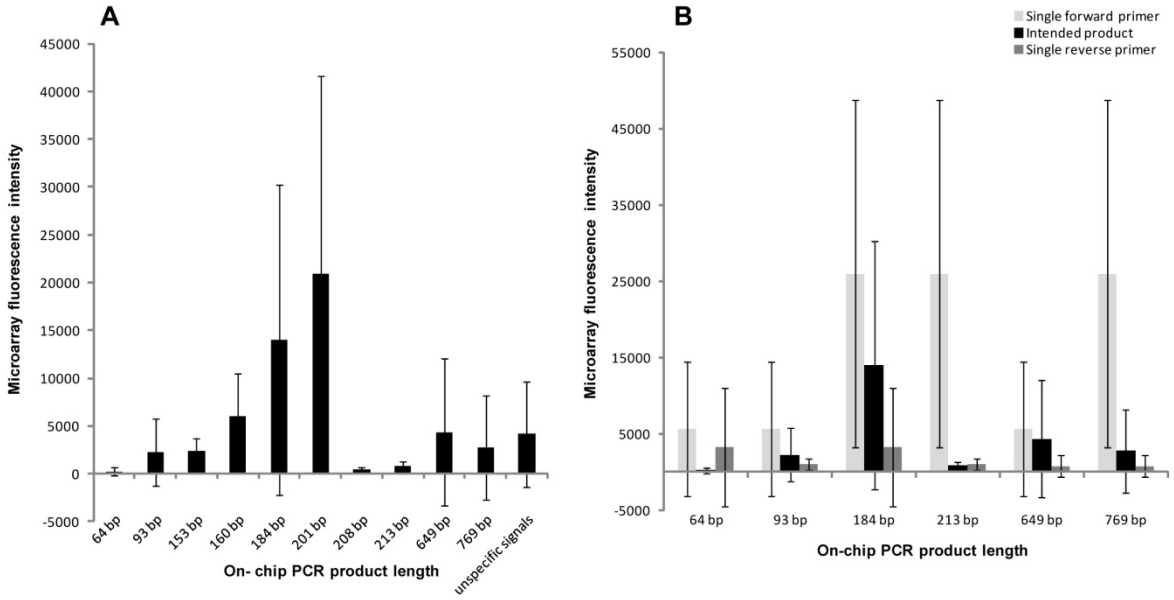


3.3.8 Impact of amplification product length

The nucleotide length of the immobilized PCR products influences the reaction mechanisms of an on-chip PCR strongly. Too short and too long on-chip PCR products cannot anneal to immobilized primers due to steric hindrance and DNA secondary structures. To determine the ideal amplification length, on-chip PCR primers were designed to amplify PCR products of different lengths. The mean signal intensities of 17 on-chip PCRs were calculated and compared (Figure 31A). The highest signal intensities were observed for PCR products with an intended length of 201 bp. The strong fluorescence signal decrease for slightly longer amplificates with 208 bp and 213 bp rose further questions about the significance of the data.

To evaluate the experiments investigating the ideal on-chip PCR product length, a forward or reverse primer was spotted on a single spot. Thus, on-chip PCR experiments included spots where only one primer, either forward or reverse, was spotted. The theoretical result would have been a very weak signal at these spots as the number of amplification products cannot grow at these sites exponentially but only linearly. Interestingly, these spots showed in the experiments the highest signal intensities (Figure 31B). These signals have to be interpreted as unintended PCR products.

Figure 31. On-chip PCR results showing the mean signal intensities of the on-chip PCR products with different nucleotide length obtained with the PCR products of bla_{TEM-1} as DNA template in the reaction. Error bars indicate the standard deviation. (A) "Unspecific signals" represents the mean fluorescence intensity of all microarray spots with immobilized primers targeting genes of SHV-1, OXA-1, CTX-M-1, or the ribosomal 16S gene. (B) On-chip PCR slides were also produced with spots containing only a single primer (forward and reverse respectively). The fluorescence signal intensities of these spots were compared to those with both primers immobilized.



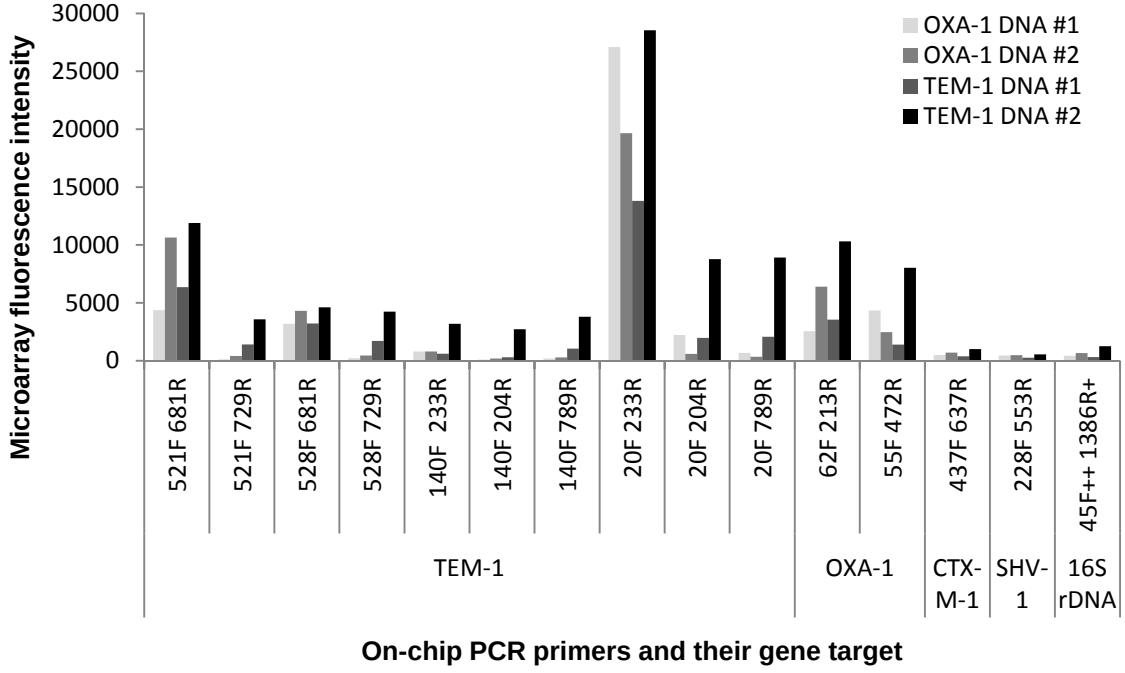
3.3.9 Specificity of on-chip PCR

The specificity of on-chip PCR was tested with sequenced PCR products and genomic DNA of *E. coli* and *K. pneumoniae*. The impact of the previously tested parameters on specificity was monitored during those experiments. Reagent composition of the PCR kits, annealing temperature, primer length, on-chip PCR product length and primer concentration could not be adjusted to amplify a specific on-chip PCR product. The

problem is illustrated in an experiment using PCR products of the β-lactamase genes of OXA-1 and TEM-1 as DNA templates (Figure 32). Regardless of the introduced DNA template, microarray signals showed a similar pattern. These findings were also observed using genomic DNA. The concentration of template DNA had no significant effect on specificity.

To determine which on-chip PCR products were amplified on the chip, hybridization probes were used. Labelling of the on-chip PCR products was performed with dCTPs fluorescent at a wave length of 532nm. Hybridization probes were designed to bind to the on-chip amplification products and show fluorescence at 635nm. After on-chip PCR, they were hybridized to the slide surface, and subsequently slides were stringently washed. In case of specific amplification during on-chip PCR, fluorescence signals should have been obtained at both wave lengths; at 532nm because of amplified on-chip PCR products and at 635nm because of hybridization probes annealing to those. Although in the experiments, fluorescence at 532nm was detected, there was never fluorescence detectable at 635nm. Thus, I conclude that the amplified DNA on the on-chip PCR slides was not the target DNA.

Figure 32. On-chip PCR results using two different PCR products as DNA templates. The horizontal axis shows the fluorescence intensity with the used forward and reverse primer combination and their gene affiliation.



3.4 Ligation based solid phase detection

Knowledge in modern medicine increases rapidly since new molecular mechanisms responsible for various diseases are revealed weekly. Much of this data is genetic information. It is important for clinicians to retrieve this disease-related information fast, reliable and in a cost-effective way. Some molecular methods such as PCR, Maldi-TOF, etc. are already used in routine diagnostics. The main limitation of many of these detection methods is their poor multiplex ability. Alternative approaches that overcome this limitation such as next generation sequencing are still too expensive for routine diagnostics. Thus, the development of fast and economic multiplex detection methods is still an important issue.

The introduction of microarrays was a promising step in reaching this goal. Several microarrays and similar hybridization based methods were developed for diagnostic purposes, although problems regarding unspecific hybridization remained. The need for pre-amplification of DNA with PCR posed a problem for the microarray technology, because the bottleneck concerning multiplex ability was PCR, allowing maximal parallel amplification of approximately twelve genes. An advance in multiplex detection was made when ligation dependent methods were presented. Assays based on padlock probes were developed enabling the amplification of thousands of genes in parallel. However, these methods were dependent on solid support based detection such as microarrays, bead arrays or sequencing, which extended the detection time of these methods dramatically.

Consequently, methods were developed that combined the very specific ligation reaction with oligonucleotides immobilized on a solid support. Unfortunately, these methods had also problems regarding unspecific reactions. In this work, I present new ligation dependent detection methods that are based on probes with double-stranded DNA spacers and use RCA for DNA amplification. For proof of concept, a group of clinically important antibiotic resistance genes were used. The resistance genes belong to the genetically very diverse group of β-lactamases.

3.4.1 Proof of principle

The multiplex ligation assays with immobilized probes on a glass slide were tested with bacterial genomic DNA and PCR products of the β-lactamase genes of CTX-M-1, CTX-M-9, OXA-1, TEM, and SHV. The slides were incubated with a ligation mixture containing the respective probes and processed according the particular protocols (Figure 12 to Figure 15). In all methods, detection oligonucleotides were used for ligation product detection and analysis was performed with a microarray scanner.

Microarray fluorescence signals were successfully obtained with all tested methods except the one presented in Figure 13. In this method, the immobilized linear probe had first to be elongated by a polymerase until the polymerase reached the specificity probe, which subsequently had to be ligated to the immobilized amplification product. Modification of annealing temperature and varying nucleotide distances between specificity probe and the immobilized probe did not result in a positive fluorescence signal.

3.4.2 Specificity using immobilized probes and specificity probes

The ligation method shown in Figure 12 is based on an immobilized probe and a specificity probe. During the reaction, a DNA target molecule anneals to the immobilized probe and serves as template strand for the specificity probe, which binds next to the immobilized probe. A ligase connects the two probes. Annealing temperatures ranging from 55°C to 65°C were tested for this method but a specific signal could not be obtained. Also different ligation reaction concentrations of the specificity probes ranging from 1 pM to 10 µM were tested. The combination of immobilized probe TEM 140F and the specificity probe TEM 204R was the only one that produced a ligation product which was detectable by the detection oligonucleotide. Unfortunately, the specificity probe TEM 204R was designed to target the complementary strand of the one to which immobilized probe TEM 140F binds. These two probes can anneal to each other and have an overlap of 14 bp. An experimental setup in which other specificity probes than TEM 204R were ligated to a solid support probe could not be established.

3.4.3 Capture probes concentration

Capture probes are oligonucleotides hybridizing to a DNA target and an immobilized nucleotide probe at the same time. They are used in the ligation reactions described in Figure 33 and Figure 34. The concentration of capture probes used during the ligation reaction is an important parameter influencing specificity and sensitivity. Too high concentrations of capture probes facilitate unspecific hybridization to DNA targets and immobilized probes. Lower concentrations improve specificity but reduce the sensitivity of the assay. To determine the ideal concentration of capture probes, experiments were performed with capture probe concentrations ranging from 1 pM to 1 µM per reaction. PCR products were used as gene targets in those experiments.

Figure 33. Schematic overview of the ligation process using looplock probes. Yellow sequences indicate regions responsible for looplock and capture probe recognition, blue indicates the region on the capture probe detecting a specific DNA target, and red where detection oligonucleotides bind. After annealing of capture probes, the looplock probe and the DNA target to each other, a ligase concatenates the ligated capture probes to the looplock probe. During a stringent washing step using boiling water, all unligated capture probes are removed. Detection oligonucleotides are used to validate if the ligation reactions were successful.

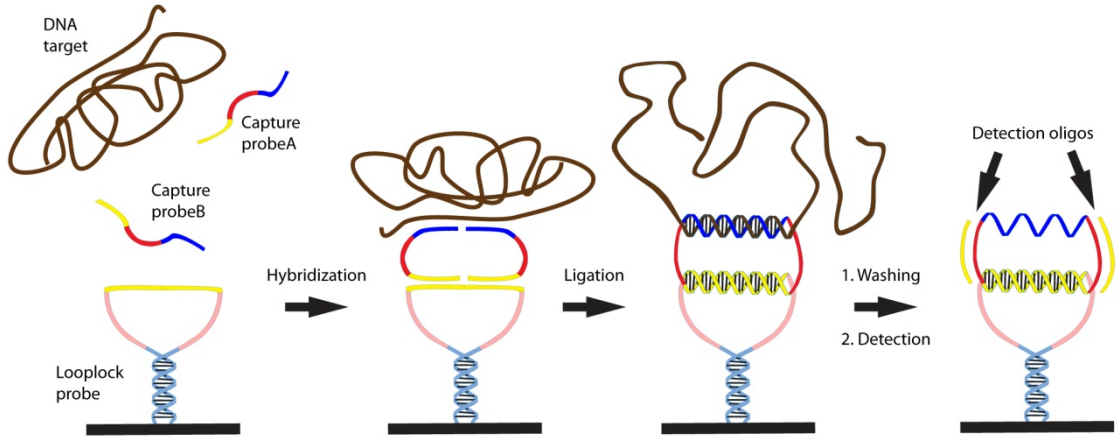
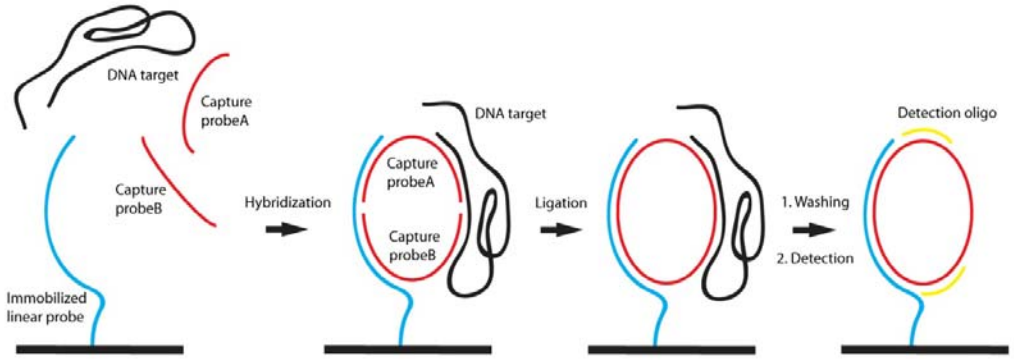
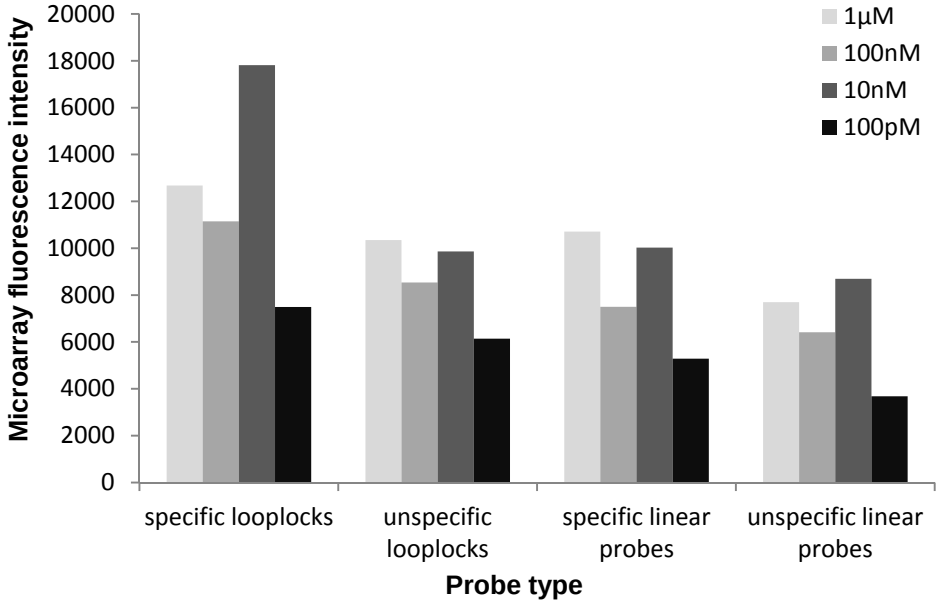


Figure 34. Schematic overview of the ligation process using an immobilized linear probe to bind capture probes. The process is analogous to the one presented in Figure 33 except that no boiling water is used for washing but a less stringent washing buffer.



Fluorescence signals in experiments with 10 pM capture probes or less were not detectable. Capture probe concentrations starting from 100 pM to 1 µM were used in subsequent experiments (Figure 35). An increase of signal intensity with increasing concentrations of capture probes was detected.

Figure 35. Mean fluorescence intensities of the on-chip ligation reactions described in Figure 33 and Figure 34. Both immobilized probe types described in the two figures were spotted on every slide. Different concentrations of the capture probes were used in these ligation reactions. The data was separated according the type of immobilized probe (immobilized linear probes or looplock probes) and specificity of the signals. PCR products of the TEM-1 gene were used as DNA templates in the ligation reactions.

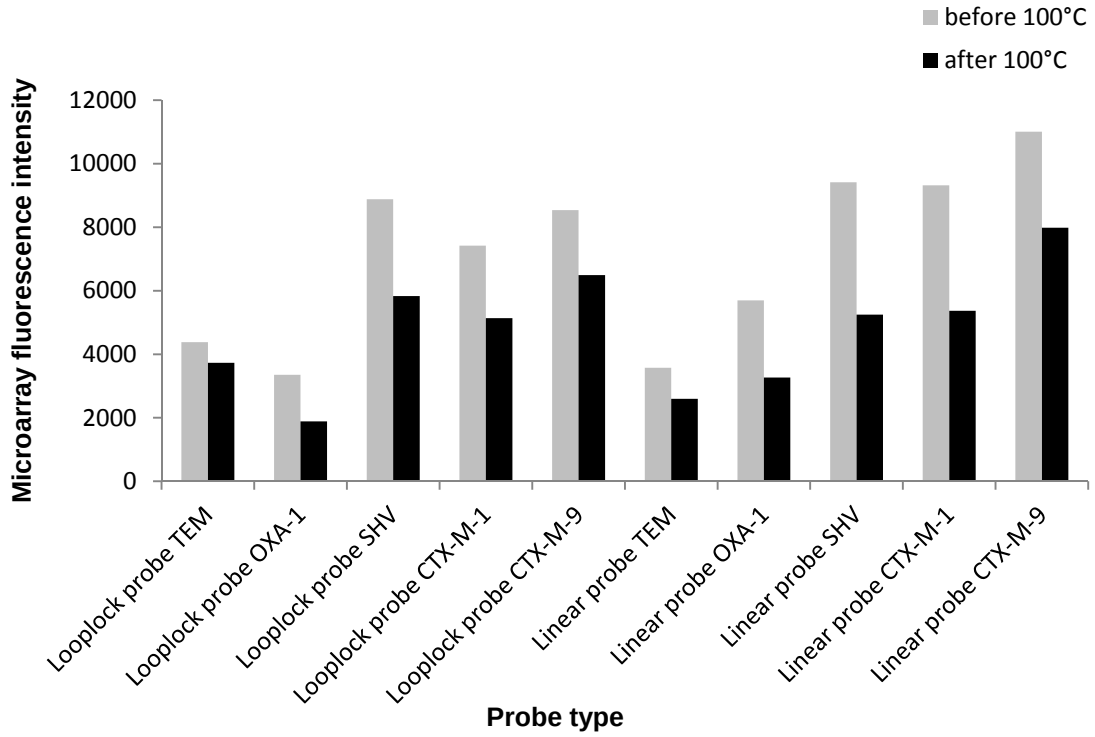


3.4.4 Looplock vs. linear probes

Most methods in which DNA was hybridized to single stranded immobilized oligonucleotides face the problem of unspecific binding. The goal in this study was to design oligonucleotides that are less prone to this phenomenon. Looplock probes were covalently attached with their 3' end and their 5' end to a solid support. A feature of the looplock probes was the double stranded DNA spacer increasing the distance of the recognition sequence from the surface. Single stranded DNA probes can form secondary structures which results in a decreased distance of the binding sequence from the surface, and thus make them less accessible to target DNA. The main idea behind looplock probes was the elimination of unspecific hybridization. The used enzyme ampligase can recognize perfectly matching DNA more specifically than DNA polymerases. Additionally, the integration of a total denaturation and a washing step after the ligation reaction into the protocol should result in the improvement of current methods. Capture probes were designed to concatenate with their corresponding looplock probe after ligation, and thus, could not be washed away. In contrast, the analogous immobilized linear probes helped to ligate capture probes in the same way, but due to their free 3' end, under very stringent washing conditions ligated capture probes could be washed away.

Results of an experiment comparing the two immobilized types of probes under stringent washing conditions are illustrated in Figure 36. Both types of probes were spotted on the same slide. The ligation reaction was performed as described in Figure 14 and Figure 15. After the ligation and first incubation with detection oligos, spots with looplock probes and immobilized linear probes showed, as expected, fluorescence signals. Washing in boiling ddH_2O and subsequent scanning confirmed that all detection oligos and non-covalently bound DNA were washed away (data not shown). The last incubation with detection oligos had a surprising result since not only spots with looplock probes but also the ones with immobilized linear probes showed fluorescence signals. The most probable explanation of this phenomenon was that capture probes were covalently connected to the free 3' ends of the immobilized linear probes. To test this hypothesis, immobilized linear probes with an analogous dideoxynucleotide (ddNTP) at their 3' ends were ordered. The ddNTP prevented the ligation of capture probes at the 3' end.

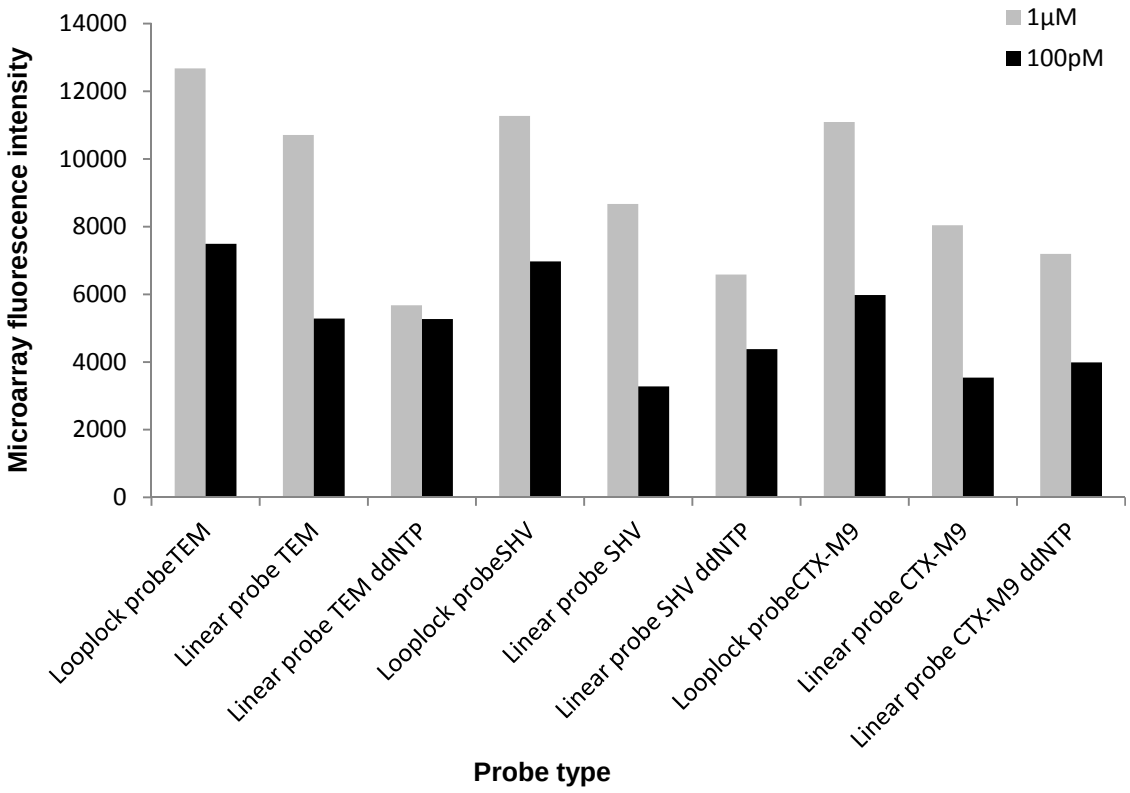
Figure 36. Mean fluorescence intensities of an on-chip ligation experiment containing both immobilized probe types described in Figure 33 and Figure 34. The slide was scanned before washing in boiling ddH_2O and afterwards. In both scenarios, the slide was incubated with a detection oligonucleotide and washed before scanning. PCR products of the TEM-1 gene were used as DNA templates in the ligation reactions.



3.4.5 Immobilized linear probes with dideoxynucleotide at the 3' end

Looplock probes, linear probes with and without a dideoxynucleotide at its 3' end were spotted onto a glass slide. Capture probes were added to the ligation reaction in the concentrations 1 µM, 100 nM, 10 nM and 100 pM per capture probe. After ligation, slides were washed in boiling ddH_2O and subsequently incubated with detection oligos. Then, slides were washed and analysed. At high capture probe concentrations, a significant decrease in signal fluorescence intensity of spots with linear probes with a ddNTP in comparison to normal immobilized linear probes could be observed (Figure 37). This data confirmed the hypothesis of unspecific binding of capture probes to free 3' ends. Interestingly, at the lowest tested capture probe concentration of 100 pM, spots with linear probes with a ddNTP showed equal or even slightly higher fluorescence signal intensities.

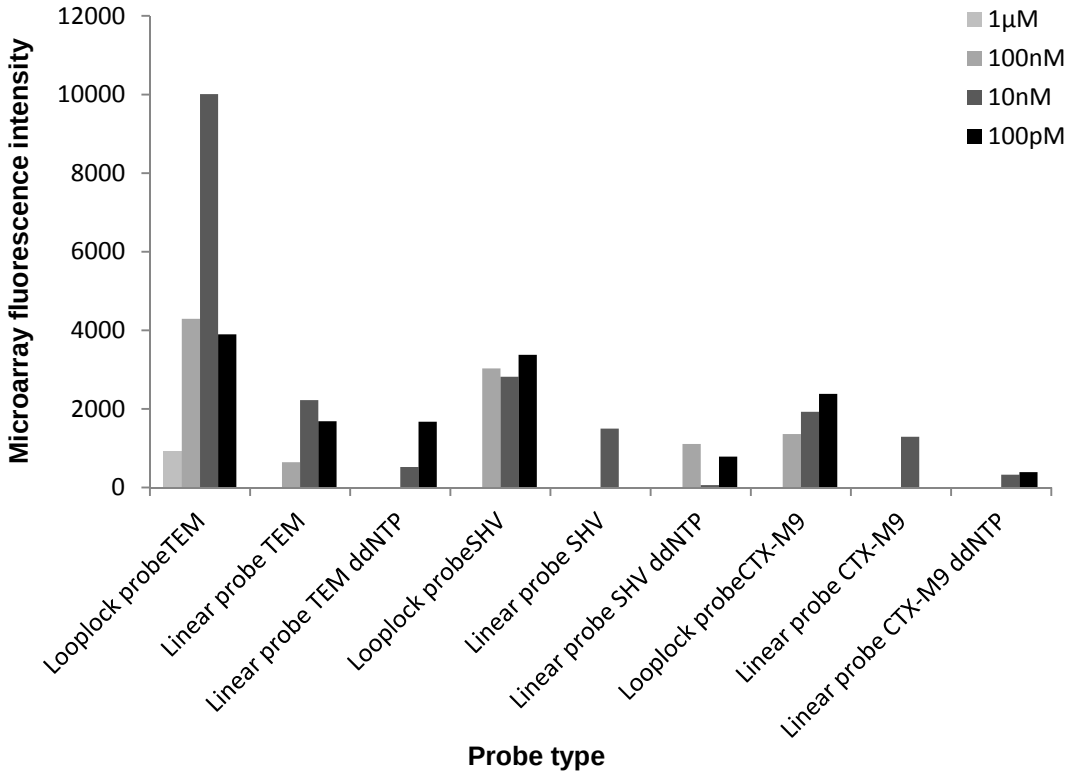
Figure 37. Mean fluorescence intensities of the on-chip ligation experiments containing three immobilized probe types on a single slide (Looplock probes, linear probes with and without a dideoxynucleotide at their 3' end). The reactions described in Figure 33 and Figure 34 were conducted on the slides. Two different concentrations of capture probes were used and are shown in grey and black columns. PCR products of the TEM-1 gene were used as DNA templates in the ligation reactions.



3.4.6 Specificity of on-chip ligation

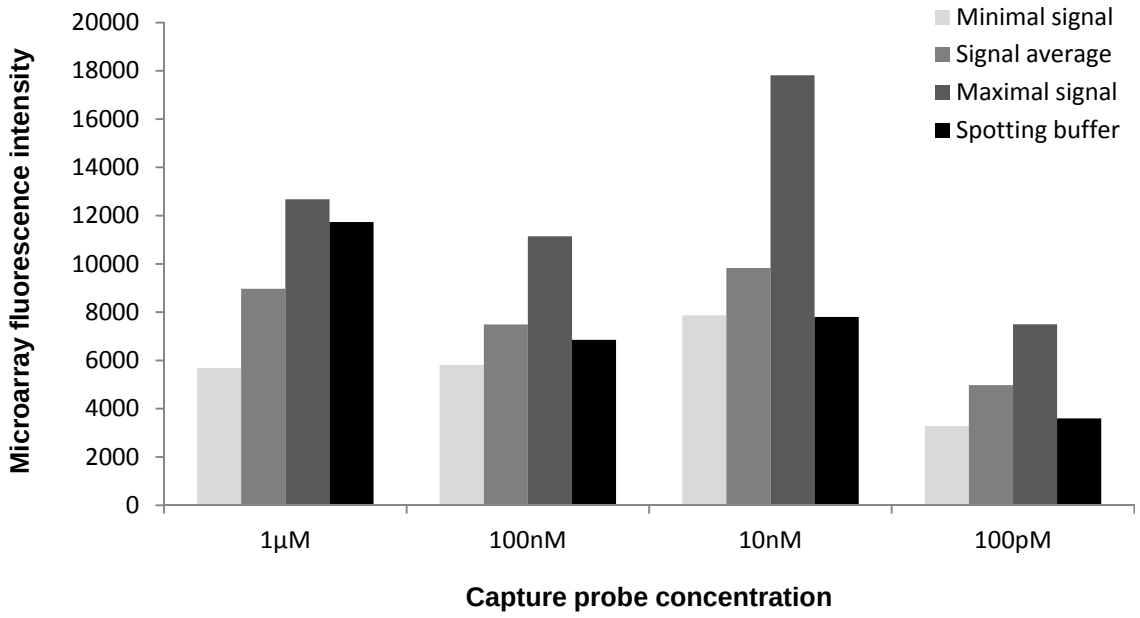
The specificity of the used ligation reactions was tested with PCR products and genomic bacterial DNA. The problem of unspecific ligation to the free 3' ends of the immobilized linear probes could not be totally resolved. Thus, the interpretation of unspecific signals remained difficult. Nevertheless, specific looplock probes produced slightly higher signal intensities than unspecific ones using PCR products as DNA templates (Figure 38). Ligation reactions with genomic bacterial DNA were unspecific.

Figure 38. Mean fluorescence intensities of four on-chip ligation experiments using the PCR product of the β-lactamase gene of TEM-1 as template. The reactions described in Figure 33 and Figure 34 were conducted on the slides. Three different immobilized probe types were spotted on a single slide (Looplock probes, linear probes with and without a dideoxynucleotide at their 3' end). Four different capture probe concentrations were used in the four ligation reactions to monitor the impact of the capture probe concentration on the specificity.



A possible source contributing to unspecific fluorescence signals was the spotting buffer containing betaine and SSC. It was spotted in all experiments as negative control additionally on the slides. However, in some experiments, fluorescence intensities obtained from spots made only with spotting buffer exceeded the fluorescence intensities from spots containing immobilized probes (Figure 39). This phenomenon occurred on several occasions, although new spotting buffer and spotting solutions were prepared after realization of the problem.

Figure 39. Mean fluorescence intensities of the ligation experiments with different concentrations of capture probes. Columns show spots with the weakest and strongest fluorescence intensity. Also the average signal intensity including all spots on the slide is illustrated. Black columns show the signal intensity from spots on which only the spotting buffer was spotted.



3.5 Multiplex detection of β-lactamases in liquid phase using padlock probes

In parallel to the development of the on-chip ligation based detection methods described in the previous section, a ligation based assay in liquid phase was also developed. The assay uses padlock probes that allow a high degree of multiplexing. These probes anneal to a DNA target, circularize, and a ligase connects their 5' and 3' ends. The most popular method to detect the subsequently amplified and labelled padlock probes are microarrays.

In this study, I further optimized multiplex genotyping using padlock probes and developed an assay with nearly 100 padlock probes. The used padlock probes contained a DNA target recognition site, a sequence region for C2CA, and a barcode sequence tag for microarray based detection (Figure 16) (80). Ligated padlock probes were amplified with C2CA. This amplification method consisted of two RCAs intercepted by monomerization, circularization and ligation of the products from the first RCA. These circularized products were amplified in the second RCA and subsequently fluorescently labelled with a linear PCR (Figure 19). The assay was designed to identify 33 different β-lactamase genes. Genomic DNA from clinical samples, PCR products and synthetic DNA templates were used to evaluate specificity and sensitivity of the method.

3.5.1 Evaluation of padlock probes using synthetic templates

I first tested the functionality and specificity of the padlock probes with short synthetic DNA templates: synthetic templates corresponding to the same gene were pooled and used in a single ligation reaction. Six of the 96 padlock probes (MIR-1 P147, OXA-1 P540, OXA-48 P448, OXA-51 P439, OXA-51 P712 and SHV-1 P734) produced no microarray signal (Figure 40). In particular, the detection of bla_{MIR-1}, bla_{OXA-1}, bla_{OXA-48} and bla_{SHV-1} might be problematic when using the padlock probes described herein, and a single functional probe was not sufficient for bla_{OXA-51} confirmation.

Because a barcode sequence microarray containing 299 probes was used to identify the 96 padlock probes, two-thirds of the microarray probes had no corresponding padlock probe to bind.

Nonetheless, we observed nonspecific hybridization of the labelled DNA to these microarray probes in all the experiments (Figure 41). Moreover, three microarray

probes of the 96 that targeted the padlock probe amplification products had fluorescence signals in more than 71% of the experiments. We confirmed that these false-positive signals were the result of the nonspecific hybridisation of labelled DNA rather than the nonspecific ligation and amplification of padlock probes, as illustrated in Figure 41. Four microarray probes lacking any corresponding padlock probe were also positive in the majority of our experiments due to the nonspecific binding of the labelled DNA. To discriminate false positives from true positives, each gene had to be targeted at least by three padlock probes to be scored as positive.

Figure 40. Heatmap of the microarray signals from 33 experiments using the synthetic DNA as template in the ligation reaction. Two experiments with negative controls are included. On the vertical axes microarray probes are named according their corresponding padlock probe. The number after the letter P in the probe name indicates the nucleotide position on the gene. The fluorescence intensities were normalized and transformed to log(2) values.

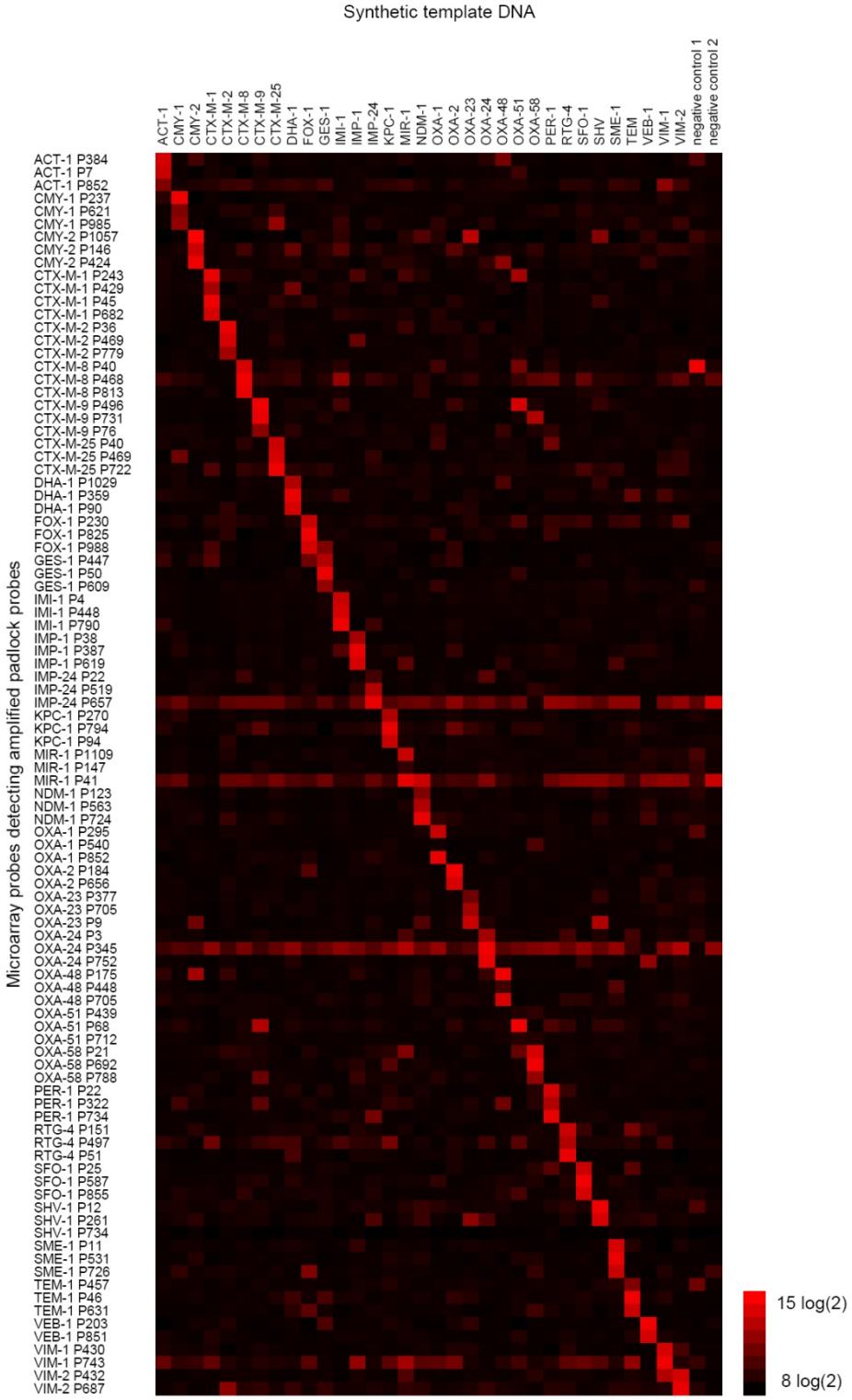
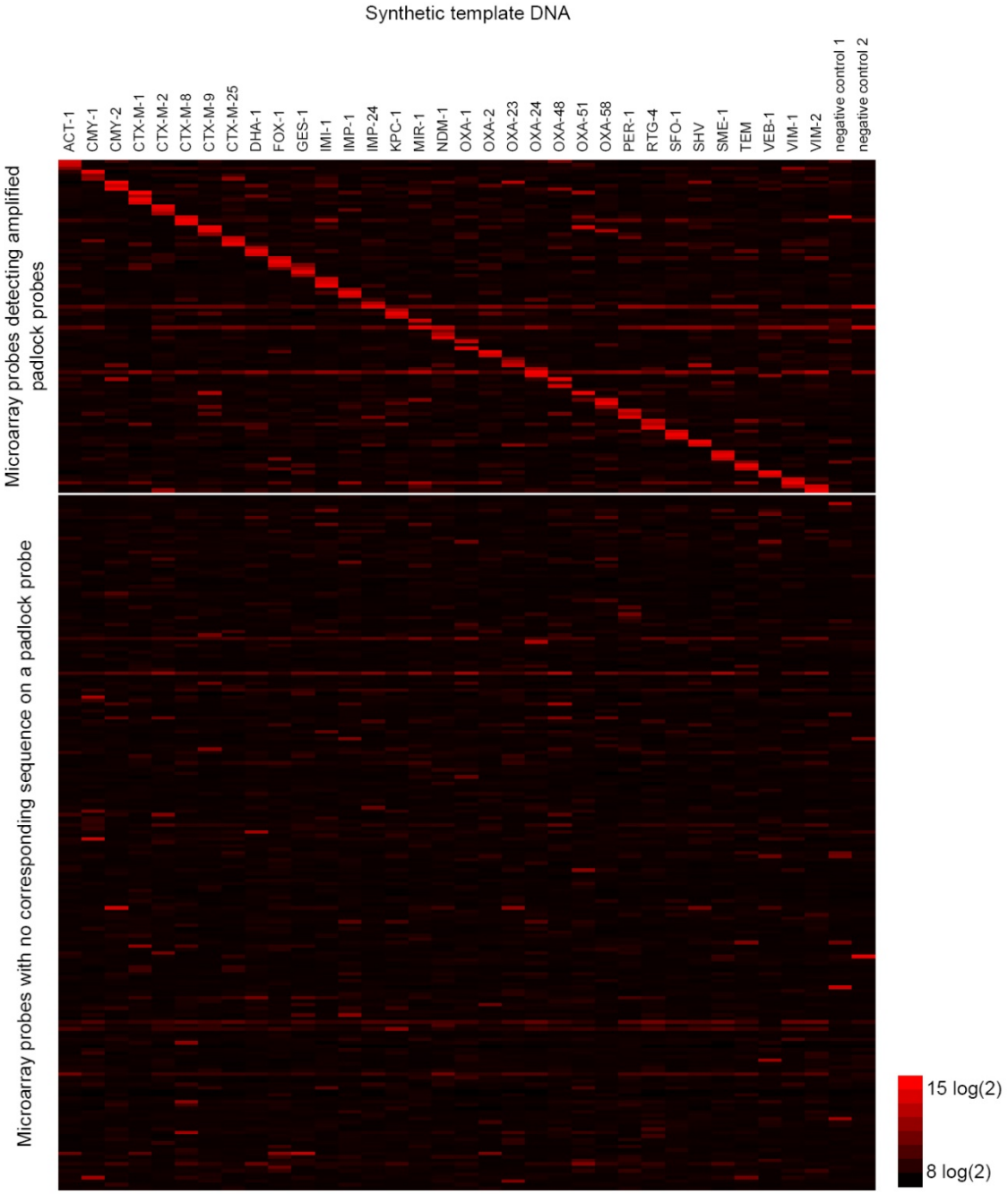


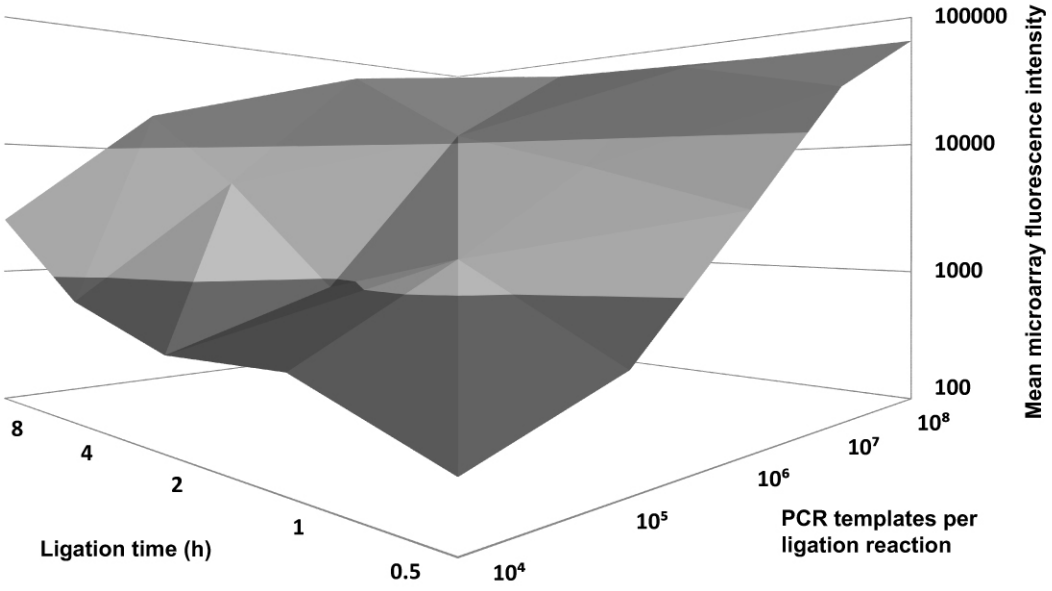
Figure 41. Heatmap of the microarray signals from 33 experiments using the synthetic DNA as template in the ligation reaction. Two experiments with negative controls are included. The upper part of the figure shows microarray probes which have a complementary sequence to bind the padlock probe amplificates. The part below shows the fluorescence intensity of the microarray probes with no corresponding padlock probe. The fluorescence intensities were normalized and transformed to log(2) values.



3.5.2 Test of the assay using PCR products

To determine the specificity and sensitivity of our approach, five sequenced PCR products for the clinically most prevalent β-lactamases were used as templates. The β-lactamase assay successfully detected the PCR products of $bla_{CTX\text{-}M\text{-}1}$, $bla_{CTX\text{-}M\text{-}9}$, $bla_{OXA\text{-}1}$, $bla_{SHV\text{-}1}$ and $bla_{TEM\text{-}1}$. The evaluation of the sensitivity of the assay was performed with 1:10 dilution series of these PCR products from 10^0 to 10^9, and the impact of the ampligase ligation time on the sensitivity was also examined. The detection limit of the method for the PCR products was found to be 10^4 DNA molecules per ligation reaction. The 3D-diagram in Figure 42 shows an increase in sensitivity with longer padlock probe ligation times. The microarray signal intensity increased with increasing PCR product concentration in the padlock probe ligation reaction, and longer ligation times during this reaction step had a similar effect on the microarray signals. Interestingly, the increase in the microarray signal intensities to saturation was approximately linear with respect to the increases in the template concentration and ligation time. These results suggest that the assay is at least semi-quantitative.

Figure 42. Microarray results from multiplex detection experiments using different template concentrations of PCR products of β-lactamase gene of CTX-M-9. The impact of ligation time on sensitivity during the annealing of the padlock probes is also illustrated.



3.5.3 Specificity of genotyping

To investigate the specificity of the assay, genomic DNA samples from 70 characterized clinical strains were used as templates, with 141 β-lactamase gene sequences being present (Figure 43). These sequences encode 8 different β-lactamases, comprising 25 different subtypes. Because the assay was not conceptually designed to detect SNPs, it was not possible to identify all 25 subtypes but the assay could specifically detect their associated genes. The microarray results for a representative clinical isolate showing the mean gene values is presented in Figure 44. All β-lactamase genes present were correctly detected in 97% of the investigated bacterial isolates. In the two isolates with a false negative result, one gene (bla_{OXA-1} and bla_{SHV-11}, respectively) was not detected as a direct result of dysfunctional padlock probes. The mean gene values remained below the cut-off in these two cases. In total, 98.6% of all the β-lactamase genes present in the tested clinical isolates were correctly detected with our assay.

The correlation of the β-lactam resistance phenotypes of the clinical isolates and their β-lactamase genotypes deduced from the results of the detection assay was also evaluated. The β-lactamase phenotype could be precisely confirmed with the developed assay for 62 (88.6%) of the 70 tested clinical isolates (Figure 43). In four of the remaining eight clinical strains, potential ESBL (extended spectrum β-lactamase) genes were identified, providing a possible mechanism for the observed resistance phenotype; however, due to the lack of SNP subtyping, the narrow-spectrum β-lactamases (bla_{TEM-1}, bla_{SHV-1}) could not be distinguished from ESBLs (eg. bla_{TEM-7}, bla_{SHV-2}, bla_{SHV-12}). Therefore, the precise confirmation of the observed phenotype was not possible in these cases. In four of the eight isolates, the molecular mechanisms responsible for the resistance phenotype were previously not known and could not be determined with our method.

Figure 43. Results of the evaluation of the multiplex detection method using genomic DNA from 70 clinical isolates. Grey squares indicate the correct identification of a gene. Black squares are false negatives. In the isolates illustrated in bold letters, the genetic mechanism responsible for the resistance phenotype was not known. In the italicized ones, confirmation of the resistance phenotype was not possible with our method due to missing SNP genotyping.

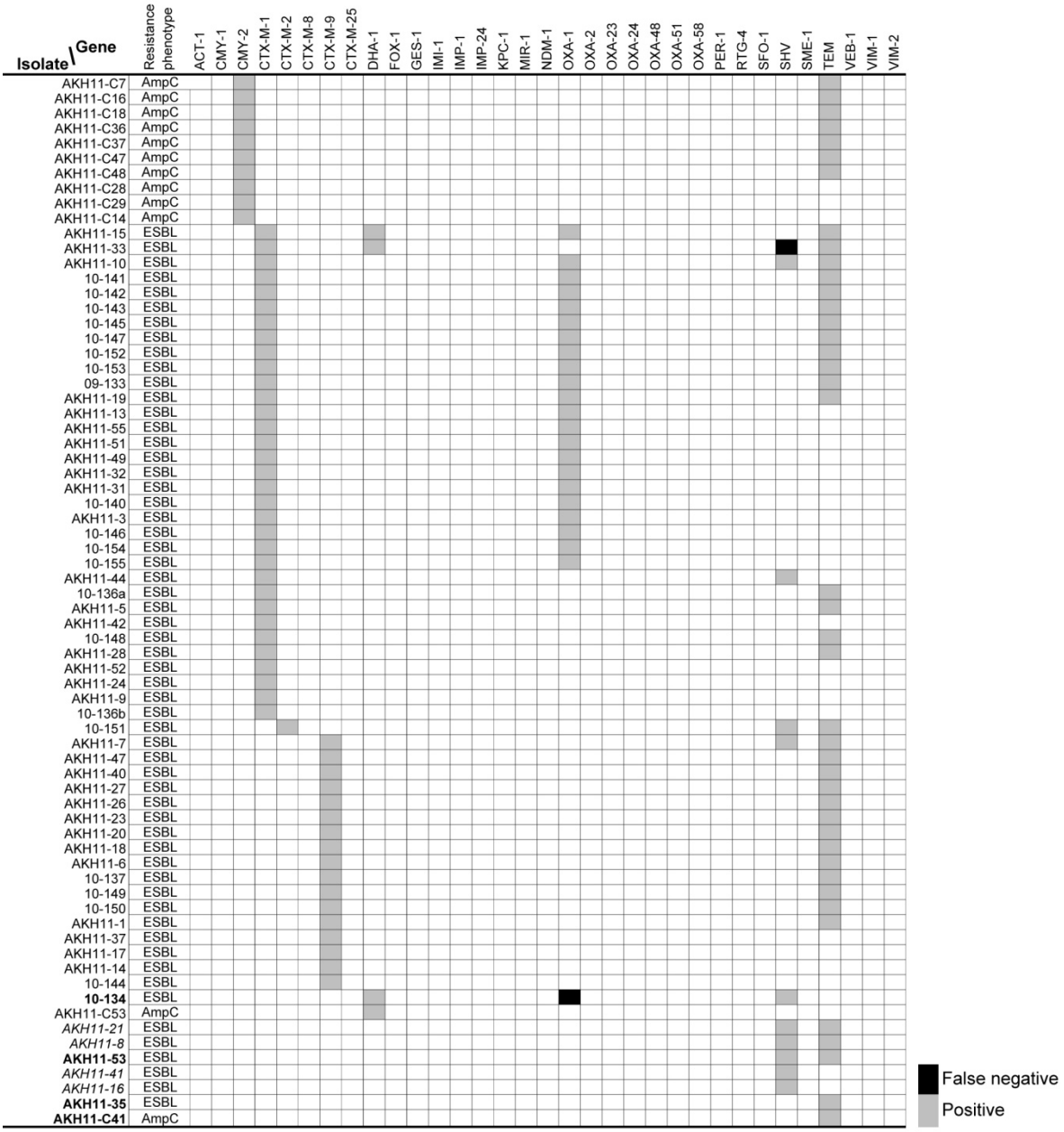
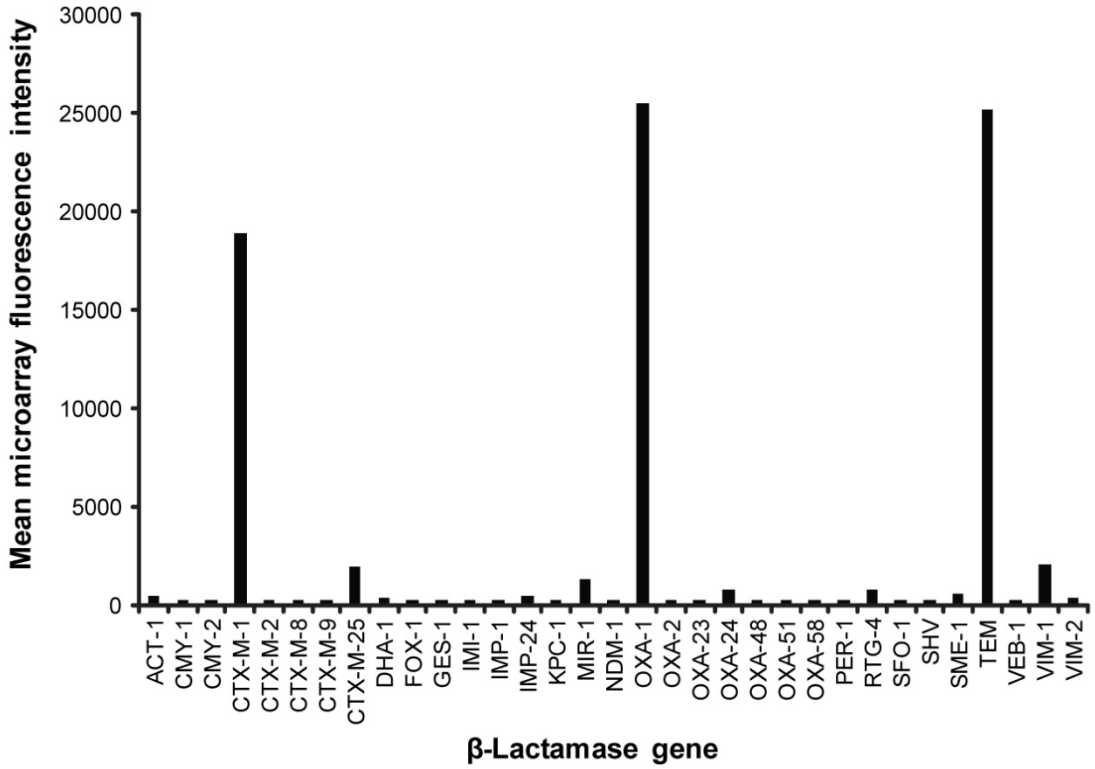


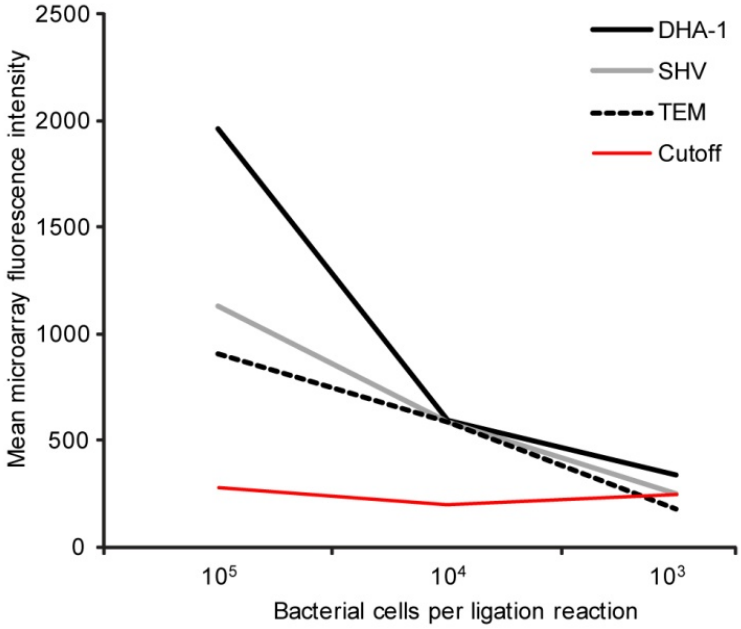
Figure 44. Microarray result of the multiplex detection experiment using genomic bacterial DNA from an overnight culture. Genes of CTX-M-1, OXA-1 and TEM-1 were present in the isolate and successfully detected with the β-lactamase assay.



3.5.4 Sensitivity of β-lactamase detection assay

Clinical samples were the source of the genomic DNA used to determine whether the detection limits were clinically relevant. To this end, a 1:10 dilution series of bacterial cells ranging from 10^0 to 10^9 cells/ml was prepared, and the genomic DNA was extracted. We selected a ligation time of at least 12 hours for β-lactamase detection based on a dilution of 10^9 cells/ml; the cell density was critical for the results because it was not possible to detect any genes using the 10^7 cells/ml dilution, even after a prolonged ligation time of 24 hours. However, the initial 25-multiplex PCR pre-amplification of the genomic DNA significantly improved the detection limit, and positive microarray signals were observed for the 10^7 cells/ml dilution (Figure 45). Furthermore, the ligation time could be reduced to two hours by using pre-amplified genomic DNA originating from a dilution of 10^9 cells/ml.

Figure 45. Microarray results showing the positive mean gene values using genomic bacterial DNA as template. The tested bacterial isolate harboured the β-lactamase genes of DHA-1, SHV, and TEM. The DNA extracts from bacterial dilution series were used in a multiplex PCR for DNA pre-amplification before the PCR products were added to the ligation reaction. The red line indicates the cutoff for the mean gene values.



4 Discussion

The analysis of the genetic diversity of a biological habitat is a routine procedure in microbial and general ecology. A large set of molecular and statistical tools have been developed to address this issue. The first methods were based on fingerprinting techniques such as RFLP. More sophisticated studies sequenced selected marker genes with Sanger sequencing to draw conclusions about the investigated habitat. The current state of the art uses next generation sequencing to investigate biological communities. A huge problem in microbial ecology is that the large majority of species cannot be cultivated. To circumvent this problem, all genes are sequenced from an environmental sample and then assigned to a corresponding species. This approach is summarized under the term “Metagenomics”. The huge accumulation of data resulting from the introduction of this new approach led to the development of many different diversity indices addressing particular problems in ecology. Thus, ecology has become an interdisciplinary field consisting of biology, molecular biology and statistics.

Especially for clinicians, it becomes difficult to keep track on the advances in these fields because their profession in the hospital is very time demanding. Clinical studies using only RFLP analyses are still widespread. Nevertheless, hospitals have to be considered as very dynamic and interesting microbial environments harbouring many bacterial species. The high selection pressure caused by antibiotic treatment and the global exchange of bacterial strains due to human and goods traffic enhance the evolution of pathogens dramatically. Thus, hospitals are hotspots of bacterial evolution. Due to their outstanding importance for the human health, the bacterial community is very well monitored in many hospitals, providing large datasets to researchers. The scientific advances resulting from the routine implementation of microbial ecology techniques into the clinical environments will be interesting to observe. A fundamental biological dogma, that genetic information can only be transferred from parents to

children, was already annihilated after the discovery of HGT during the investigation of antibiotic resistance.

4.1 Emergence of β-lactamases in the General Hospital of Vienna

The high occurrence of CTX-M genes in this study confirmed the prevalence of these enzymes in ESBL-positive isolates. The dissemination of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{CTX\text{-}M\text{-}14}$ is already well documented, but the spread of the second most commonly encountered CTX-M gene in this study, $bla_{CTX\text{-}M\text{-}38}$, was only observed in China so far (88;89). CTX-$bla_{CTX\text{-}M\text{-}27}$, which was present in two different variants, was so far rarely present in Europe and only detected in UK, France and Spain (90;91). The five new CTX-M genes were all encountered in this study for the first time so that their clinical importance cannot be predicted. More detailed characterizations of these genes have to be carried out in a follow-up study.

Both $bla_{SHV\text{-}26}$ and $bla_{SHV\text{-}33}$ were detected once in two different *K. pneumoniae* and both for the first time in Austria. Previously, $bla_{SHV\text{-}26}$ has been only detected in Portugal and $bla_{SHV\text{-}33}$ in Spain (92;93).

In this study, I describe for the first time the occurrence of the carbapenemase gene $bla_{OXA\text{-}72}$ in *Enterobacteriaceae*, which was only observed in *Acinetobacter baumannii* until now. It is also the first detection of $bla_{OXA\text{-}72}$ in central Europe. The gene was present in an ESBL-positive *K. pneumoniae* isolate in combination with $bla_{TEM\text{-}1}$ and $bla_{SHV\text{-}33}$.

Furthermore, CMY genes were detected for the first time in Austria. Beside the more common $bla_{CMY\text{-}2}$ and $bla_{CMY\text{-}4}$ genes, the $bla_{CMY\text{-}42}$ gene, which was only once identified in Germany, was present in this study (94). The new CMY gene with similarity to $bla_{CMY\text{-}41}$ has to be further analysed. This is also the first report of the inhibitor-resistant β-lactamase genes $bla_{TEM\text{-}32}$ and $bla_{TEML\text{-}150}$, and the β-lactamase gene $bla_{TEM\text{-}135}$ in Austria.

The emergence of $bla_{VIM\text{-}4}$ was also observed for the first time in Austria, although local studies published the presence of VIM-1 in *Enterobacter cloacae* and VIM-2 in *Pseudomonas aeruginosa* (95;96). The presence of KPC in *Klebsiella pneumoniae* and *Klebsiella oxytoca* was already confirmed in south-eastern Austria but no sequence

analyses were performed so that it remained unclear whether KPC-2 is really new in Austria (97;98).

In two isolates (C8 and C21), there was a discrepancy between phenotype (metallo-β-lactamase, due to synergism of meropenem with dipicolinic acid) and genotype as no gene could be found that would explain the observed phenotype. However, the metallo-β-lactamase phenotype could not be reproduced on re-testing in one of the two cases. In contrast, in the other isolate (C8), the metallo-β-lactamase phenotype remained unexplained. This strain was additionally tested with a ligation-dependent multiplex method detecting the carbapenemase genes of IMI-1, IMP-24, OXA-48 and SME-1 and the commercial kit Check-MDR CT103 from Checkpoints (data not shown). The Check-MDR CT103 kit detected the AmpC β-lactamase ACT/MIR, but no other genes were identified with the above mentioned methods. Explanations for the absence of carbapenemase genes could be either technical errors, mutations or the presence of rare or new carbapenemases. This strain has been chosen as a target for whole genome sequencing in a new project to understand mechanisms behind the phenotype.

4.1.1 Analysis of β-lactamase diversity

To my best knowledge, this is the first report in which the genetic diversity of β-lactamases in a hospital was statistically estimated. My data showed that the majority of β-lactamase gene-groups present in the General Hospital of Vienna in the year 2011 was detected in this study. Approximately 55% of the β-lactamase genes estimated to be present in the hospital were identified. Interestingly, the total number of different genotypes has still remained unclear. Many different combinations of the detected β-lactamase sequences seem to be possible and present. Rarefaction curve analyses also revealed that the genetic diversity of AmpC-positive isolates is much lower than the one of ESBL-positive. Especially the high occurrence of different variants of the same genes in ESBL-positive isolates was notable. These trends are either the result from higher mutation rates in ESBL-positive *Enterobacteriaceae* or that an ESBL-phenotype is more easily developed by *Enterobacteriaceae* with higher mutation rates.

4.2 Analysis of genetic diversity in south-eastern Austria

In recent reports, a wide distribution of CTX-M genes was found in Austrian *Enterobacteriaceae* isolates; however, the presence of the three CTX-M groups 1, 2, and 9 in a single study set was not observed so far (87;99;100). Similar to those studies, the wide distribution of CTX-M genes was confirmed in this study. Eisner et al. investigated the prevalence of CTX-M genes in the identical laboratory and found CTX-M genes in 58% of all ESBL-positive isolates in 2004 (87). Compared to that data, a significant increase in CTX-M prevalence was detected in this study.

Recently, NDM-1, TEM-1, OXA-1, and a SHV subtype with 99% homology to SHV-5 and SHV-12 were also identified in isolates of *Enterobacteriaceae*; however, the spread of other β-lactamases has not been published in Austria yet (99;101). Except for NDM-1, all β-lactamase genes, observed in isolates of *Enterobacteriaceae* in Austria so far, were present in the investigated strains. The distribution of bla_{SHV-1}, bla_{SHV-11}, and bla_{DHA-1} was not described until now.

Although the Class C β-lactamase DHA-1 was already isolated in isolates of *Enterobacteriaceae* from several continents, it was not found in Austria yet (102-104). In 2009, Shi et al. published a *K. pneumoniae* isolate from China that contained the β-lactamase genes of DHA-1, OXA-1, SHV-28 similar to the isolate 10/134. In this Chinese isolate, the genes of TEM-1, CTX-M-9, and CTX-M-55 were additionally identified. Shi et al. also tested the resistance genes transfer of 18 isolates of *K. pneumoniae* to those of *E.coli* and found five of eight transconjugants that harboured bla_{DHA-1} (105). The dissemination of DHA-1 in combination with OXA-1 and SHV was also observed by Menezes *et al.* who found a similar resistance genotype with bla_{DHA-1}, bla_{OXA-1}, bla_{SHV-12} and bla_{TEM-1} in *Salmonella* serovar Agona from India (106). The only metallo-β-lactamases identified in Austria were NDM-1 in isolates of *K. pneumoniae*, IMP-13, IMP-22 and VIM-2 in isolates of *Pseudomonas aeruginosa* (101;107). The resistance genes of those enzymes could not be detected among the investigated ESBL-positive isolates of *Enterobacteriaceae*.

Rarefaction curves and the richness estimators ACE and Chao1 were used to estimate the diversity and number of species in the samples (108). To my best knowledge, these tools have not been used in clinical environments to estimate the diversity of antibiotic resistance genotypes respectively resistance genes. The analyses of resistance gene

patterns revealed a high diversity in this study. Due to the almost linear slope of the rarefaction curve, it was not possible to estimate the number of resistance gene patterns with the present data set correctly.

In contrast, analysing the diversity of β-lactamases revealed that most of the screened genes present among the ESBL-positive isolates of *E.coli* and *K. pneumoniae* were identified. The ACE and the Chao1 models calculated similar values. Characterizing new isolates with the used primer set should unveil only a few new β-lactamases and subtypes, which is confirmed by the flattening shape of the rarefaction curve.

In conclusion, this data supports recent data showing that the TEM, OXA, SHV, and CTX-M genes are widely disseminated in isolates of *Enterobacteriaceae* and that $bla_{CTX\text{-}M\text{-}15}$ and $bla_{TEM\text{-}1}$ may be the most abundant genes of these enzymes. As no phenotypically carbapenem resistant isolates were analysed, it was not surprising that metallo-β-lactamase genes or the serine β-lactamase gene of KPC were absent. The high number of different β-lactamase genotypes may indicate a vivid exchange of β-lactamase genes in the bacterial environment. To reduce the spread of new β-lactamases, the hotspots of these clinically relevant horizontal gene transfers should be identified and prevention strategies should be defined.

4.3 On-chip PCR

The concept of PCR was a revolutionary invention in life sciences. It was the fundament of modern molecular biology because DNA could be detected and amplified in a simple reaction for the first time. Unfortunately, the new technique had also limitations. Some of them such as the possibility to quantify DNA could be resolved with the introduction of real time PCR. Others, such as the parallel detection and amplification of high numbers of genes still remained. Thus, many investigations addressed this issue. A theoretically straightforward and elegant idea was the spacial separation of PCR primers and products in one reaction camber. With the introduction of microarrays it became possible to immobilize thousands of separated primers onto a surface and create a reaction chamber for the PCR. Although researchers faced constant drawbacks in their attempts to immobilize the PCR, the persuasion remained that only the right reaction conditions remained to be found. A boost in this field was the introduction of the next generation sequencer from Solexa. The commercial product used immobilized primers to amplify DNA on a chip for subsequent sequencing. After this success, the research field on on-chip PCR was revived. Several publications presented advances and further developments using new surfaces and binding chemistries.

4.3.1 Annealing temperature

The major problem regarding on-chip PCR in this and other studies was the poor specificity of the reaction. The attempts performed in this study to resolve this problem included adaption of annealing temperature, primer sequence composition, primer length, primer concentration, spacer composition of primers, surface chemistry, reaction reagents, and on-chip PCR product length. In general, the most used parameter to adjust the specificity in a liquid phase PCR is the annealing temperature. In my experiments, the increase of annealing temperature until 76°C showed no improvement regarding specificity, but only a loss in fluorescence signal intensity with increasing temperature. In liquids, the Brownian motion of molecules increases with temperature. This is the reason why it possible to adjust specificity with an increase in temperature. The increase of Brownian motion of primers and template DNA leads to the melting of double stranded DNA since the hydrogen bonds cannot withstand the increasing thermal motion. AT-rich DNA sequences have lower melting temperatures than GC-rich

regions, because their nucleotides are connected only with two hydrogen bonds per nucleotide. Guanine and cytosine use three hydrogen bonds, and thus have higher binding energies. The immobilization of primers may influence the Brownian motion of the primers dramatically. On one hand on-chip PCR primers cannot rotate at their 5' end as their counterparts in liquid phase. On the other hand, through the covalent attachment to a glass surface, the primers absorb the thermal motion of the glass slide, which could be altering their own thermal motion. More systematic investigations have to be conducted to estimate the impact of these two factors on the molecular system, but the mentioned factors should play a key role in new attempts to gain specificity in on-chip PCR.

4.3.2 Primer spacer

An important difference between the primers used in the liquid phase PCR and the on-chip PCR is the presence of a primer spacer in the on-chip PCR. The spacer is necessary to grant the accessibility of the large DNA polymerase molecules and their catalytic centres to the primer sequence. Furthermore, primers without spacers have a decreased annealing capability (109;110). The spacers in this study consisted of two elements. One element was necessary to covalently bind the primers exclusively at their 5' ends to the glass slide. The binding had to be highly thermostable and was accomplished with crosslinker molecules consisting of carbon chains. The second element was a decamer nucleotide spacer. Two sequence variants of the nucleotide spacer were tested in this work but no difference was observed. The combination of the two spacer elements resulted in a relative long spacer molecule. Unfortunately, this length could be a cause for the unspecificity of on-chip PCR. Tests with the on-chip PCR primers and the same primers lacking the spacer were performed in a liquid phase PCR. The reactions lacking the primers with spacers were specific, whereas in reactions with primer spacers unspecific amplification products could be observed (data not shown). Especially the nucleotide spacer increased the primer length, which is known to be a critical parameter in liquid phase PCR because too long primers increase the probability of unspecific hybridization. Thus, new on-chip PCR approaches should exclude the nucleotide spacers which are widely used in DNA microarrays. Also a possible interaction of the

DNA polymerase with the organic spacers, necessary for the covalent attachment of the primers to the surface, should be investigated in more detail.

4.3.3 On-chip PCR chamber

As no thermostable reaction chambers were commercially available that could be used in standard equipped clinical laboratories, an appropriate chamber had to be developed. Restraints were that the analysis in a microarray scanner should be possible, implying that a standard glass slide had to be used as substrate for the primers. Due to the used thermal cycler, also the maximal thickness was restricted to 2.5 times a glass slides. A critical point during on-chip PCR represents the molecular environment in which the reaction takes place. The glass slides were coated with aminosilane resulting in a nonpolar surface. Polar surfaces such as the ones present on aldehyde slides can bind polar biomolecules to their surface. These aldehyde slides need to be blocked with BSA or urea prior PCR. This procedure and possible source of contamination was successfully avoided with the choice of a nonpolar surface (Figure 25).

One of the greatest challenges in the development of the reaction chamber was the control of evaporation of the reaction mixture during thermal cycling which resulted in increased ion concentrations. Positively charged ions like Mg^{2+} or Na^{+} increase the binding energy of double stranded DNA because they intercalate with the negatively charged phosphate backbone of DNA. The ions stabilize the opposite DNA strands through electrostatic interactions. Thus, if evaporation due to thermal cycling occurs, there is a decrease in PCR specificity after every cycle due to unspecific primer binding to target DNA. The addition of nonpolar reagents like SolutionQ (Qiagen) or Self-Sealing Solution (LTF Labortechnik) to the reaction mixture and sealing of the chamber with PCR-foil reduced evaporation significantly. Nevertheless, all used reaction chambers in this study were susceptible to a certain degree to evaporation. New approaches of on-chip PCR have to develop a slide that is compatible with microarray spotters, microarray scanners and also integrates a reaction chamber that can be sealed and reopened several times.

4.3.4 Surface chemistry of on-chip PCR slides

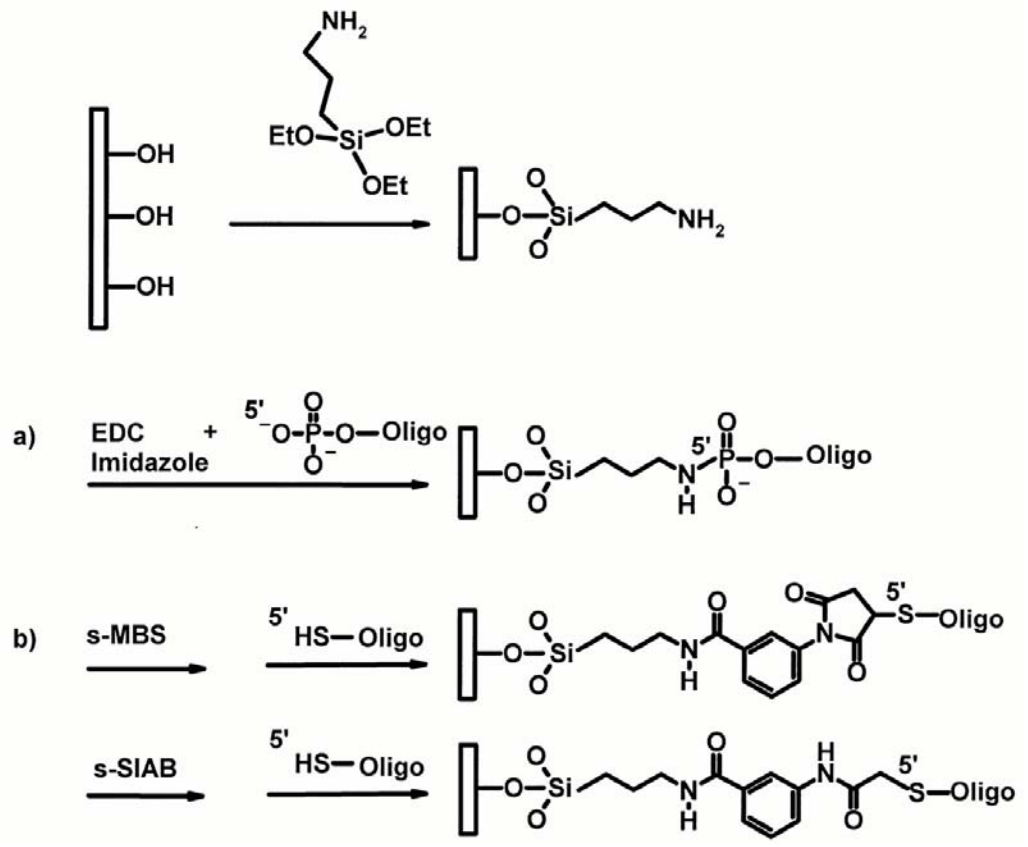
In this study, the coating of the glass slides with aminosilane was chosen as the best surface. The advantage of this surface over aldehyde and epoxy slides is that the aminosilane surface is non-polar. Thus, polar molecules like DNA and proteins are not trapped by electrostatic interactions on the surface. Another important prerequisite is that the covalent binding between the surface and the oligonucleotide is thermostable. Selective binding of the primer at its 5' end to the surface is also necessary, as well as a certain surface density of primers.

Especially the specific attachment of the primers at their 5' end was subject of several investigations. Epoxy silane-derivatised slides were used to generate a covalent binding with 5' amino modified primers (111;112). Thiol-derivatised glass slides were used in combination with 5' disulfide modified primers (113). 5' succinylated oligonucleotides can be attached to amino-derivatised glass slides (114). Also, crosslinker molecules can be used such as those in the present study, which increase the distance of the primer to the surface and influence the surface properties (Figure 46). Beside the crosslinker used in this study, many others have been tested. Maleic anhydride, phenyldiisothiocyanate, carbodiimide hydrochloride (EDC), sulfosuccinimidyl(4-iodoacetatyl)aminobenzoate (s-SIAB), sulfosuccinimidyl 4-(*N*-maleimidomethyl)-cyclohexane-1 carboxylate (s-SMCC), *N*-(γ-maleimidobutyryloxy)sulfo succinimide ester (s-GMBS) and sulfosuccinimidyl 4-(*p*-maleimidophenyl)-butyrate (s-SMPB) also mediated the covalent linkage of the 5' end of primers (115;116). The crosslinker chemistry is not limited to glass supports. The carboiimide chemistry was chosen together with latex beads, dextran supports, polystyrene microwells and amino controlled-pore glass (117-121). In addition, many other solid supports were tested for their suitability for on-chip PCR. Oligonucleotides were also immobilized in polyacrylamide gels and the PCR conducted within the gel (122;123).

Adessi *et al.* performed extensive experiments evaluating various binding chemistries regarding their loading density, the 5' end selectivity of the binding reaction and the thermal stability of the covalent bond (116). The binding chemistry in this study was chosen based on those results preferring the one with the highest thermal stability, high loading density and 5' end selectivity. Retrospectively, it can be said that those factors were not among the most important ones to have a good specificity of the on-chip PCR.

The focus during the development of new binding and surface chemistries has to be put on better emulation of the liquid phase primer behaviour than other factors.

Figure 46. Illustration of the different primer attachment chemistries taken from Adessi *et al.* (116). The processes to covalently attach nucleic acid molecules to a glass support are shown. Hydroxylated glass surfaces were silanised using aminosilane reagents exemplified here by ATS. EDC and imidazole reagents lead to the formation of a phosphoramidate linkage between amino-derivatised glass surfaces and 5' end phosphate modified oligonucleotides in a one-step reaction (**a**). For cross-linker chemistry (**b**), an amide bond was formed between amino groups of the surface and the succinimidyl ester moiety of the cross-linker compounds exemplified with s-MBS or s-SIAB reagents. 5' end thiol modified oligonucleotides reacted with either the maleimide portion of s-MBS-like compounds or the iodoacetamide portion of s-SIAB.



4.3.5 On-chip primer design

Primers are short, initiating DNA strands that bind to target DNA and are further elongated by DNA polymerases. Critical for the specificity of a PCR is that the primer matches the complementary DNA strand perfectly. Nevertheless, DNA polymerases can also elongate primers that do not perfectly match to their target DNA (124). Thus, it is

important during primer design to consider various factors. The stability of the 3' end of the primer, amplification product length, primer length, melting temperature and GC content are important parameters. Possible mismatches should be located at the 3' end (125-127). DNA secondary structures formed by primers such as hairpins, palindroms, primer dimers and cross-dimers reduce PCR efficiency significantly (128).

Primer name	Spacer	Sequence 5' 3'	Length	GC content	Melting temperature
Solid TEM 20F	TTTTTTTTTT	GTGTCGCCCTTATTCCCTTTTT	22	50.0%	52°C
Solid TEM 789R	TTTTTTTTTT	TTCATCCATAGTTGCCTGACTCCC	24	45.5%	53°C
Solid TEM 233R	TTTTTTTTTT	GCCCGGCGTCAACACGGGATAATACCG	27	50.0%	52°C
Solid TEM 140 F	TTTTTTTTTT	TGGATCTCAACAGCGGTAAGATCCTTGAGAG	31	47.6%	52°C
Solid TEM 204R	TTTTTTTTTT	CCACATAGCAGAACTTTAAAAGTGCTCA	28	37.5%	52°C
Solid TEM SS 521 F	TATTATTATT	ACGACGAGCGTGACACCACGATGCC	25	64.0%	64°C
Solid TEM SS 681 R	TATTATTATT	CCAGCCGGAAGGGCCGAGCGCA	22	73.9%	66°C
Solid TEM SS 528 F	TATTATTATT	GCGTGACACCACGATGCCTGC	21	66.7%	60°C
Solid TEM SS 729 R	TATTATTATT	CCGCGAGACCCACGCTCACC	20	75.0%	62°C

Table 17. Primers targeting the TEM gene designed for on-chip PCR

On-chip primer design differed only slightly from the one in liquid phase. The main difference represented the spacer sequence that was meant not to interact with target genes. To identify important primer parameters that influence on-chip PCR, nine primers were designed targeting the TEM gene (Table 17). Sixteen different primer combinations and PCR products were possible with these primers. The primers differed in size (18bp – 31bp), GC content (37.5% - 75%) and melting temperature. Surprisingly, none of these parameters had a detectable impact on the specificity of the on-chip PCR.

A parameter that influences the reaction mechanism of the on-chip PCR massively is the amplification product length (129;130). Too short and too long immobilized products cannot bind to the immobilized primers due to steric hindrance. Thus, the optimal amplification product length was tested experimentally (Figure 31). It was expected that at the optimal product length the microarray signal intensities are highest and that they are decreasing with shorter and longer amplification product lengths. Indeed, the signal intensity increased constantly with its maximum at 201bp product length, but at 208bp and 213bp there was a sharp decrease in signal intensity. The two

following longer on-chip PCR products (649bp, 769bp) showed higher signal intensities than the ones with 201bp and 208bp (Figure 31). This irregular pattern in the signal intensities indicates that the major factors responsible for the observed different signals intensities are not the varying on-chip PCR product lengths, because such a strong decrease in signal intensities cannot be explained by significant steric differences between DNA molecules with 201bp, 208bp and 213bp length. Failure to detect the intended immobilized on-chip PCR products with specific detection oligos, although microarray signals were present (3.3.9 Specificity of on-chip PCR), indicates that the observed microarray signals were not resulting from the intended fluorescently marked on-chip PCR products. The unspecific amplification of DNA on the chip surface could be an explanation for the irregular pattern observed with different amplification product lengths.

In summary, no parameter important during liquid phase primer design was identified to be also important in on-chip PCR design under the tested reaction conditions.

4.3.6 Conclusions regarding on-chip PCR

On-chip PCR is a promising technology aiming to detect and amplify thousands of genes in a single reaction. The method has gained popularity in the scientific community with the introduction of microarrays since it became possible to immobilize thousands of primers onto a small area. This miniaturization was also economically interesting because the downsizing of many parallel amplification reactions to a small volume promised a wide application of the technology. Although the first publications were presented in the early 90s, the achieved progresses regarding this technology were poor so far.

In this study, a simple reaction chamber was developed that can be used in standard equipped laboratories. Furthermore, the PCR mechanism could be established with primers immobilized on a glass solid support. An aminosilane surface, s-MBS as crosslinker and 5' end thiol-modified primers were chosen as surface and binding chemistry. The efficiency of on-chip PCR was strongly dependent on the used PCR kit. The Qiagen products Multiplex PCR kit and TypeIt kit led in combination with fluorescence labelling with $Vent_R$(exo-) polymerase, incorporating Atto532-dCTPs into the on-chip PCR products, to the highest signal intensities. To adjust the specificity of

on-chip PCR, several parameters were tested. Variation of annealing temperature, reaction reagents, primer composition, amplification products length, primer concentration, blocking procedures, PCR cycling protocols and other parameters could not improve specificity to a desirable degree.

New approaches have first to investigate the molecular interactions on the chip surface. The differences between liquid phase and solid phase PCR have to be understood before alternative on-chip PCR setups are to be developed. The new surface chemistry should grant accessibility of target DNA and enzymes to the immobilized primers without the necessity of a nucleotide spacer.

4.4 Ligation based solid phase detection

Microarrays used to be a promising technology in molecular biology and diagnostics because of their multiplex ability. A huge set of microarrays for many different applications was developed but false positive signals remained a main limitation of this technique. Nevertheless, clever design and extensive testing of a microarray probe panel can decrease the presence of false signals to an acceptable degree. An interesting application of microarrays was their use for the detection of padlock probes. Padlock probes are approximately 100 base pair long nucleotides that bind to a gene target and are ligated to a circular molecule. Every padlock probe can be identified on the microarray through a unique sequence tag. This combination allows the detection of up to 1000 different targets in a single reaction. The logical consequence of these results was the immobilization of the very specific padlock probe reaction to a microarray surface. To my knowledge, the achievements obtained from liquid phase experiments could not be reproduced with immobilized probes so far, probably due to the different hybridization behaviour of immobilized probes. Thus, I developed a new kind of immobilized probe, the looplock probe, which allowed the elimination of some factors contributing to unspecific signals.

4.4.1 Ligation methods using immobilized probes and specificity probes

The proof of concept of the ligation method that uses a polymerase to fill up the gap between the immobilized probe and the specificity probe prior ligation failed (Figure 13). Later it was realized that a systematic error may be responsible. The polymerase used to fill up the gap was $Vent_R$(exo-) polymerase lacking 5' end exonuclease activity. It was thought that the polymerase reaches the 5' end of the specificity probe and detaches from the DNA target strand. This was an false assumption because the $Vent_R$(exo-) polymerase is capable of strand displacement. $Vent_R$(exo-) polymerase can detach the specificity probe from the DNA target strand and no ligation would be possible. The detection oligonucleotide would have no sequence to bind to. A solution for this problem would be the use of the Stoffel fragment as alternative polymerase. The Stoffel fragment is lacking 5' end exonuclease but also strand displacement activity.

With the other, simpler solid phase ligation method shown in Figure 12, which also uses an immobilized linear probe and a specificity probe, a microarray fluorescence signal

was obtained (data not shown). The problem with this method was that the reaction was not specific. The immobilized probe TEM 140F and the specificity probe TEM 204R were ligated, although they share none common DNA template strand but bind to the complementary sequences. The two probes have an sequence overlap of 14bp and thus can bin to each other. This overlap might facilitate the unspecific ligation process although the 3' end of immobilized probe TEM 140F and the 5' end of specificity probe TEM 204R is 16bp apart.

Since no DNA amplification steps were conducted, poor sensitivity of both methods might be considered as reason for negative fluorescence signals. However, the high concentrations used in the experiments of up to 10 μM of probes and 10^{12} DNA copies in the reaction mixture should have resulted in sufficient ligation products for detection without a DNA amplification step.

4.4.2 Looplock probes

The encouraging results from the proof of principle experiments showed that looplock probes are suited for immobilization onto a glass surface and subsequent enzymatic reactions (Figure 35 - Figure 39). An advantage of these probes over conventional microarray probes is the possibility to include a maximal stringency washing step into the protocol. This feature may be helpful in resolving the problem of unspecific hybridization, which is faced by microarray probes. The closed structure of the looplock probe is also of advantage due to the unexpected unspecific ligation of capture probes to the 3' ends of linear immobilized probes. This result was highly surprising because ampligase was thought to recognize and ligate highly specific perfectly matching DNA. The usage of linear immobilized probes with a dideoxynucleotide at their 3' end reduced fluorescence signals significantly, but not completely. An explanation for the persistence of fluorescence signals could be the poor purification of oligonucleotide after synthesis with remaining shorter oligonucleotides lacking the terminal dideoxynucleotide. The specificity of ligases mentioned in publications was the main reason why they were chosen over polymerases in recent solid support methods. In combination with polymerases capable of strand displacement (Φ29 polymerase, *Bst* polymerase and $Vent_R$(exo-) polymerase), the amplification of DNA molecules circularized through ligation should be highly selective.

Another difference of the looplock probes in comparison to conventional microarray probes concerns the spacer. Moiseev *et al.* showed that immobilized single stranded DNA is due to its conformation closer located to the surface than double stranded DNA. Thus, double stranded DNA was used as spacer to grant better accessibility of the enzymes to the immobilized probes. A possible problem regarding looplock probes concerns the ring strain that emerges with their circularization. It is known from padlock probes that too short molecules cannot be ligated. In this study, two circularized DNA molecules (ligated capture probes and looplock probes) had to hybridize and overcome the accumulated ring strains of both molecules. Unintended secondary structures with reduces ring strain energies could be a result.

4.4.3 Capture probes

The concentration of probes or primers in an enzymatic hybridization reaction is of particular importance. High concentrations lead to a high probability of hybridization to a DNA target and high sensitivity, but also decreased specificity due to unspecific binding. Low concentrations of oligonucleotides reduce sensitivity and hybridization events, but make them highly specific. The ideal concentration is a trade-off between specificity and sensitivity, and has to be determined experimentally. The impact of capture probe concentrations on the signal intensity and hence sensitivity is illustrated in Figure 35 confirming the above mentioned model. Interestingly, signal intensities did not increase in the same order of magnitude as did capture probe concentrations. The reason for this was that the ligation process was also influenced by factors such as DNA template concentration, enzyme concentration and other parameters. The biggest difference in signal intensity between unspecific and specific probes was observed at a capture probe concentration of 10 nM (Figure 38). This concentration approximately matches the ones used in padlock probe experiments were ligation products were specifically separated by capture probes immobilized to magnetic beads.

4.4.4 Specificity of on-chip ligation assay

As in all multiplex methods, the specificity of the on-chip ligation assay is the most important parameter. Unintended ligation to 3’ ends was identified as factor contributing to unspecificity. Remarkable and unclear was the fluorescence pattern

obtained with oligonucleotides with a terminal ddNTP using low concentrations of capture probes (Figure 37). Oligonucleotides with a terminal ddNTP showed equal or even higher fluorescence signal intensities than the same probes with a normal 3' end. This may be an indication for further unintended reactions on the surface. Also the spotting buffer was identified in some experiments as possible contributor to fluorescence signals. In some cases, the combination of SSC in the spotting buffer with a detection oligonucleotide showed fluorescence at 532 nm, even if the detection oligonucleotide used carried only a fluorophore emitting at 635 nm (data not shown). A reason why this phenomenon occurred was not found yet.

In conclusion, a promising new type of probe was developed. Further improvements of the protocol will include the implementation of polymerases with strand displacement capability. These polymerases will take advantage of the circularized capture probes amplifying them via rolling circle amplification, thus improving sensitivity and specificity of the assay. The successful immobilization of the ligation reaction onto the detection platform microarray will reduce the time to obtain data for at least two hours in comparison to analogous liquid phase methods. This is especially important in clinical environments where the correct treatment of the patient has to be induced as soon as possible. Thus, further effort will be put in the improvement and development of multiplex detection methods such as this on-chip ligation assay.

4.5 Multiplex detection of antibiotic resistance genes with padlock probes

In this work, a multiplex DNA detection method for antibiotic resistance characterization based on padlock probes is presented. The main advantage over current procedures is the multiplex capability of the developed assay including the possibility to detect SNPs, which can alter the susceptibility to antibiotics. Results from studies using similar ligation dependent assays indicate that with the presented method up to 10,000 DNA targets might be detected in a single assay (131;132). With these features, it is possible to design an assay targeting all known antibiotic resistance mechanisms.

The optimization of several parameters was necessary to obtain a high specificity with the developed assay. The most critical one was concerning the concentration of padlock probes. High concentrations of DNA in a solution increase the number of events of specific and unspecific hybridization. Although mismatching double stranded DNA has a lower melting temperature than perfect matching DNA and denaturates fast, the short living intermediate can be modified by enzymes. Unspecific PCR products are the most prominent representatives of these unintended reaction products. But also other enzymes than polymerases are prone to this phenomenon. As shown in the previous sections, ligases which are thought to have better proof-reading properties than polymerases also ligate mismatching DNA hybrids. Thus, limiting the amount of padlock probes in a reaction mixture decreases the events of unspecific hybridization and ligation. In my first experiments using padlock probes, their initial concentration was 10 nM and 1 nM per padlock probe respectively (data not shown). The subsequent microarray analyses revealed that all padlock probes had been ligated, although only one DNA target was present. The 1000-fold reduction of the initial padlock probe concentration to 1 pM resulted in specific signals when using the same protocol. This finding was considered as essential for the optimization of specificity for ligation based reactions. Due to parallel development of the padlock probe assay and the on-chip ligation assays presented in the previous sections, the ideal concentration of probes was under intense evaluation in the on-chip ligation based methods.

4.5.1 Sensitivity of padlock probe assay

Unfortunately, a low padlock probe concentration does not only decrease unspecific hybridization events but also the specific ones. This factor has a strong impact on sensitivity. However, a high sensitivity is for clinical applications of outstanding importance. Some diseases such as sepsis need diagnostic tests capable to detect one cell per ml. Others, such as urinary tract infections need less sensitive tests (10^4 cells/ml). In clinical routine, cultivation based methods are used that can theoretically detect up to one cell per ml. Nevertheless, some studies have shown that these tests can also produce false negative and false positive results (52;53). An advantage of DNA based tests over cultivation based ones is that these tests can also detect non viable cells and free DNA in blood and other sample types. An optimized PCR assay is able to detect ten DNA molecules per reaction, although commercially available sepsis detection kits specify a detection limit of 30 to 300 viable cells. Explanations for this suboptimal sensitivity are inhibitory substances in biological samples and inefficient DNA extractions.

The sensitivity of the developed padlock probe assay was 10^4 PCR product molecules per test. If genomic DNA was used, a multiplex PCR for pre-amplification of DNA was necessary to detect at least 10^7 cells/ml. This large difference between sensitivity using PCR products and genomic DNA may be caused by several factors. A poor DNA extraction protocol results in low amounts of target DNA in the sample. Furthermore, the number of non target DNA molecules in genomic DNA is much higher than in PCR products, where theoretically every molecule should be a target. The non target DNA is partly competing with target DNA for hybridization events with padlock probes and thus reducing the probability for stable target-padlock probe hybridizations.

The number of antibiotic resistance gene copies in a bacterial cell can also vary dramatically. They can be located on high copy number plasmids or on the genome as single copy. However, in many cases the presence or expression of antibiotic resistance genes represents a reduction of fitness. Thus, not all surviving bacteria in a culture have antibiotic resistance genes, because enough enzymes are transported into the extracellular space by some members of the culture to deactivate antibiotics. This illustrates the problem to estimate the exact copy number of a resistance gene from a sample with known cell numbers.

An increase of the sensitivity of the assay can be achieved by several measures. First, the DNA extraction protocol for genomic DNA should be optimized. Furthermore, the ideal time point during bacterial cultivation, when the copy number of antibiotic resistance genes is highest, has to be determined. Only DNA from cultures taken at this time point should be analysed. Another possibility to increase the sensitivity is the introduction of more RCA steps into the C2CA procedure. The fluorescence labelling of C2CA products is performed in a linear PCR at this point. This reaction has a very low efficiency. The use of two primers in the labelling PCR would increase the number of fluorescently labelled molecules exponentially with every cycle. The combination of these measures should result in an improved sensitivity of the assay.

4.5.2 Specificity

The high specificity of the β-lactamase assay is the most remarkable property of the method. It was achieved by a whole cascade of discriminatory enzymatic reactions. The ampligase is thought to have significantly lower error rates than DNA Taq polymerases. The circularization of the padlock probe after ligation produces a very unique molecule, which is approximately only 90 base pairs long. The next steps take advantage over this structure and use the phi29-polymerase to amplify DNA. This polymerase has a strand displacement capability and can only amplify circular molecules efficiently due to missing denaturation steps. The linear PCR using only one primer results in a low probability of formation of unspecific PCR products.

Furthermore, the high specificity was also achieved by the assay design philosophy. Every gene was detected by multiple padlock probes. As the microarray technology has limitations regarding specific target hybridization, it was necessary to design more padlock probes per gene in order to identify false positive microarray signals. Figure 41 shows the evaluation of the padlock probes with synthetic templates and clearly illustrates this problem. Thus, at least three padlock probes per gene were necessary for definite information on the presence of β-lactamase genes.

4.5.3 State of the art

Molecular methods for ligation-dependent multiplex detection of β-lactamases are commercially available but limited only to a few genes per assay (Check-MDR CT101, CT102, CT103; Check-Points, Netherlands). Multiplex-PCR-based assays were also developed as were microarrays for SNP characterization of β-lactamases (54;55). Nevertheless, none of these assays can detect all β-lactamases in a single test.

Alternative approaches to characterize pathogens are based on mass spectrometry. The low cost per analysis (€0.40) has made this technology attractive in routine diagnostics. The characterization is based on large databases where every pathogen is characterized with molecular biological tools in detail regarding its antibiotic resistance and strain information. Subsequently, the strain is analysed with a mass spectrometer, and the spectrum fingerprint is deposited in a reference database. In clinical routine, bacterial isolates are analysed with mass spectrometry and the resulting spectrum fingerprint is compared with the reference database.

With decreasing prices, sequencing technologies are also becoming more interesting for hospitals. Yet, no assays are available for routine diagnostics. Beside bureaucratic aspects caused by missing certifications for these technologies, also other factors make them little attractive for clinicians. Fast data retrieval is the most important argument for clinicians to use molecular diagnostic tools. The long duration time to obtain the sequencing results makes the current sequencers almost meaningless in a clinical setting. Another aspect concerns the produced data flood. Clinicians are interested in very specific issues concerning the investigated pathogen and have little interest in additional information. However, a very promising and revolutionary technology might change medical diagnostics. The Oxford Nanopore is presented as technology capable of single nucleotide sequencing within minutes or hours. The system should have been commercially available at the end of 2012 but its release was postponed.

4.5.4 Future outlook for the β-lactamase detection assay

Along with the technological advantages, our novel approach also offers cost benefits. We estimate the costs for consumables, including the self-made microarrays, to be approximately $25 per analysis. In addition, the majority of clinical laboratories are equipped with the required state-of-the-art technology and can easily adapt this method

to their routine analyses. The automation of the multiplex assay or integration into a lab-on-chip device represent further advances for this technology. Additionally, a linear increase in the microarray signal intensity corresponding to a gradually elevated template concentration or ligation time was observed until saturation of the microarray signal (Figure 42). This can be used for two different purposes. As signal intensity is dependent on the ligation time of padlock probes, the sensitivity of the assay can be optimized by increasing the ligation time. The more import conclusion concerns a possible new application of the method. Introducing internal standards with defined concentrations into the β-lactamase assay might enable the semi-quantitative detection of gene targets. The concept is analogous to the one used in real time PCR. To our knowledge, no semi-quantitative multiplex detection methods were published so far. In summary, we developed a novel high-throughput and cost-effective multiplex assay to identify antibiotic resistance genes that can yield semi-quantitative results.

5 Abstract

In the present study, the issue of antibiotic resistance in clinically relevant bacteria has been discussed intensively. Due to the large number of different resistance mechanisms, the focus was on one of the most important classes of resistance genes, the β-lactamases. It is a protein category, which is defined by its enzymatic activity. These enzymes cleave β-lactam antibiotics, and thus, deactivate them. β-Lactams are the most important bactericides against human pathogens which mainly belong to the *Enterobacteriaceae*. β-Lactams share a structural similarity with D-alanyl-D-alanine, which is a component of the bacterial cell wall. It is involved in the molecular cross-linking of the bacterial murein by a peptidase. β-Lactams bind irreversibly to the peptidase, which provokes after a cascade of further reactions, bacterial cell lyses.

More than 950 different naturally occurring β-lactamases are already known and divided into four phylogenetically different classes (A-D). Although they have a similar enzymatic activity, the catalytic sites and protein sequences differ strongly between these classes. One goal of this work was to obtain an overview of the distribution of β-lactamase genes in Austria's largest urban areas Vienna and Graz. For this purpose, clinical isolates taken from patients and phenotypically determined as β-lactamase-positive were analysed genetically. The first step was the creation of a β-lactamase DNA sequence database. Based on this data, 25 primer pairs were designed targeting the clinically most important β-lactamase genes. To double check, also 24 already published primer pairs were used in the analysis. All 33 clinical isolates from Graz and 108 from Vienna were analysed with these 49 primer pairs by PCR twice. The detected genes were amplified, sequenced and used for further statistical analyses. The Chao1, ACE and Rarefaction curve algorithms, which are widely used in ecology, have been applied in a clinical study for the first time. They were used to estimate the number of β-lactamase genes in the investigated hospitals. In the present study, a large number of genes have been described in Austria and Europe for the first time. Members of all four classes of β-lactamases were among the detected genes. The statistical analyses showed that the majority of β-lactamase genes in clinical isolates of *Enterobacteriaceae* were identified in the surveyed hospitals. The number of different β-lactamase genotypes could not be determined due to the high diversity among the genotypes.

The second objective of this work was the development of a multiplex detection method. The immobilization of the PCR on a glass slide was an obvious approach for this purpose. A primer pair was covalently bound with a microarray spotter to a nonpolar surface. Based on this reaction environment, many different parameters such as reaction mixture, annealing temperature, primer sequence, product length, etc. were evaluated. The DNA was amplified on a glass surface successfully during the on-chip PCR, but it was not possible to determine parameters for specific amplification. Based on the experience obtained from on-chip PCR experiments, a multiplex ligation reaction with immobilized probes was developed. A newly developed type of probes (looplock probes) was compared with linear probes. Parameters that were essential for the specificity of the on-chip ligation reaction were studied systematically. The proof-of-principle with the new probes and the on-chip ligation reaction could be shown, but for routine use, further optimization of specificity is required.

In addition to the tested surface-based methods, also a liquid phase multiplex detection method was developed based on padlock probes. These padlock probes were ligated in the presence of a target sequence resulting in single-stranded, circular DNA molecules. The ligated padlock probes were amplified in two successive rolling circle amplification reactions and then fluorescently labelled in a linear PCR. The labelled amplification products were detected with a microarray. With this method, nearly 100 different target sequences could be determined in parallel. An assay detecting all clinically important β-lactamase genes in a single reaction was developed. The assay was successfully evaluated with clinical isolates and produces results with excellent sensitivity and specificity. Compared to existing products on the market, the developed assay can detect three to four times more genes in parallel. In addition, it has a very high development potential because the detection of all antibiotic resistance genes in a single assay is possible with this method.

6 Zusammenfassung

In der vorliegenden Arbeit wurde das Thema der Antibiotikaresistenzen in klinisch relevanten Bakterien intensiv behandelt. Aufgrund der hohen Anzahl an unterschiedlichen Resistenzmechanismen wurde der Fokus auf eine der wichtigsten Klassen von Resistenzgenen gelegt, die β-Laktamasen. Es handelt sich hierbei um eine Proteinkategorie, die über ihre enzymatische Aktivität definiert wird. Die Enzyme sind in der Lage β-Laktam Antibiotika zu spalten und damit zu deaktivieren. Die β-Laktame sind die wichtigsten Bakterizide gegen die meisten humanen Pathogene. Die Wirkung der β-Laktame basiert auf einer strukturellen Ähnlichkeit mit einem Bestandteil der bakteriellen Zellwand, dem D-Alanyl-D-Alanin, welches in Kombination mit einer Peptidase für die molekulare Vernetzung des bakteriellen Mureins verantwortlich ist. β-Laktame binden irreversibel an die Peptidase, wodurch nach einer Kaskade von weiteren Reaktionen das Bakterium sich schließlich selbst lysiert.

Über 950 unterschiedliche, natürlich vorkommende β-Laktamasen sind bereits bekannt und werden in vier Klassen (A-D) eingeteilt, die zwar ähnliche enzymatische Aktivität, aber unterschiedliche katalytische Zentren und Proteinsequenzen haben. Ein Ziel dieser Arbeit war es einen detaillierten Überblick über die Verbreitung der β-Laktamasen in Österreichs größten Ballungszentren Wien und Graz zu erlangen. Zu diesem Zweck wurden klinische, phänotypisch β-Laktam resistente Stämme genetisch untersucht. Nach dem Erstellen einer β-Laktamase DNA Sequenzdatenbank wurden 25 Primerpaare entworfen mit denen die 25 wichtigsten β-Laktamasegene identifiziert werden konnten. Zur Doppelkontrolle wurden auch 24 bereits publizierte Primerpaare verwendet. Dreiunddreißig klinische Isolate aus Graz und 108 aus Wien wurden jeweils zweimal mit diesen 49 Primerpaaren analysiert. Die mittels PCR amplifizierten Gene wurden sequenziert und für statistische Auswertungen verwendet. Die Rarefaction Curve, Chao1 und ACE Algorithmen wurden genutzt um die Anzahl von β-Laktamasegenen in den untersuchten Spitälern abschätzen zu können. In der vorliegenden Arbeit wurde

eine große Anzahl von Genen zum ersten Mal in Österreich bzw. Europa beschrieben. Darunter befinden sich Gene aus allen vier Klassen der β-Laktamasen. Die statistischen Auswertungen ergaben, dass der Großteil der β-Laktamasegene in den klinischen Isolaten der *Enterobacteriaceae* in den untersuchten Spitälern identifiziert wurde. Die genaue Anzahl an unterschiedlichen β-Laktamase Genotypen konnte aufgrund der hohen Diversität nicht bestimmt werden.

Das zweite Ziel dieser Arbeit war die Entwicklung einer Methode mit der viele unterschiedliche Gene in einer Reaktion detektiert werden können. Die Immobilisierung der PCR auf einem Objektträger war eine naheliegende Herangehensweise bei der Primerpaare mit einem Microarrayspotter an eine unpolare Oberfläche kovalent gebunden wurden. Unterschiedliche Parameter wie Reaktionsgemisch, Annealing Temperatur, Primersequenz, Produktlänge, etc. wurde getestet. Die DNA konnte zwar in der On-Chip-PCR auf der Oberfläche vervielfältigt werden, aber es gelang nicht spezifische Reaktionsbedingungen zu bestimmen. Darauf aufbauend wurde eine Multiplex-Ligationsreaktion mit immobilisierten Sonden getestet. Ein neu entwickelter Typ von Sonde (looplock probe) und diverse Parameter wurden systematisch untersucht. Die Ligationsreaktion konnte mit den neuen Sonden auf der Objektträgeroberfläche zwar etabliert werden, aber eine weitere Optimierung der Spezifität ist notwendig.

Neben oberflächenbasierenden Methoden wurde auch ein Multiplex-Detektionsverfahren in Lösung entwickelt. Dabei wurden Padlock Sonden verwendet, die bei Vorhandensein eines Zielgenes zu zirkularen DNA Molekülen ligiert werden. Die ligierten Padlock Sonden werden in zwei aufeinanderfolgenden Rolling-Circle-Amplication-Reaktionen vervielfältigt und anschließend in einer linearen PCR fluoreszenzmarkiert. Die Detektion der markierten DNA erfolgte mit einem Microarray. Mit dieser Methode konnten knapp 100 verschiedene Zielsequenzen parallel bestimmt werden. Der Assay kann alle wichtigen β-Laktamasegene in einer Reaktion identifizieren und wurde mit klinischen Isolaten erfolgreich evaluiert. Er zeichnet sich durch eine hervorragende Spezifität und Sensitivität aus. Im Vergleich zu bestehenden Produkten am Markt kann er drei bis vier Mal mehr Gene parallel detektieren und hat ein sehr hohes Entwicklungspotential, da eine Erweiterung des Assays um eine Vielzahl von zusätzlichen Zielgenen möglich ist.

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8 Supplemental material

Supplementary Table 1. Padlock probes designed in this study. Probe names contain information of the gene target and the nucleotide position where the padlock probes anneal.

Padlock probe name	Sequence 5'-3'	Length
PL ACT1 Pos.384	AGGTTGCCAGATCCAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTACAAGACTCCAGCCTGCCAAACCTCCTGCGGTAT	80
PL ACT1 Pos.7	CCAATACCGAGCAGGGCGCGTGTATGCAGCTCCTCGAGTAGCCGACCCCCCTCTACAAGACTCCATCTGACATCGGGGTAG	81
PL ACT1 Pos.852	CCTGATACATGGCCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCATCCATCAGCGACACCCGATTTCCCAGCCTAACC	79
PL CMY1 Pos.237	AACAGGGTCTGCTCGGCGCGTGTATGCAGCTCCTCGAGTAGCCGACTTCGCAACTCGTTGTACTTCCCCACGGATCCTATCTCG	84
PL CMY1 Pos.621	GGTGTGGTGCATGCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTGCGAGTAACGTCAATCGCCTCGGCACATTGACATA	80
PL CMY1 Pos.985	AAGAGCACCTGGCTCGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCTCTCTGTGCTACCGCTCGGAGCCGGTCTTGTTG	79
PL CMY2 Pos.1057	ACGATGCCAAGGTTTGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCATGAGGCCCGAAGGTCTTGTTTGCCAGCATC	77
PL CMY2 Pos.146	AGATAACGGCAACGGGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCGTCACCCGAGAGCAAGCAGGGTTTTCCCTGGT	78
PL CMY2 Pos.424	CTAACGTCATCGGGGGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCAGCCTGCCAGTTTCGAATGAATGCGGCTTTATCC	80
PL CTX-M1 Pos.429	GGCCGCCAACGTGAGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCCATCAGGCAGATCCCCATACGGTGACGCTAGCCG	79
PL CTX-M1 Pos.243	GCGGCCATCACTTTACGCGCGTGTATGCAGCTCCTCGAGTAGCCGTGGGATTGGCTTACCGTCGCCAGCACCGCGGCC	78
PL CTX-M1 Pos.45	AACAGCGTGACGGTTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTTGACACCTTCCTCCCGACTCGGCACACTTCCTAAC	81
PL CTX-M1 Pos.682	CAGGAAGCAGGCAGTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCATCTGACTCAATCAACCGCCTGTTATCCCCCACAACC	82
PL CTX-M2 Pos.36	ATAGCAGGGGTAGCGGCGCGTGTATGCAGCTCCTCGAGTAGCCGGTCAGCCTTTACCCCACCTACTAGCGTTGCGCTGCTAA	82
PL CTX-M2 Pos.469	TCATCACCCAACGAGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCAACCAGTTTCAGATGCAATTCCCTCCAGACGGAAGGTC	84
PL CTX-M2 Pos.779	TAGGTCACCAGAACCAGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCGTGGTAACCGTCCCCCTTGTCCGGTTGGGTAAAG	81
PL CTX-M25 Pos.40	CGAAACACAGGCTGTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTGCTTTGGTCCGTAGACGTCACTGGCCAACAGCAG	80
PL CTX-M25 Pos.469	TCATCGCCAATCGTAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTCCCCTGTGCTACCGCTCGAGCCGGAACGTG	76
PL CTX-M25 Pos.722	CGTACCATAACCGCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCACCACCTTCCTCCTCCTCGCTGCGCGATATCATTCGT	79
PL CTX-M8 Pos.40	CGAAATACAGGCCGTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTCCTCCTTCAGCGTTCTACTTGCACTCCCCAGCAACAG	83
PL CTX-M8 Pos.468	TATCCCCAATCGCACGCGCGTGTATGCAGCTCCTCGAGTAGCCGGGTCCATCTGGTAGTGATGCAAGTGGAGCCGGAAGGTGT	83
PL CTX-M8 Pos.813	CGACTTTCTGCCTTCTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTCCCCTGTGCTACCGCTCGAGCCGGAACGTG	85
PL CTX-M9 Pos.496	GGTTCAGTGCGATCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGGGCCAAGGCTTATACTCGCTGGGCGGTATTCAGCGTA	81
PL CTX-M9 Pos.731	ATCATTGGTGGTGCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCGCCAAGCCACAAGGACTCCAGATCACCGCAAT	78
PL CTX-M9 Pos.76	CGTCTGCGCATAAAGGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCCTGGTTCGCCAAAAAGGCTAGGCTGCACCGCACT	80
PL DHA1 Pos.1029	GGAATAAAGGCGACATGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTCCGGTGGAAAAAGAAGCGTGCCACCTGTTTTTCC	81
PL DHA1 Pos.359	TGTGATCCCCTTCCAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCTCTTTTTCCGGTGGAGCAAGAGCCAGATCCAGCAA	80
PL DHA1 Pos.90	ATGGTGCTGTCCACCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCAGTCAACCTAGAGTGCCCAACTGCCATCAGCGGTTTA	82
PL FOX1 Pos.230	CGAACAGGGTCTGCTGCGCGTGTATGCAGCTCCTCGAGTAGCCGATGGGATTTGCATGACCTCGCGTGACCGAGCCAATCT	81
PL FOX1 Pos.825	AGAAACCGGTATGGGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCGTCTTTCACTTTTGAACCATGCTGTCTCCCACCGAGT	83

PL FOX1 Pos.988	GTTATAGAGCCGCTGCGCGCGTGTATGCAGCTCCTCGAGTAGCCGGGGTCAGAGGATGTCAAGATTTGGGTCGAGCCCGTCTT	83
PL GES1 Pos.447	ACAGAGTCGCCAATTTGCGCGTGTATGCAGCTCCTCGAGTAGCCGATCTCTAGAGGGGTCAGAGGATGTCGGTCTAGCCGACTC	84
PL GES1 Pos.50	ATTTTTCCGACGCATGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCACTCCTCTTTCCAATTGAGTGCAATCGGTCTTGAAGGTTA	86
PL GES1 Pos.609	CGATCAGCCACCTCTGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCCATGCGGCATAAACTGTTATGCCTCCCGTTTGGTTTC	83
PL IMI1 Pos.4	CTTGGTTTTACATTAAGTGAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCCGAAAGCGCCTTTCACTCTTTAAACAAGATGGCTATTCTA	91
PL IMI1 Pos.448	CCGCATGAATTTAGTCATGCGCGTGTATGCAGCTCCTCGAGTAGCCGGGACGTTCAGTTACTAACGTCCTTGAAATCTTTATCTCCAATCGA	92
PL IMI1 Pos.790	TCGTTTTTTGTAGTGTATACGCGCGTGTATGCAGCTCCTCGAGTAGCCGAACCATGCGGTTCAAACAACCATCCTCATGCTTGGCTTCTTTT	92
PL IMP1 Pos.38	CGGTAGCAATGCTGCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCCGAAGGGGACGTCCTATCTCTAGGCAAAGACTCTGCTG	84
PL IMP1 Pos.387	TTTGTGGCTTGAACCTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCGTTCGCAACTCATCCGGAGAAGAACTCCGCTAAATGAA	84
PL IMP1 Pos.619	TTTGCCTTACCATATTTGGGCGCGTGTATGCAGCTCCTCGAGTAGCCGACCGTTCGCGACTCAAGAAAACACGACTTGGAACAACCAGT	90
PL IMP24 Pos.22	CAAAGGAAGCATACACAGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCCAGACTTTCCTGACCGCCTGGCGGCAGTAATGCTA	83
PL IMP24 Pos.519	ACAAAACAACCACCGAGCGCGTGTATGCAGCTCCTCGAGTAGCCGAGCATACTATGGTTAAGCCACAGCCAGACCGTCCGGTTTA	85
PL IMP24 Pos.657	AGAGTGATGCGTCCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCTCCTCGTCTGTTCGCTCGACTTCCCATGTACGTTTCA	83
PL KPC1 Pos.270	GGATGGGTGTGTCCAGCGCGTGTATGCAGCTCCTCGAGTAGCCGGTCGTACTCCCCCAGGCGGAGGCATTTTTGCCGTAAC	81
PL KPC1 Pos.794	ATCCTTGTTAGGCGCGCGCGTGTATGCAGCTCCTCGAGTAGCCGACTTATAGATGGATCCGCGCTGCATCTCGCTGTGCTTGTC	84
PL KPC1 Pos.94	CGAGTTTAGCGAATGGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCCGCTTTCATCCGTGGATCGTATCGCCAAAGTCCTGTT	84
PL MIR1 Pos.1109	GTATGCCGCCTCAACGCGCGTGTATGCAGCTCCTCGAGTAGCCGAATCAGCCATCGGCCAACCCTAGCGTCGAGGATACG	80
PL MIR1 Pos.147	AAATTACCGCCACCGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCAGTTCGCGCCGCAGGGGTGGCTGACCCTGAT	76
PL MIR1 Pos.41	GCAGAACTGGCGACGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCGACGGATCTTTGCTCCTCGTGGTGCGGCGAAT	77
PL NDM1 Pos.123	CGTTGGTCGCCAGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCAGACCGTACGGGCCGACCAGATCGCCAAAC	74
PL NDM1 Pos.563	GTCACTGGTGTGGCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGGTAACACTCCCGTACGCTGCCTTTCCCAACGGTGATATT	83
PL NDM1 Pos.724	TCATGCTGGCCTTGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCATCCCCATCCTCCACCGATGAAATGGCTCATCACGA	80
PL OXA1 Pos.295	CTCCATTCCTTTGGGGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCGGCAGGTTATCCCACAGTAATGGTGATTGCTGTTCCAGAT	86
PL OXA1 Pos.540	GCGGCCATCACTTTACGCGCGTGTATGCAGCTCCTCGAGTAGCCGCGCCAGAGTTAAACCCCAACCCCCAGCACCGCGGCC	81
PL OXA1 Pos.852	AAGTGCGGACACAAAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCAGTCCCGCACTTTCATCTTCCGCCCCAAGTTTCCTGT	82
PL OXA2 Pos.184	GTTTCTTCGATCGCACGCGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCTACCTCCCTCTGACACACTCGAATGCAGGCGAGTAGC	84
PL OXA2 Pos.656	CCATCCTACCCACCAGCGCGTGTATGCAGCTCCTCGAGTAGCCGAGAGTTCCCGAAGGCACTCCCAGTCGGCCACTCAAC	80
PL OXA23 Pos.377	AGACTGGGACTGCAGAGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCCACTCGTCAGCAGAGCAGCGCAAGTTCCTGAT	79
PL OXA23 Pos.705	TTCCATCTGGCTGCTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCACTTTGTGAGGTCCGCTTGCTGCAAAAGCGACAATTT	82
PL OXA23 Pos.9	CCACATAGCAAGTAAAATATGCGCGTGTATGCAGCTCCTCGAGTAGCCGCGATAGTGAGAGGCCCGAAGGTCAGAAAGAAAAAGAGAAGCAA	92
PL OXA24 Pos.3	TATAGGAAGTATAAATTTTTTCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCACATCCGATGGCGTGAGGCACTAGAATAGAAATGCTGAA	91
PL OXA24 Pos.345	AAAGTCATATCTTTCTCCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCGATAGTGCAAGGTCCGAAGAGCCTGCCATTGCCTCACCT	88
PL OXA24 Pos.752	AGACATTCCTTCTTTCATTTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCAAGGGCACAACCTCCAAGTAGACAATTTCATTACGAATAGAACC	94
PL OXA48 Pos.175	GTTCGCCCGTTTAAGGCGCGTGTATGCAGCTCCTCGAGTAGCCGAAGACTCAAGCCTGCCAGTTTCGAACGGGTAAAAATGCTTG	85
PL OXA48 Pos.448	TCTACATTGCCCGAAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCTCCCCGCTGAAAGTGCTGTCGAGCCAGAAACTG	79

PL OXA48 Pos.705	TCCATATTCATCGCAAGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCCACTGCAGTTCCCAGGTTGACCGATGTGGGCATA	81
PL OXA51 Pos.439	AACACGCTTCACTTCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGATCAAAAAGATGGACCGGCCAGCGTGCATTGCCATAACC	84
PL OXA51 Pos.68	ATTTGGATTAGCAGTCAGCGCGTGTATGCAGCTCCTCGAGTAGCCGAGGCTTATACTCGCTGGCTCCGTTTTGAAGCGCTGTG	83
PL OXA51 Pos.712	ATTCCCTTGAGGCTGGCGCGTGTATGCAGCTCCTCGAGTAGCCGATCAAAAAAGATGGACCGGCCAGCAGGGAGAACGCTACAAT	85
PL OXA58 Pos.21	GCTTAAGCAAACTAAACTCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCAGAAAGATGGACCGGCCAACCACAAGCCCCAATACTTAT	88
PL OXA58 Pos.692	CCACATACCAACCCAGCGCGTGTATGCAGCTCCTCGAGTAGCCGGGTTGGGTCCAGTACGCATCAGATGCCTTTTCAACAAAAC	84
PL OXA58 Pos.788	CTAAAGACAATTGTTTACGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCAAGCCACAAGGACTTGGGGTTCCAACTTATCTAGCACAT	88
PL PER1 Pos.22	CGAGGCAGTAACTACAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCGGGTGCATTCCAGTACACGAAGATACCATCAGTAGCGT	85
PL PER1 Pos.322	ATTATCGGAGCCCAGGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCCTGGTTCGCCCGAGGGTCCCTGATACGCTTTTC	78
PL PER1 Pos.734	TTTTCCGGCTTTGATGCGCGTGTATGCAGCTCCTCGAGTAGCCGGTGCTTACCATAGGTAGACCGTCCGATTAGTGGCCGCAGT	84
PL RTG4 Pos.151	CCAAATCATGCGCAGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCATAGAAACACCGCCCGATCCCAACGTTTTCCCGTTT	81
PL RTG4 Pos.497	TGTTTCCCAGCGATCGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCCGAGCGGGTCATCATAGAAACACCCGCTTCGTTAAGTTC	85
PL RTG4 Pos.51	GATAGCGTGAGACATAAAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCAGTGAAGGCCATGAGATTCCCCTGTTGCATTAGCATTTAAT	90
PL SFO1 Pos.25	GACCATCAGGGTGGTGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCATACAGGACCACCAGGAGGTCGGCATAACCGTAGC	81
PL SFO1 Pos.587	TAGGGTTTGCAGGCTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCAAGACAGTGCACTCCGTGAGGAGCCTTGCCCAAAGT	81
PL SFO1 Pos.855	TTTAGCGGCAGCAGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCCAAATCGACACCGTTTACAGCGTCCCTTCGGTGACAAT	83
PL SHV1 Pos.12	GAGATAATACACAGGCGAGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCTAGCGATCGTCGGCTTGGTAGGGGTGGCTAACAGG	84
PL SHV1 Pos.261	GGATCTTTCGCTCCAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCAATAGTGCGAGGCCCGAAGGTCCTGCTGGCGATAGT	81
PL SHV1 Pos.734	GTTATTCGGGCCAAGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCGCCACCGAAGTGTAAACACCCAATGCGCTCTGCTTT	81
PL SME1 Pos.11	AAGCCGTTTTAAAATTTACTGCGCGTGTATGCAGCTCCTCGAGTAGCCGTGTCTCCACTAACCGCGACCGACAAACACTAAACAAAAATG	90
PL SME1 Pos.531	CGTTTATCTCCTGGGAGCGCGTGTATGCAGCTCCTCGAGTAGCCGAGGCTTTCCTCCCCAACTAAAGTGCGGCGTTGAAGTGTCA	85
PL SME1 Pos.726	GTACCTATAGCCCCACGCGCGTGTATGCAGCTCCTCGAGTAGCCGGGCACGGATCCCAAGGAAGGAACGGCATAATCATTCGCA	84
PL TEM1 Pos.457	ACATGATCCCCCATGGCGCGTGTATGCAGCTCCTCGAGTAGCCGCGAACAACGCGACAAACCACCCGATCAAGGCGAGTT	82
PL TEM1 Pos.46	AGGAAGGCAAAATGCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCGACACGGAAGCGGTGGAACCCTGGGTGAGCAAAAAC	81
PL TEM1 Pos.631	GCAACTTTATCCGCCCGCGCGTGTATGCAGCTCCTCGAGTAGCCGTCCACCCACAGAGGATGCCCTCGCAGAAGTGGTCCT	80
PL VEB1 Pos.203	TCGGAAATTTCATAACGCGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCGGATAACGCTTGCGACCTATGTACAAAACGGCTAAAGCAA	89
PL VEB1 Pos.851	TTTTGCAATGTCTGAAATAAGCGCGTGTATGCAGCTCCTCGAGTAGCCGCACCCGTCATGCGACAGGCAAATAGTAATTCCACGTTAT	88
PL VIM1 Pos.430	GTTCCCCTCTGCCTCGCGCGTGTATGCAGCTCCTCGAGTAGCCGAACACTCGCTCTCGGCCGCCAAGATGCGTGGGAATCTC	82
PL VIM1 Pos.743	CTGTGTGCTGGAGCAAGCGCGTGTATGCAGCTCCTCGAGTAGCCGAATAAAGTTGGGTGTCGGCTGGCGCTGCTTTGACAACGTTCG	87
PL VIM2 Pos.432	TCGTTCCCCTCTACCGCGCGTGTATGCAGCTCCTCGAGTAGCCGGCGCCGGCCAATCCTCTACAGAGCATGAGTGCGTGGGAATC	83
PL VIM2 Pos.687	GTGCTTCCGGGTAGTGCGCGTGTATGCAGCTCCTCGAGTAGCCGCCGTGCTTGAGGGCAGCAATGACCGGAATGACGAACT	81

Supplementary Table 2. Synthetic templates used in this study to evaluate the ligation process of padlock probes. Names of templates are corresponding to names of padlock probes.

Synthetic template name	Sequence 5' 3'	Length
tar ACT1 Pos.384	TGGATCTGGCAACCTATACCGCAGGAGGTT	30
tar ACT1 Pos.7	CCTGCTCGGTATTGGCTACCCCGATGTCAGA	31
tar ACT1 Pos.852	GGGCCATGTATCAGGGGTTAGGCTGGGAAA	30
tar CMY1 Pos.237	CGAGCAGACCCTGTTCGAGATAGGATCCGTG	31
tar CMY1 Pos.621	GCATGCACCACACCTATGTCAATGTGCCGA	30
tar CMY1 Pos.985	GAGCCAGGTGCTCTTCAACAAGACCGGCTC	30
tar CMY2 Pos.1057	AAACCTTGGCATCGTGATGCTGGCAAACAA	30
tar CMY2 Pos.146	CCGTTGCCGTTATCTACCAGGGAAAACCCT	30
tar CMY2 Pos.424	CCCCGATGACGTTAGGGATAAAGCCGCATT	30
tar CTX-M1 Pos.429	TCACGTTGGCGGCCCGGCTAGCGTCACC	28
tar CTX-M1 Pos.45	AACCGTCACGCTGTTGTTAGGAAGTGTGCCG	31
tar CTX-M1 Pos.682	ACTGCCTGCTTCCTGGGTTGTGGGGGATAA	30
tar CTX-M2 Pos.36	CGCTACCCCTGCTATTTAGCAGCGCAACG	29
tar CTX-M2 Pos.469	CTCGTTGGGTGATGAGACCTTCCGTCTGGA	30
tar CTX-M2 Pos.779	CTGGTTCTGGTGACCTACTTTACCCAACCGGA	32
tar CTX-M25 Pos.40	ACAGCCTGTGTTTCGCTGCTGTTGGCCAG	29
tar CTX-M25 Pos.469	TACGATTGGCGATGACACGTTCCGGCTC	28
tar CTX-M25 Pos.722	GGCGGTTATGGTACGACGAATGATATCGCG	30
tar CTX-M3 SNP243	GTAAAGTGATGGCCGCGGCCGCGGTGCTG	29
tar CTX-M8 Pos.40	ACGGCCTGTATTTCGCTGTTGCTGGGGAGT	30
tar CTX-M8 Pos.468	GTGCGATTGGGGATAACACCTTCCGGCTC	29
tar CTX-M8 Pos.813	AGAAGGCAGAAAGTCGTCGTGACGTACTCGC	31
tar CTX-M9 Pos.496	GGATCGCACTGAACCTACGCTGAATACCGC	30
tar CTX-M9 Pos.731	GGCACCACCAATGATATTGCGGTGATCTGG	30
tar CTX-M9 Pos.76	CTTTATGCGCAGACGAGTGCGGTGCAGC	28
tar DHA1 Pos.1029	ATGTCGCCTTTATTCCGGAAAAACAGGTGGC	31
tar DHA1 Pos.359	TGGAAGGGGATCACATTGCTGGATCTGGCT	30
tar DHA1 Pos.90	GGTGGACAGCACCATTAAACCGCTGATGGC	30
tar FOX1 Pos.230	AGCAGACCCTGTTCGAGATTGGCTCGGTCA	30
tar FOX1 Pos.825	CCCATACCGGTTTCTACTCGGTGGGAGACA	30
tar FOX1 Pos.988	GCAGCGGCTCTATAACAAGACGGGCTCGAC	30
tar GES1 Pos.447	AAATTGGCGACTCTGTGAGTCGGCTAGACCG	31
tar GES1 Pos.50	ATGCGTCGGAAAAATTAACCTTCAAGACCGAT	32
tar GES1 Pos.609	AGAGGTGGCTGATCGGAAACCAAACGGGAG	30
tar IMI1 Pos.4	TCACTTAATGTAAAACCAAGTAGAATAGCCATCTTGTTTA	40
tar IMI1 Pos.448	ATGACTAAATTCATGCGGTCGATTGGAGATAAAGATTT	38
tar IMI1 Pos.790	GTATACACTACAAAAAACGAAAAAGAAGCCAAGCATGA	38
tar IMP1 Pos.38	GCAGCATTGCTACCGCAGCAGAGTCTTTGCC	31
tar IMP1 Pos.387	AGGTTCAAGCCACAAATTCATTTAGCGGAGTT	32
tar IMP1 Pos.619	CCAAATATGGTAAGGCAAAACTGGTTGTTCCAAGTC	36
tar IMP24 Pos.22	TGTGTATGCTTCCTTTGTAGCATTACTGCC	30
tar IMP24 Pos.519	TCGGTGGTTGTTTTGTTAAACCGGACGGTCT	31
tar IMP24 Pos.657	GGGACGCATCACTCTTGAAACGTACATGGGA	31
tar KPC1 Pos.270	TGGACACACCCATCCGTTACGGCAAAAATGC	31
tar KPC1 Pos.794	GCGCCTAACAAGGATGACAAGCACAGCGAG	30
tar KPC1 Pos.94	CCATTCGCTAAACTCGAACAGGACTTTGGCG	31
tar MIR1 Pos.1109	GTTGAGGCGGCATACCGTATCCTCGACGC	29

tar MIR1 Pos.147	CGGTGGCGGTAATTTATCAGGGTCAGCCAC	30
tar MIR1 Pos.41	GTCGCCAGTTCTGCATTCGCCGCACC	26
tar NDM1 Pos.123	CTGGCGACCAACGGTTTGGCGATCTGGT	28
tar NDM1 Pos.563	GGCCACACCAGTGACAATATCACCGTTGGGA	31
tar NDM1 Pos.724	CAAGGCCAGCATGATCGTGATGAGCCATT	29
tar OXA1 Pos.295	CCCAAAGGAATGGAGATCTGGAACAGCAATCA	32
tar OXA1 Pos.540	GTAAAATTATTAATCACAATCTCCCAGTT	29
tar OXA1 Pos.852	TTTGTGTCCGCACTTACAGGAAACTTGGGG	30
tar OXA2 Pos.184	GTGCGATCGAAGAAACGCTACTCGCCTGCAT	31
tar OXA2 Pos.656	TGGTGGGTAGGATGGGTTGAGTGGCCGACT	30
tar OXA23 Pos.377	TCTGCAGTCCCAGTCTATCAGGAACTTGCGC	31
tar OXA23 Pos.705	AGCAGCCAGATGGAAAAATTGTCGCTTTTGC	31
tar OXA23 Pos.9	ATATTTTACTTGCTATGTGGTTGCTTCTCTTTTTCTTTCT	40
tar OXA24 Pos.3	GAAAAAATTTATACTTCCTATATTCAGCATTTCTATTCTAGT	42
tar OXA24 Pos.345	GGGAGAAAGATATGACTTTAGGTGAGGCAATGGCA	35
tar OXA24 Pos.752	AAATGAAAGAAGGAATGTCTGGTTCTATTCGTAATGAAAT	40
tar OXA48 Pos.175	CTTAAACGGGCGAACCAAGCATTTTTACCCG	31
tar OXA48 Pos.448	TTCGGGCAATGTAGACAGTTTCTGGCTCGAC	31
tar OXA48 Pos.705	TTGCGATGAATATGGATATGCCCACATCGG	30
tar OXA51 Pos.439	GGAAGTGAAGCGTGTTGGTTATGGCAATGCA	31
tar OXA51 Pos.68	TGACTGCTAATCCAAATCACAGCGCTTCAAAA	32
tar OXA51 Pos.712	CAGCCTCAAGGGAATATTGTAGCGTTCTCCCT	32
tar OXA58 Pos.21	GAGTTTAGTTTGCTTAAGCATAAGTATTGGGGCTTGT	37
tar OXA58 Pos.692	TGGGTTGGTATGTGGGTTTTGTTGAAAAGGCA	32
tar OXA58 Pos.788	CGTAAACAATTGTCTTTAGATGTGCTAGATAAGTTGG	37
tar PER1 Pos.22	TGTAGTTACTGCCTCGACGCTACTGATGGTATC	33
tar PER1 Pos.322	CTGGGCTCCGATAATGAAAGCGTATCAGGGA	31
tar PER1 Pos.734	ATCAAAGCCGGAAAAACTGCGGCCACTAAT	30
tar RTG4 Pos.151	TGCGCATGATTTGGAAACGGGAAAACGTT	29
tar RTG4 Pos.497	GATCGCTGGGAAACAGAACTTAACGAAGCGG	31
tar RTG4 Pos.51	TTTATGTCTCACGCTATCATTAAATGCTAATGCAACAG	38
tar SFO1 Pos.25	ACCACCCTGATGGTCGCTACGGTTATGCCG	30
tar SFO1 Pos.587	AGCCTGCAAACCCTAACTTTGGGCAAGGC	29
tar SFO1 Pos.855	CTGCTGCCGCTAAAATTGTCACCGAAGGG	29
tar SHV1 Pos.12	TCGCCTGTGTATTATCTCCCTGTTAGCCACCC	32
tar SHV1 Pos.261	TGGAGCGAAAGATCCACTATCGCCAGCAGG	30
tar SHV1 Pos.734	CTTGGCCCGAATAACAAAGCAGAGCGCATT	30
tar SME1 Pos.11	AGTAAATTTTAAAACGGCTTCATTTTTGTTTAGTGTTTGT	40
tar SME1 Pos.531	TCCCAGGAGATAAACGTGACACTTCAACGCC	31
tar SME1 Pos.726	GTGGGGCTATAGGTACTGCGAATGATTATGCCG	33
tar VEB1 Pos.203	GCGTTATGAAATTTCCGATTGCTTTAGCCGTTTTGT	36
tar VEB1 Pos.851	TTATTTCAGACATTGCAAAAATAACGTGGAATTACTATTT	40
tar VIM1 Pos.430	GAGGCAGAGGGGAACGAGATTCCCACGCAT	30
tar VIM1 Pos.743	TTGCTCCAGCACACAGCGAACGTTGTCAAAGCA	33
tar VIM2 Pos.432	GGTAGAGGGGAACGAGATTCCCACGCACTC	30
tar VIM2 Pos.687	ACTACCCGGAAGCACAGTTCGTCATTCCGG	30
tarTEM1 Pos.457	CATGGGGGATCATGTAACTCGCCTTGATCG	30
tarTEM1 Pos.46	GCATTTTGCCTTCCTGTTTTTGCTCACCCAG	31
tarTEM1 Pos.631	GGCGGATAAAGTTGCAGGACCACTTCTGCG	30

Supplementary Table 3. Synthetic templates used in this study to evaluate the ligation process of looplock probes. Names of templates are corresponding to names of looplock probes.

Synthetic template name	Sequence 5' 3'	Length
synt c.pneu. P108	GCGGAAGGGTTAGTAGTACATAGATAATCTGCCCT	35
synt c.pneu. P646	CCCAAGTCAGCATTTAAAACTATCTTTCTAGAGGATAG	38
synt c.pneu. P191	AGTGTAATTAGGCATCTAATATATATTAAAGAAGGGGATC	40
synt b.per. P737	CTGTTTGCTCCCCACACTTTCGTGCATGAGCG	32
synt b.per. P574	GTTAAGCCCTGGGATTTCACATCTTTCTTTCCGAA	35
synt b.per. P46	AGTCGGACGGCAGCACGGGCTTCGGCCT	28
synt k.king. P79	ACGGCAGCCACGAGTACTTGTACTTGGTGGC	31
synt k.king.P1008	CATGTACGGAAGAGAGTAGAGATACTCTTGTGCC	34
synt k.king. P455	TGTTAGGGAAGAAAAGGTTGATGCTAATATCATTAACTG	39
synt m.hom.P1001	CCATCTGTCACTCCGATAACCTCCACTATATCTCT	35
synt m.hom. P170a	ACAACGGAACCATGCGGTTCCATGCGTATCCG	32
synt m.hom. P170b	GGATACGCATGGAACCGCATGGTTCCGTTGTG	32

Supplementary Table 4. Microarray oligonucleotides used in this study and their corresponding padlock probes.

Name	Sequence 5' 3'	Padlock target	Length
Bsrev	TTTTTTTTTTTTAAAACGACGGCCAGTGAGC	Hybridization control	21
ecl6	TTTTTTTTTTCTACAAGACTCCAGCCTGCCA	PL ACT1 Pos.384	21
ecl7	TTTTTTTTTTTACCCCCCTCTACAAGACTCCA	PL ACT1 Pos.7	22
efa2	TTTTTTTTTTCATCCATCAGCGACACCCGA	PL ACT1 Pos.852	20
efa3	TTTTTTTTTTACTTCGCAACTCGTTGTACTTCCC	PL CMY1 Pos.237	24
ena4	TTTTTTTTTTTCTGCGAGTAACGTCAATCGCC	PL CMY1 Pos.621	22
kpn1	TTTTTTTTTTGCTCTCTGTGCTACCGCTCG	PL CMY1 Pos.985	20
kpn2	TTTTTTTTTTGCATGAGGCCCGAAGGTC	PL CMY2 Pos.1057	18
klo1	TTTTTTTTTTTCGTCACCCGAGAGCAAGC	PL CMY2 Pos.146	19
klo2	TTTTTTTTTTCCAGCCTGCCAGTTTCGAATG	PL CMY2 Pos.424	21
mom2	TTTTTTTTTTGCCATCAGGCAGATCCCCATAC	PL CTX-M1 Pos.429	22
mom3	TTTTTTTTTTCTTGACACCTTCCTCCCGACT	PL CTX-M1 Pos.45	21
mom4	TTTTTTTTTTCATCTGACTCAATCAACCGCCTG	PL CTX-M1 Pos.682	23
pmi3	TTTTTTTTTTGTCAGCCTTTACCCCACCTACTAG	PL CTX-M2 Pos.36	24
pmi5	TTTTTTTTTTCCAACCAGTTTCAGATGCAATTCCC	PL CTX-M2 Pos.469	25
psa6	TTTTTTTTTTCGTGGTAACCGTCCCCCTTG	PL CTX-M2 Pos.779	20
pvu2	TTTTTTTTTTCTGCTTTGGTCCGTAGACGTCA	PL CTX-M25 Pos.40	22
sem1	TTTTTTTTTTCTCCCCTGTGCTACCGCTC	PL CTX-M25 Pos.469	19
sem2	TTTTTTTTTTCACCACCTTCCTCCTCGCTG	PL CTX-M25 Pos.722	20
sma3	TTTTTTTTTTTGGGATTGGCTTACCGTCGC	PL CTX-M3 SNP243	20
spn1	TTTTTTTTTTCTCCTCCTTCAGCGTTCTACTTGC	PL CTX-M8 Pos.40	24
spn3	TTTTTTTTTTGGTCCATCTGGTAGTGATGCAAGTG	PL CTX-M8 Pos.468	25
spy2	TTTTTTTTTTCTGGTTAGTTACCGTCACTTGGTGG	PL CTX-M8 Pos.813	25
cal1	TTTTTTTTTTGGCCAAGGCTTATACTCGCTGG	PL CTX-M9 Pos.496	22
cgl1	TTTTTTTTTTCCGCCAAGCCACAAGGACT	PL CTX-M9 Pos.731	19
cpa1	TTTTTTTTTTTCCTGGTTCGCCAAAAAGGCTA	PL CTX-M9 Pos.76	22
efa51	TTTTTTTTTTCTCCGGTGGAAAAAGAAGCGT	PL DHA1 Pos.1029	21
efa52	TTTTTTTTTTCTCTTTTTCCGGTGGAGCAAG	PL DHA1 Pos.359	21
sta2	TTTTTTTTTTCAGTCAACCTAGAGTGCCCAACT	PL DHA1 Pos.90	23
sta4	TTTTTTTTTTATGGGATTTGCATGACCTCGCG	PL FOX1 Pos.230	22
sar1	TTTTTTTTTTCCGTCTTTCACTTTTGAACCATGC	PL FOX1 Pos.825	24
sep2	TTTTTTTTTTGGGTCAGAGGATGTCAAGATTTGG	PL FOX1 Pos.988	24
sep3	TTTTTTTTTTATCTCTAGAGGGGTCAGAGGATGT	PL GES1 Pos.447	24
efc1	TTTTTTTTTTCCACTCCTCTTTCCAATTGAGTGCA	PL GES1 Pos.50	25
efc2	TTTTTTTTTTGCCATGCGGCATAAACTGTTATGC	PL GES1 Pos.609	24
efc3	TTTTTTTTTTCCCGAAAGCGCCTTTCACTCTT	PL IMI1 Pos.4	22
efc4	TTTTTTTTTTGGACGTTCAGTTACTAACGTCCTTG	PL IMI1 Pos.448	25
bsu2	TTTTTTTTTTAACCATGCGGTTCAAACAACCATCC	PL IMI1 Pos.790	25
bsu4	TTTTTTTTTTCCCGAAGGGGACGTCCTATCTCTA	PL IMP1 Pos.38	24
smi1	TTTTTTTTTTCGTTCGCAACTCATCCGGAGAAG	PL IMP1 Pos.387	23
smu1	TTTTTTTTTTACCCGTTCGCGACTCAAGAAAACAC	PL IMP1 Pos.619	25
smu3	TTTTTTTTTTTCCAGACTTTCCTGACCGCCTG	PL IMP24 Pos.22	22
ssa2	TTTTTTTTTTAGCATACTATGGTTAAGCCACAGCC	PL IMP24 Pos.519	25
sca1	TTTTTTTTTTGCTCCTCGTCTGTTCGCTCGACT	PL IMP24 Pos.657	23
sap3	TTTTTTTTTTGTCGTACTCCCCCAGGCGGAG	PL KPC1 Pos.270	21
sau1	TTTTTTTTTTACTTATAGATGGATCCGCGCTGCAT	PL KPC1 Pos.794	25
buc1	TTTTTTTTTTCCCGCTTTCATCCGTGGATCGTAT	PL KPC1 Pos.94	24

buc2	TTTTTTTTTTAATCAGCCATCGGCCAACCCTA	PL MIR1 Pos.1109	22
pbu2	TTTTTTTTTTCAGTTCGCGCCGCAGGG	PL MIR1 Pos.147	17
pbu1	TTTTTTTTTTCCGACGGATCTTTGCTCCTCGT	PL MIR1 Pos.41	22
pbu3	TTTTTTTTTTCCAGACCGTACGGGCCG	PL NDM1 Pos.123	17
pin3	TTTTTTTTTTGTAACACTCCCGTACGCTGCCTT	PL NDM1 Pos.563	23
pin1	TTTTTTTTTTCATCCCCATCCTCCACCGATGA	PL NDM1 Pos.724	22
pme1	TTTTTTTTTTTCGGCAGGTTATCCCACAGTAATGG	PL OXA1 Pos.295	25
hin3	TTTTTTTTTTCGCCAGAGTTAAACCCCAACCCC	PL OXA1 Pos.540	23
hin1	TTTTTTTTTTCAGTCCCGCACTTTCATCTTCCG	PL OXA1 Pos.852	23
nme3	TTTTTTTTTTTCTACCTCCCTCTGACACACTCGA	PL OXA2 Pos.184	24
esa3	TTTTTTTTTTAGAGTTCCCGAAGGCACTCCC	PL OXA2 Pos.656	21
esa1	TTTTTTTTTTGCCACTCGTCAGCAGAGCA	PL OXA23 Pos.377	19
pag3	TTTTTTTTTTCACTTTGTGAGGTCCGCTTGCT	PL OXA23 Pos.705	22
pag1	TTTTTTTTTTCGATAGTGAGAGGCCCGAAGGTC	PL OXA23 Pos.9	23
yen1	TTTTTTTTTTCACATCCGATGGCGTGAGGC	PL OXA24 Pos.3	20
pmi11	TTTTTTTTTTCGATAGTGCAAGGTCCGAAGAGCC	PL OXA24 Pos.345	24
sen2	TTTTTTTTTTCAAGGGCACAACCTCCAAGTAGACA	PL OXA24 Pos.752	25
sen1	TTTTTTTTTTAAGACTCAAGCCTGCCAGTTTCGAA	PL OXA48 Pos.175	25
kpn11	TTTTTTTTTTCCTCCCCGCTGAAAGTGCT	PL OXA48 Pos.448	19
sma11	TTTTTTTTTTTCCACTGCAGTTCCCAGGTTGA	PL OXA48 Pos.705	22
calx1	TTTTTTTTTTATCAAAAAGATGGACCGGCCAGC	PL OXA51 Pos.439	23
calx3	TTTTTTTTTTAGGCTTATACTCGCTGGCTCCG	PL OXA51 Pos.68	22
cpax2	TTTTTTTTTTATCAAAAAAGATGGACCGGCCAGC	PL OXA51 Pos.712	24
ctrx1	TTTTTTTTTTCAGAAAGATGGACCGGCCAACC	PL OXA58 Pos.21	22
ctrx2	TTTTTTTTTTGGTTGGGTCCAGTACGCATCAGA	PL OXA58 Pos.692	23
cglx2	TTTTTTTTTTCAAGCCACAAGGACTTGGGGTT	PL OXA58 Pos.788	22
cglx1	TTTTTTTTTTCCGGGTGCATTCCAGTACACGAA	PL PER1 Pos.22	23
iorx2	TTTTTTTTTTTCCTGGTTCGCCCGAGGG	PL PER1 Pos.322	18
iorx1	TTTTTTTTTTGTGCTTACCATAGGTAGACCGTCCG	PL PER1 Pos.734	25
aspx1	TTTTTTTTTTTCATAGAAACACCGCCCGATCCC	PL RTG4 Pos.151	23
aspx2	TTTTTTTTTTGCCGAGCGGGTCATCATAGAAACAC	PL RTG4 Pos.497	25
anix1	TTTTTTTTTTCCAGTGAAGGCCATGAGATTCCC	PL RTG4 Pos.51	23
fnex2	TTTTTTTTTTGCATACAGGACCACCAGGAGGT	PL SFO1 Pos.25	22
fnex1	TTTTTTTTTTCAAGACAGTGCACTCCGTGAGGA	PL SFO1 Pos.587	23
aac2	TTTTTTTTTTCCCAAATCGACACCGTTTACAGCGT	PL SFO1 Pos.855	25
aac1	TTTTTTTTTTGCTAGCGATCGTCGGCTTGGTAG	PL SHV1 Pos.12	23
bor1	TTTTTTTTTTCAATAGTGCGAGGCCCGAAGGT	PL SHV1 Pos.261	22
bru1	TTTTTTTTTTCGCCACCGAAGTGTAAACACCC	PL SHV1 Pos.734	22
bru2	TTTTTTTTTTTGTCTCCACTAACCGCGACCG	PL SME1 Pos.11	21
fra1	TTTTTTTTTTAGGCTTTCCTCCCCAACTAAAGTGC	PL SME1 Pos.531	25
myc2	TTTTTTTTTTGGCACGGATCCCAAGGAAGGAA	PL SME1 Pos.726	22
myc1	TTTTTTTTTTCACGAACAACGCGACAAACCACC	PL TEM1 Pos.457	23
twh2	TTTTTTTTTTCGACACGGAAGCGGTGGAACC	PL TEM1 Pos.46	21
twh1	TTTTTTTTTTTCCACCCACAGAGGATGCCCT	PL TEM1 Pos.631	21
myc2	TTTTTTTTTTCCGGATAACGCTTGCGACCTATGT	PL VEB1 Pos.203	24
mfl1	TTTTTTTTTTCACCCGTCATGCGACAGGC	PL VEB1 Pos.851	19
ckru1	TTTTTTTTTTAACACTCGCTCTCGGCCGCCAAG	PL VIM1 Pos.430	23
afum1	TTTTTTTTTTAATAAAGTTGGGTGTCGGCTGGCGC	PL VIM1 Pos.743	25
anig1	TTTTTTTTTTGCGCCGGCCAATCCTACAGAGCAT	PL VIM2 Pos.432	24
afum2	TTTTTTTTTTCCGTGCTTGAGGGCAGCAATGA	PL VIM2 Pos.687	22
aba1	TTTTTTTTTTCAAGCTACCTTCCCCCGCT		19

aba2	TTTTTTTTTTGTAACGTCCACTATCTCTAGGTATTAACTAAAGTAG	36
aba4	TTTTTTTTTTGCAGTATCCTTAAAGTTCCCATCCGAAAT	29
ajo2	TTTTTTTTTTTCCCAGTATCGAATGCAATTCCTAAGTT	28
ajo3	TTTTTTTTTTGAAAGTTCTTACTATGTCAAGACCAGGTAAG	31
ajo4	TTTTTTTTTTCTTAACCCGCTGGCAAATAAGGAAAA	26
alw1	TTTTTTTTTTGAGATGTTGTCCCCCACTAATAGGC	25
alw2	TTTTTTTTTTTGACTTAATTGGCCACCTACGCG	23
alw3	TTTTTTTTTTCCCATACTCTAGCCAACCAGTATCG	25
ara1	TTTTTTTTTTCGCTGAATCCAGTAGCAAGCTAC	23
ara2	TTTTTTTTTTGTCCACTATCCTAAAGTATTAATCTAGGTAGCCT	34
ara3	TTTTTTTTTTCCGAAGTGCTGGCAAATAAGGAAA	24
cif1	TTTTTTTTTTGCTCCTCTGCTACCGTTCG	19
cif2	TTTTTTTTTTCCACAACGCCTTCCTCCTCG	20
cif3	TTTTTTTTTTTCTGCGAGTAACGTCAATCGCTG	23
cik1	TTTTTTTTTTCGGGTAACGTCAATTGCTGTGG	22
cik2	TTTTTTTTTTCGAGACTCAAGCCTGCCAGTAT	22
ecl4	TTTTTTTTTTGCGGGTAACGTCAATTGCTGC	21
efa1	TTTTTTTTTTCAAGCTCCGGTGGAAAAAGAAGC	23
ena2	TTTTTTTTTTGGTTATTAACCTTAACGCCTTCCTCCT	27
ena3	TTTTTTTTTTCAATCGCCAAGGTTATTAACCTTAACGC	28
pmi4	TTTTTTTTTTGGGTATTAACCTTATCACCTTCCTCCC	27
pmi6	TTTTTTTTTTGTTCAAGACCACAACCTCTAAATCGAC	27
psa4	TTTTTTTTTTGATTTCACATCCAACTTGCTGAACCA	26
psa5	TTTTTTTTTTTCTCCTTAGAGTGCCCACCCG	21
pvu4	TTTTTTTTTTTTCCCGAAGGCACTCCTCTATCTCTA	26
sem3	TTTTTTTTTTGAGTAACGTCAATTGATGAGCGTATTAAGC	30
sma1	TTTTTTTTTTAGCTGCCTTCGCCATGGATGTTC	23
spn5	TTTTTTTTTTTCTTGCACTCAAGTTAAACAGTTTCCAAAG	30
spy1	TTTTTTTTTTATTACTAACATGCGTTAGTCTCTCTTATGCG	31
spy3	TTTTTTTTTTTTCTCCAGTTTCCAAAGCGTACATTG	26
efa42	TTTTTTTTTTCCGTCAAGGGATGAACAGTTACTCTCATCCTTGTTCTTC	39
efa43	TTTTTTTTTTATTAGCTTAGCCTCGCGACTTCGCAACTCGTTGTACTTC	39
sta1	TTTTTTTTTTCTCTATCTCTAGAGCGGTCAAAGGAT	26
sta3	TTTTTTTTTTAGCTGCCCTTTGTATTGTCCATT	23
sar2	TTTTTTTTTTAGCTAATGCAGCGCGGATC	19
sar3	TTTTTTTTTTTGCACAGTTACTTACACATATGTTCTT	27
sep1	TTTTTTTTTTAAGGGGAAAACTCTATCTCTAGAGGG	26
eco2	TTTTTTTTTTGTAACGTCAATGAGCAAAGGTATTAACTTTACTCCCTTCC	40
eco3	TTTTTTTTTTCCGAAGGCACATTCTCATCTCTGAAAACTTCCGTGGATG	39
bsu7	TTTTTTTTTTTGCACTCAAGTTCCCCAGTTTCCAATGA	28
bat1	TTTTTTTTTTCTATTTGAACGGCACTTGTTCTTCCCTAACA	31
bat2	TTTTTTTTTTCCCTATTTGAACGGCACTTGTTCTTCCC	28
cko5	TTTTTTTTTTACTCAAGCCTGCCAGTATCAGATGCA	26
cfr5	TTTTTTTTTTCAGTTTCGGATGCAGTTCCCAGGTTG	26
cpe3	TTTTTTTTTTTCCTCGATTAAGAGTAATGCAAGGGATGTCA	31
cpe1	TTTTTTTTTTTGCTCCTTTGGTTGAATGATGATGCCATCTTTC	33
cpe2	TTTTTTTTTTACCGTCATTATCTTCCCCAAAGACAGAGC	29
cdi2	TTTTTTTTTTGTCCATCCTGTACTGGCTCACCTTTGAT	28
cdi3	TTTTTTTTTTTCCGATTAAGGAGATGTCATTGGGATGTCA	30
cdi1	TTTTTTTTTTTGCCTCTCAAATATATTATCCCGTATTAGCATACCT	36
lmo1	TTTTTTTTTTGCCGAAACCATCTTTCAAAAGCGTGG	26

lmo3	TTTTTTTTTTCTTCGCGACCCTTTGTACTATCCATTGTAG	30
lmo2	TTTTTTTTTTGGTTAGATACCGTCAAGGGACAAGCAG	28
sag2	TTTTTTTTTTCCACTCCTACCAACGTTCTTCTCTAACA	29
sag1	TTTTTTTTTCCGTTCGCAACTCATCAGTCTAGTGTAAAC	30
sag3	TTTTTTTTTTCACTTCTGCTCCGAAGAGAAAGCCTAT	28
sdy3	TTTTTTTTTCGAAGGGAAAGCCTATCTCTAGACCGG	27
sdy1	TTTTTTTTTTCTGGTTAGTTACCGTCACATGGTGGATT	29
sdy2	TTTTTTTTTCTCCAGTTTCCAAAGCGTACAATGGTTGA	29
smi2	TTTTTTTTTCTGGTAGTGATGCAATTGCACCTTTTAAGC	30
smi3	TTTTTTTTTTTCTGGTAAGATACCGTCACAGTGTGAACT	30
smu2	TTTTTTTTTTGCGCTTGCATCTTTCAATCAATTATCATGCA	32
ssa3	TTTTTTTTTACTCAAGTTAAACAGTTTCCAAAGCATACT	30
ssa1	TTTTTTTTTCCATCTGGTAGTGATGCAATTGCATCTTTCAATT	34
sca2	TTTTTTTTTCGCTAACGTCAGAGGAGCAAGCTC	24
sha1	TTTTTTTTTAGTGATAGCAAAACCATCTTTCACTATCGAACCA	34
sha2	TTTTTTTTTACACGTATGTTCTTCCCTAATAACAGAGTTTTACGA	36
sha3	TTTTTTTTTTCAAGACGTGCATAGTTACTTACACGTATGTTCTTC	36
sho1	TTTTTTTTTGAAGTTATCCCAGTCTTATAGGTAGGTTACCTACGT	36
sho3	TTTTTTTTTGCAGGTCCATCTATAAGTGATAGCAGAGCC	30
sho2	TTTTTTTTTATGCGGTTCAACATATTATCCGGTATTAGCTCC	33
slu2	TTTTTTTTTACGTTTGTTCTTCCCTAATAACAGAGTTTTACGATCCTAAGA	42
slu1	TTTTTTTTTAGTGATAGCAAAACCATCTTTCACTATTGAACCA	34
sap1	TTTTTTTTTTGCGGTTCYAAATGTTATCCGGTATTAGCTCC	32
swa1	TTTTTTTTTACAGCAAAGCCGCCTTTCACTATTGA	26
swa2	TTTTTTTTTGATCAGGTACCGTCAAGATGTGCACA	26
sxy1	TTTTTTTTTGCGGTTCTAAATGTTATCCGGTATTAGCTCC	31
sxy3	TTTTTTTTTCCTAATAACAGAGTTTTACGAGCCGAAACCC	31
sxy2	TTTTTTTTTTCACTTTAGAACCATGCGGTTCCAAATGT	29
sau2	TTTTTTTTTTATGTGCACAGTTACTTACACATATGTTCTTCCCTAAT	37
ste1	TTTTTTTTTAAGTGACAGCAAAACCGTCTTTCACTATTGA	31
ste3	TTTTTTTTTCTATCTCTAGAGGGGTCAGAGGATGTCAAGA	31
ste2	TTTTTTTTTTGATTAGGTACCGTCAAGACGTGCATAGT	29
bce2	TTTTTTTTTACTGAATGATGGCAACTAAGATCAAGGGTT	30
bce1	TTTTTTTTTAGGTGCCAGCTTATTCAACTAGCACT	26
buc3	TTTTTTTTTGGGGATTTCACATCGGTCTTAGCAAACC	28
pin2	TTTTTTTTTTACTCCCCAACAAAAGCAGTTTACAACCC	29
pme2	TTTTTTTTTATCCATTACCGATAAATCTTTACTTCAAATCTGATGCCG	39
pme3	TTTTTTTTTTGAGCGTCTACATTTCACAACACACTTAATCTC	33
hin2	TTTTTTTTTTCGGTGCTTCTTCTGTATTTAACGTCAATTTGATGT	35
nme1	TTTTTTTTTCGATCAGTTATCCCCCACTACTCGGT	26
nme2	TTTTTTTTTTATTAGCAACAGCCTTTTCTTCCCTGACA	28
bfr2	TTTTTTTTTACATACAAAACAGTATACATACTGCACTTTATTCT	35
bfr3	TTTTTTTTTTTTCCACATCATTCCACTGCAATTTAAGCCC	30
bfr1	TTTTTTTTTACCTCACCAACAACCTAATGGAACGC	26
cje1	TTTTTTTTTACACCGAAAAACTTTCCCTACTCAACTTGT	30
cje2	TTTTTTTTTCCCAACAACTAGTGTACATCGTTTAGGGC	29
cje3	TTTTTTTTTAGCACCCTCTTATCTCTAAAAGGTTCTTAGGA	32
cam1	TTTTTTTTTTCAAGGGCACAACCTCCAAATAGACA	26
cam2	TTTTTTTTTCTGCCAAGTTCTGTGGATGTCAAGACC	27
esa2	TTTTTTTTTGCAGGATTCTCTGGATGTCAAGACCA	26
pag2	TTTTTTTTTGCCATTACCCCGCCTACTAGCT	22

yen3	TTTTTTTTTTACAGACTTTATGTGGTCCGCTTGCTC	26
yen2	TTTTTTTTTTTGCTTCTTCTGCGAGTAACGTCAATCCA	28
yps2	TTTTTTTTTTGCGTATTAAACTCAACCCCTTCCTCCT	27
yps1	TTTTTTTTTTCCCTAAGGTCCCCCACTTTGCT	22
aca2	TTTTTTTTTTGCACCTCAGCGTCAGTGTTAGGC	23
aca1	TTTTTTTTTTTCTCCCACACTCTAGCTAACCAGTATCG	28
aca3	TTTTTTTTTTGGAAAGTTCTTACTATGTCAAGGCCAGGT	29
eik2	TTTTTTTTTTATTTCACATCCTGCTTAAATAACCGTCTGCG	31
eik3	TTTTTTTTTTACTCTAGCTATCCAGTTCAGAACGCAGT	28
eik1	TTTTTTTTTTACCCCACCAACTAGCTAATCAGTTATCG	28
pmi12	TTTTTTTTTTAGGGTATTAACCTTATCACCTTCCTCCCC	29
pvu11	TTTTTTTTTTCGTAGACGTCATGCGGTATTAGCYACC	27
pvu12	TTTTTTTTTTCATATGGGTTCATCCGATAGCGCAAG	26
aba12	TTTTTTTTTTTCCTCTCCCATACTCTAGCTCACCA	25
aba11	TTTTTTTTTTACATCCGACTTAATAAGCCGCCTACG	26
eco11	TTTTTTTTTTAGACTCAAGCTTGCCAGTATCAGATGCA	28
psa11	TTTTTTTTTTCCCACTTTCTCCCTCAGGACGT	22
calx2	TTTTTTTTTTTCAAAGTAAAAGTCCTGGTTCGCCATAAATGG	32
cpax3	TTTTTTTTTTGCTGGGTCCAGTACGCATCAAAAAAGAT	28
cpax4	TTTTTTTTTTAGTAAAAGTCCTGGTTCGCCAAAAAGG	27
car2	TTTTTTTTTTGTCAGTATCGACCCAGAAAGCTGCC	25
car1	TTTTTTTTTTTCACTGCTACACTGGAAATTCCGCTTT	27
eik2	TTTTTTTTTTGCTATCCAGTTCAGAACGCAGTTCCC	26
eik1	TTTTTTTTTTCAACTAGCTAATCAGTTATCGGCCGCT	27
kin1	TTTTTTTTTTTCTTCCCTAACAAAAGTCCTTTACAACCCGA	31
kin2	TTTTTTTTTTGGTGCTTATTCTTTAGGTACCGTCATCAGTTAATGA	36
bor2	TTTTTTTTTTGCTTATTCTGCAGGTACCGTCAGTTGC	27
bar1	TTTTTTTTTTGGGATTTCACCTCTGACTTAAATATCCGCCTAC	33
bar2	TTTTTTTTTTATTTTACCTCTACACTCGGAATTCCACTCACC	32
fra2	TTTTTTTTTTCGCTACACCAGAAATTCCCCATTCCT	26
cbu1	TTTTTTTTTTACGTCAACGCCCAAGGATATTACCCT	26
cbu2	TTTTTTTTTTCAGGGCTTTCACATCCGACTTAAATATCCAC	31
myc1	TTTTTTTTTTGCTCCATCAAGCTTTCGCTCATTGTG	26
mpn1	TTTTTTTTTTCAATCTTAAAGACCTTCATCGTTCACGCGG	30
mpn2	TTTTTTTTTTTCGTCACTTATTCAAAATGGTACAGTCAAACTCTAGC	37
leg2	TTTTTTTTTTCACTACCCTCTCCCATACTCGAGTCAA	27
leg1	TTTTTTTTTTCTTCAAGGCATATTCCTACGCGTTACTCAC	30
cpn1	TTTTTTTTTTGGTTGAGCCCCAAAATTTAACATCTAACTTTCCT	34
cpn2	TTTTTTTTTTTCCTCTAGAAAGATAGTTTTAAATGCTGACTTGGGGT	37
cps1	TTTTTTTTTTAGCCAATCTCTCTTATTCCCAAGCGAAAGT	30
cps2	TTTTTTTTTTGTTGAGCCCCAAGATTTAACATCTAACTTTCCT	33
atu1	TTTTTTTTTTCGGTATTAATTCCAGTTTCCCGGAGCTATTCC	32
atu2	TTTTTTTTTTACCCCGAATGTCAAGAGCTGGTAAG	25
mfl2	TTTTTTTTTTCGGTCGAAAACATGTCAAGCCTTGGT	26
ric2	TTTTTTTTTTAGGAATTCCATCATCCCCTACTACACTCT	29
ric1	TTTTTTTTTTGGGGCTTTTTCTGCAAGTAACGTCATTATCT	31
calb1	TTTTTTTTTTTTGAAGATATACGTGGTAGACGCTACCGCCGCA	33
ctro1	TTTTTTTTTTTATGAAATAAATTGTGGTGGCCACTAGCAAAATAAGCGTT	40
cpar1	TTTTTTTTTTTTGAGGTCGAATTTGGAAGAAGTTTTGGAGTTTGTAC	37
cgla1	TTTTTTTTTTACTTATCCCTCCCTAGATCAACACCGAGTTGGTAAAACCTA	41
afla1	TTTTTTTTTTGGTCAACCTGGAAAAAGATTGATTTGCGTTCGGC	34

cneo1	TTTTTTTTTTGAGATGGTTGTTATCAGCAAGCCGAAGACTACCCCATAGG	40
uniF1	TTTTTTTTTTTTAAGTTCAGCGGGTATYCCTACCTGAT	28
ctro2	TTTTTTTTTTTTGGATAAACCTAAGTCGCTTAAAATAAGTTTCCACGTT	39
cpar2	TTTTTTTTTTACCTATCCATTAGTTTATACTCCGCCTTTCTTTCAAGC	38
ckru2	TTTTTTTTTTCTAGTTCGCTCGGCCAGCTTCG	22
cgla2	TTTTTTTTTTACTCAAAAACGAGAGTATCACTCACTACCAAACACA	36
afla2	TTTTTTTTTTCGATGATTCACGGAATTCTGCAATTCACACTAGTT	35
anig2	TTTTTTTTTTACCTGGAAAAAATGGTTGGAAAACGTCGG	29
cneo2	TTTTTTTTTTGGTAATCACCTTCCCACTAACACATTTAAGGCG	33
calz1	TTTTTTTTTTACCAAGTTTGACCAGCTTCTCGGTT	25
cpaz1	TTTTTTTTTTACTAGCTTCTCGGTTCCAAGATGGAGTT	28
ctrz1	TTTTTTTTTTTCTCGGTTCCAGAATGGAGTTGCC	24
cglz2	TTTTTTTTTTTGAAGTGGAGTCGCCCCCTCTT	22
cglz1	TTTTTTTTTTCGGCCAAGGTTAGACTCGCTGG	22
ckrz1	TTTTTTTTTTCGGGCCTCTCGACCAAGGTT	20
ckrz2	TTTTTTTTTTAGATTCTCGGCCCCATGGGAAAGT	24
afuz1	TTTTTTTTTTCTCTGGGGAGTCGTTGCCAACTC	23
aflz2	TTTTTTTTTTCCTCCTGGGCCAGTCCGA	18
aflz1	TTTTTTTTTTCAAACGCGGACCGGGCTATTT	21
aniz1	TTTTTTTTTTGCCAACCCTCCTGAGCCAGT	20
fnez2	TTTTTTTTTTAACGCCAATCCGGAGATCTCACTAAGC	27
fnez1	TTTTTTTTTTCTGGTGACTCACGCTTACTAGGTATTCCT	29
FW45	TTTTTTTTTTCGYTCGACTTGCATGTRTTARGC	23

9 Acknowledgments

I want to thank my supervisor at the Medical University Vienna Prof. Tim Skern for giving me the freedom to conduct my research in such a wide scientific field. Also my supervisor at the Austrian Institute of Technology Herbert Wiesinger-Mayr allowed me to realize my ideas, which is why I am deeply grateful to him. During the whole time, both supervisors gave me the feeling that our professional relationships are based on friendship.

Special thank belongs to all my colleagues in the laboratory and the office. They helped with advises, smiles, jokes and sweets when failed experiments were becoming frustrating or the feeling of being sabotaged spread. It was very nice and amusing to work with all of you.

I want to single out my former boss Christa Nöhammer who was permanently trying hard to resolve problematic issues and help when it was necessary. Without her, all the research presented in this thesis would not have been conducted.

The brightest star in my life, my wife Elli, was the biggest support. She was understanding when I had the chance to conduct parts of my research in Sweden and always enthusiastic when positive results were obtained. Volim te više nego ikad.

And of course my family has to be mentioned. My brothers, father and mother helped to cultivate an intellectual culture that strongly facilitated to work as scientist. The importance of an open mind, the permanent quest for truth, moral integrity, and love were chosen by my parents as pillars for our education. I hope their effort is partly reflected in this work.

10 Curriculum Vitae

Personal information

Name	Ivan Barišić
Birth date	May 24, 1982
Birth place	Zagreb, Croatia
Nationality	Austria

Education

1988 – 1992	Elementary school, Vienna
1992 – 2000	Gymnasium, Vienna
2000 – 2001	Biology, University of Vienna
2001 – 2008	Molecular biology, University of Vienna

Scientific experience

4/2009 – **Doctoral thesis at the Austrian Institute of Technology (Herbert Wiesinger-Mayr)**
Seibersdorf (Austria), Antibiotic resistance mechanisms and an innovative detection method

2007 – 2008 **Development aid instead of military service, Fundación Casa de los Tres Mundos**
Granada (Nicaragua), Technical assistance in agricultural project and optimization of chemical processes for paper production in local manufacture

2005 – 2006 **Diploma thesis at the department of microbial ecology (Michael Wagner)**
Vienna, Phylogenetical analyses, distribution and activity of sulphate reducing prokaryotes in arctic marine sediments

01/2011 – 03/2011 **Collaboration with Uppsala University (Mats Nilsson)**
Uppsala (Sweden), Padlock probe detection of antibiotic resistance genes

05/2005 **Internship at the Max F. Perutz Laboratories (Alisher Touraev)**
Vienna, Research plant biology; training various experimental techniques

10/2004 – **Internship at the *Botanical institute* (Peter Schlögelhofer)**

12/2004 *Vienna*, Research plant genetics; goal was to identify genes involved in sexual reproduction

Further education

2002 – 2005 Political science (without degree), University of Vienna

Languages

Croatian	-	Mother tongue
German	-	Expert
English	-	Fluent
Spanish	-	Fluent
French	-	Basic

Posters

Antibiotic resistance mechanisms and development of an innovative detection method
Barišić, I.; Schönthaler, S.; Noehammer, C.; Wiesinger-Mayr, H.
Presented at DECHEMA in Frankfurt

Extended-spectrum beta-lactamases and their detection applying on-chip PCR
Barišić, I.; Schönthaler, S.; Noehammer, C.; Wiesinger-Mayr, H.
Presented at ECCMID in Vienna

Detection of extended spectrum beta-lactamases with on-chip PCR
Barišić, I.; Schönthaler, S.; Noehammer, C.; Wiesinger-Mayr, H.
Presented at ÖGMBT meeting in Vienna