Searching for carbapenemases in zoo animals

Degree Project in Medicine

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Gothenburg, Sweden 2018

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1 Abstract

Searching for novel carbapenemases in zoo animals

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2018, Institute of Biomedicine, Gothenburg, Sweden

1.1 Introduction

Carbapenems, a class of “last resort” antibiotics, are threatened by resistant pathogens producing carbapenemases. There is increasing evidence that the environmental/commensal bacteria act as a source of antibiotic resistance genes that can be picked up by pathogens. Sampling animal microbiota could increase the probability of finding genes that have the potential to migrate the pathogens.

1.2 Aim

To screen for novel carbapenemases in the stool of animals never purposely exposed to carbapenems.

1.3 Methods and results

By inoculating fecal samples from 43 zoo animals in meropenem-supplemented broths, 31 meropenem-resistant strains were isolated; the majority could be identified as opportunistic bacteria by MALDI-TOF. Seventeen isolates were positive in Carba NP and imipenem-EDTA synergy tests, indicating carriage of genes encoding carbapenemases of the metallo-β-lactamase (MBL) class. Total DNA was isolated from meropenem-supplemented broths with visible growth and sequenced. Through newly developed hidden Markov models, 19 novel MBLs were found, 13 novel B1 and 6 novel B3 genes, by screening the sequenced DNA.

PCR could link 6 novel B1 and 3 novel B3 MBLs to specific isolates. Samples was also pooled into five groups according to their animal origin, 11 novel B1s and 4 novel B3s could be linked to one or more of these animal groups.

1.4 Conclusion/implications

MBLs were readily detectable in the gut flora of the sampled zoo animals, including previously unknown ones, even though relatively selective methodology was applied. Their presence in gut commensals/opportunists should reduce some barriers for transfer to pathogens. Next step is to sequence the isolates to gain knowledge about mobility and possibly origin of the novel carbapenemase genes found.

1.5 Key words:

Antibiotic resistance, Carbapenemases, Metallo-β-lactamases, Resistome

2 Background

2.1 The threat of antibiotic resistance

Modern medicine is by large depending on effective antibiotics. Apart from treating primary bacterial infections, antibiotics enable other treatments and therapies, e.g. transplants, surgery, immunotherapy and cancer treatment. But many of our historically successful antibiotics have been lost due to antibiotic resistance. An estimated 700 000 lives are claimed annually due to antimicrobial-resistant infections alone. [1-3]

Multi-resistant bacteria and especially the extended spectrum β-lactamase producing bacteria, (ESBL) are considered a grave threat to the public health. The ESBLs are capable of disabling almost all of the β-lactam antibiotics – the most common family of antibiotics in use. Our last resort to combat the ESBLs and many of the other multi-resistant bacteria is often the carbapenems, the most potent antibiotic with the broadest spectrum against Gram-positive and Gram-negative bacteria discovered. [2] However, certain pathogens (Carbapenemase-producing Enterobacteriaceae, in Sweden called $ESBL_{CARBA}$) have acquired carbapenemase activity and can, as it name implies, hydrolyze carbapenems, which only leave us with drugs like colistin, tigecycline, and fosfomycin as our remaining options. Alternatives that are subject to their own resistance problems and have a high mortality rate and severe side effects (renal and liver toxicity, etc.). [4,5,6]

To guide resources to research, discovery and development of new therapies WHO, in September 2017, published a Global priority list of antibiotic resistant bacteria. The highest priority group (Critical) were all carbapenem-resistant strains, underlining the threat they pose[7].

2.1.1 A brief history of antibiotic resistance

Antibiotic molecules have existed long before humans discovered, named and started mass-producing them. For as long as there has been competition between microbes, there has also been a role for defensive countermeasures in these constant ongoing inter-microbial conflicts and it's not surprising that even in the most pristine environment thinkable, like 30 000-year-old Beringian permafrost sediments, antibiotic resistance genes have been found.[8,9]

The antibiotic resistance genes in environmental bacteria are known as the environmental resistome. There is growing evidence that this resistome acts as a huge source of genes from which human pathogens can acquire novel resistance determinants. This process, which most likely involve multiple steps, could be facilitated in the presence of a selection pressure from antibiotics.[10] It is likely that the increased consumption of antibiotics eventually leads to an increased concentration of antibiotics in the environment that speeds up the aforementioned process. The consumption of antibiotic drugs has increased by 36% globally between 2000 and 2010, and between 2010 and 2030 it is estimated to increase with another 67% to 105 000 tons per year.[11,12]

2.1.2 Acquisition of antibiotic resistance

There are two main mechanisms for a bacterium to acquire resistant genes: mutations and horizontal gene transfer (HGT):

Mutations in the DNA can either alter the molecular structure of the target site, reducing the affinity of the antibiotic molecule and thus reducing/eliminating the effect of the drug, or alter other important structures that can reduce the efficacy of the drug, such as the gene expression

of efflux pumps, or alter the number or structure of porins. Mutations tend to occur slowly and randomly.

HGT is usually divided into three subgroups:

Conjugation – where the host bacteria creates pilus that attaches to target bacteria and through which plasmids can be transferred.

Transformation – where the bacteria binds to DNA in the extracellular environment and engulfs it and incorporates it into the bacterial DNA.

Transduction – or bacteriophage – a virus containing antibiotic resistance gene DNA infests a bacteria and DNA is incorporated into the DNA of the target bacteria. [13]

2.2 Carbapenems

2.2.1 History of carbapenem

Carbapenems are a subgroup of the β-lactam family of antibiotics. The carbapenems are more resistant to enzymatic hydrolysis compared to other beta-lactams. Carbapenems were discovered roughly 50 years ago, but it took 20 years before the first drug was out on the market. [14,15] The parent compound for carbapenem antibiotics was the potent β-lactam thienamycin, a molecule produced by the soil bacteria *Streptomyces cattleya*. Unfortunately, thienamycin was too unstable to be used in vivo, but minor changes to the structure resulted in the more stable and commercially viable imipenem being launched in 1985 [16,17]. Imipenem's effect is reduced by renal dihydropeptidase (DHP-1) but today DHP-1-resistant variants have been launched like meropenem (1996), ertapenem (2001), and doripenem (2007). [7,18,19] Unlike the other carbapenems, meropenem can pass the blood-brain barrier to treat CNS infections if dosed in high enough concentrations. [20]

2.2.2 Carbapenems antibiotic action

The integrity of the bacterial cell wall is of essential importance for the bacteria and provides structure, stability, and form to the bacteria. The cell wall is autolyzed and synthesized by the bacteria's own machinery. One of the indispensable components for synthesis is the cross-linking of the peptidoglycan that stabilizes the bacterial cell wall. The cross-linking is performed by the enzyme PBP (penicillin-binding protein). PBP binds to a terminal alanine on one peptidoglycan chain and forces out an alanine on a peptidoglycan chain next to it and forms a link between the two chains. [21,22]

Carbapenems share the same fundamental mechanism as all β-lactams by acting as an analog to the terminal alanine, which makes the PBP bind irreversibly to the carbapenem thus inactivating the PBP and preventing it from cross-linking the peptidoglycan layer which in turn halts the cell wall synthesis. β-lactams have also been shown to increase the expression of autolysin which degrades the cell wall, and together with the decreased synthesis, β-lactams weakens cell wall integrity and makes the bacteria's internal osmotic pressure rupture the cell wall. The combination of these mechanisms makes β-lactams bactericidal. [23]

2.2.3 Carbapenems clinical importance

Carbapenems are in Sweden used for patients above 3 months of age with severe and complicated bacterial infections where the pathogen and susceptibility is unknown. The only real contraindication is hypersensibility to β-lactams.[24]

As a group, carbapenems have proven to be effective against many of our most aggressive pathogens: *Staphylococcus aureus* (incl those with penicillinase activity), *Enterococcus spp, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus viridans, Acinetobacter*

spp, Enterobacter cloacae, Escherichia coli, Haemophilus influenzae, Klebsiella pneumoniae, Pseudomonas aeruginosa, etc.[22]

2.3 Carbapenem resistance

2.3.1 Mechanisms of carbapenem resistance

There are four known mechanism for carbapenem resistance in bacteria:

Porins – β-lactams like carbapenems cannot penetrate the cell wall of the gram-negative bacteria. Instead, they use the porins to enter the bacteria. The bacteria can either reduce the expression of porins or alter the structure of the porins, making them more selective, both of them reducing cell wall permeability. [22,25-27]

Efflux pumps – The bacteria have efflux pumps of varying selectivity integrated into the cell membrane that allows them to pump out potentially harmful molecules and ions. Carbapenems are mostly affected by the broad specificity efflux pumps (multidrug-resistant efflux pumps – MDR). Overexpression of efflux genes is often the result of mutations.[22,25,26]

Penicillin-binding proteins with poor affinity to carbapenems (PBP) – Some resistant bacteria (gram-positive *Enterococcus spp*. among others) downregulates a majority of their regular PBPs and upregulates or acquires modified PBPs with an altered structure that makes it difficult for carbapenems to bind and have its effect.[22,28]

Carbapenemases – hydrolyzes the β-lactam ring of the antibiotic which inactivates the molecule. Genes encoding carbapenemases can be found on both chromosomes and plasmids. The origin of the β-lactamases has been speculated to be mutated PBPs.[22,25,26]

Apart from the known mechanisms above there is also the carbapenem intrinsic resistance (CIR) e.g. CarF and CarG proteins, produced by carbapenem-producing subgroups of the *Erwinia*, *Photothabdus* and *Serratia* species. Function and mechanism are unknown.[29]

2.3.2 Cost of carbapenem resistance

All biological activities come with a price tag for the organism. The expression of antibiotic-resistant genes is no exception. Carbapenem-resistant bacteria will have an obvious advantage in carbapenem-rich environments. Conversely, in an environment without the antibiotic pressure, ceteris paribus, a susceptible bacteria not carrying the overheads related to the resistance genes will outpace the growth of the resistant bacteria.[30]

In vivo, the actual cost for the resistant genes is also depending on co-selection and compensatory evolution. If the specific carbapenem resistance gene is selected together with other genes that are beneficial for the survival of the host, the fitness cost for the carbapenem-resistant gene will be balanced by the gain of the bundled genes. Compensatory mutations can over time reduce the fitness cost of the carbapenem resistance gene making them less costly. Modified versions of the produced enzymes can also turn out to have other benefits for the host that in turn compensates the original fitness cost.[31,32]

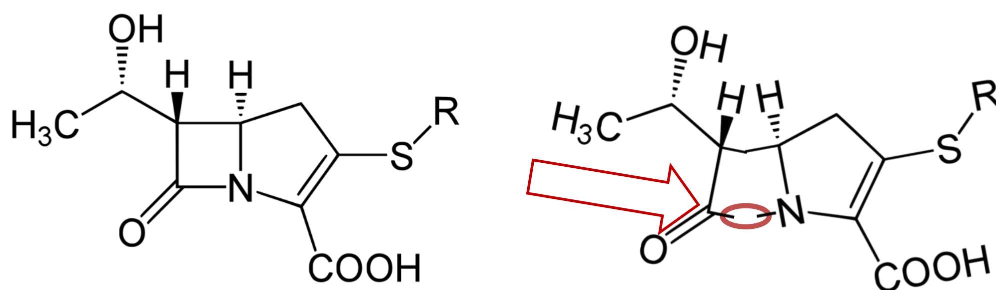
2.4 Carbapenemases

Two possible functions that can explain the evolution of carbapenemases have been proposed: to defend the integrity of the cell wall from the toxic effect of carbapenem-like molecules

produced by themselves or other microbes, and as an important protein to balance and recycle the peptidoglycan layer in the cell wall.[21]

β-lactamases is commonly divided into Ambler class A, B, C or D depending on amino acid homology. Three, A, B and D, of the classes contain carbapenemases that are clinically relevant.

Ambler class A and D have a conserved serine in the active site that mediates the hydrolysis of the β-lactam ring. [33] Members of the Ambler class B, also known as Metallo-β-lactamases (MBL), have metallic ions (often zinc) in their active sites. The ions, usually a pair of zinc ions, position a water molecule that activates and hydrolyzes the N-C bond. [33,34]. A way to clinically distinguish between serine- and metallo-β-lactamases is by adding the metal chelating agent ethylenediaminetetraacetic acid (EDTA) which inactivates the zink and thereby inhibit MBLs, but not the serine-β-lactamases.[35,36]



Source: https://commons.wikimedia.org/wiki/ File:Carbapenem.svg (modified)

***Figure** 1 - **Carbapenemases target structure.** Carbapenemases hydrolyzes the carbapenems C-N bond, weakening the structure of the β-lactam ring.*

2.4.1 Ambler class A

Class A β-lactamases hydrolyzes penicillin, cephalosporins, and carbapenems. They are partially or fully inhibited by clavulanic acid or tazobactams. They fall phylogenetically into

five groups: KPC (Klebsiella Pneumoniae Carbapenemase), IMI (Imipenem-hydrolyzing β-lactamase), GES (Guiana Extended-Spectrum β-lactamase), NMC (Not Metalloenzyme Carbapenemase), and SME (Serratia Marcescens Extended-spectrum β-lactamase). [37,38]

Class A carbapenemase genes can be chromosomal, often as gene cassettes in class 1 integrons. But they can also be part of transposable elements on plasmids.[39] The class A carbapenemases are most frequently found in *Enterobacteriaceae* and *P. aeruginosa*. [37,38]

2.4.2 Ambler class B – Metallo-β-lactamases

Metallo-β-lactamases (MBL) are considered a major challenge to the public health.[40] They can hydrolyze all beta-lactams, excluding aztreonam, and are often co-selected with other resistance genes making the host pan-resistant. The MBLs can appear both on chromosomes and plasmids. They are known to disseminate rapidly between bacterial strains as well as continents.[41,42] The MBLs are not inhibited by clavulanic acid or tazobactam but they do show inhibition when exposed to metal chelators like EDTA[36]. The most widely distributed among pathogens are the NDM (New Delhi metallo-β-lactamase), IMP (Imipenemresistance), and VIM (Verona integron-encoded metallo-β-lactamase).

Important pathogens that will express the MBLs include *E. coli*, *P. aeruginosa*, *K. pneumoniae*, *A. baumannii,* all quite common in the clinical setting.[35,43] The MBLs are classified into three groups, B1-B3. Among the MBLs the, by far, most identified type is the B1. B1 and B3 have a broader resistance spectrum and have two zinc ions in the active site, whereas the B2 only have one.[44,45]

2.4.3 Ambler class D - Oxcillinases

Class D β-lactamases are commonly known as the Oxacillinases (OXA) due to their hydrolytic activity for cloxacillin and oxacillin. The OXA types include more than 400 enzymes, but the most likely subgroups to cause concern are enzymes that can be found in bacterial pathogens and can hydrolyze carbapenems.[46,47]. A majority of the OXA enzymes have been identified in *Acinetobacter spp*, but some OXAs, like the OXA-48, have also be found in *K. pneumoniae, P. Aeruginosa* as well as *E. coli*.[38,47]

2.5 Spreading mechanisms of carbapenemases

The expression and the spreading pattern of a carbapenemase gene is affected by the location of the gene, chromosomal DNA or plasmid, and the surrounding regions.

The chromosomal DNA contains all the information needed for the bacteria's essential household protein synthesis. Since the chromosomal DNA is essential for the basic functions and thus the survival of the bacteria, any changes to the chromosomal DNA comes with a higher risk for the bacteria.

Carbapenemases spread predominately through plasmids, which in comparison to the chromosomes are smaller and contains fewer genes. Genes on plasmid can help bacteria to better survive environmental factors, deal with high concentrations of substances that might be harmful, like metals or antibiotics, to outcompete other bacteria, to kill off other bacteria etc. Plasmids can be picked up, ejected and transferred to other bacteria. [48]

2.6 Where do carbapenemases come from?

Tim Walsh sampled New Delhi water sources and found that bacteria from 166 out of 171 seepage samples and 14 out of 50 tap water samples grew on mereopenem supplemented agar plates, suggestive of carbapenemase activity. Two of the water samples and 51 of the seepage samples were positive for NDM-1. In total 20 different bacterial strains were found carrying the NDM-1, including species like *Shigella boydii*, *Vibrio cholera*, *E. coli*, *P. aerguinosa* and a *K. pneumoniae* but also strains like *Sutonella indologenes, Stenotrophomonas maltophilia, Achromobacter* spp, *Kingella denitrificans* and other *Pseudomonas spp*. NDM-1 is frequently found in clinical samples in India, but the study showed that it is also common in the environment. [49]

Environmental bacteria can thus serve as a source of carbapenemases (and other resistance genes) that under the right circumstances can be transferred to pathogenic bacteria. Members of the *Shewanella* genus, a family of gram-negative bacillus found in marine and freshwater environments, have been suggested to carry the progenitors of numerous β-lactamases and quinolone resistance genes found in pathogens today. The chromosome of *Shewanella xiamenensis* is likely the origin of the OXA-181 gene (a class D carbapenemase), identified on plasmids of several bacterial species, including common pathogens like *Klebsiella pneumoniae* and *Enterobacter cloacae*. Based on the similarity (only 4 amino acids differ) and the nucleotide sequences next to the genes it is also highly likely that the *S. xiamenensis* OXA-181 gene is the origin of OXA-48 gene. The plasmid-harboured OXA-48 is spreading into many enterobacterial species and caused numerous hospital outbreaks of carbapenemase-producing Enterobacteriacae.[50]

Another example of genes moving from the chromosome of an environmental bacterial to the plasmid of pathogenic bacteria is the commensal *Acinetobacter radioresistens* that has found to be the source of the chromosomal OXA-23 gene that can be found on plasmids of the substantially more virulent *Acinetobacter baumannii.*[51]

With this in mind the mobility of carbapenemase genes from environmental bacteria to pathogens, is a threat to be taken seriously. By identifying the environmental carbapenemases we stand prepared if they show up in the clinic, enabling us to the take right course of action.

2.7 Barriers for genetic migration

One can argue for two barriers preventing genes in environmental bacteria to migrate into pathogens: the genetic and the ecological.[52]

Genetic – If the genes are chromosomal and not associated with mobile elements, or if they are mobile but require specific cellular machinery that is not compatible with pathogens, there is less probability of successful migration.

Ecologic – If the genes flourish in a certain bacterial species that thrives in an environment far from what the pathogens can survive in, it is less likely for the genes to get a possibility to migrate to the pathogens.

3 Aim

With the long-term goal to protect the public health, it is important that we identify as many carbapenemases as possible to be able to screen for it in the clinic but also to understand how, where and when the carbapenem resistance emerges and spreads.

> *" What this implies is that for any antibiotic used in medicine or agriculture, the spectrum of resistance elements in the environment must be cataloged and monitored for possible mobilization to the clinic. Such studies will provide an early warning system for the predictable development of resistance in pathogens."*
>
> *Waglechner and Wright* [53]

By considering barriers for genetic migration and collecting samples from an environment similar to that in and on the human body, we increase our chances of identifying genes that eventually might end up in human pathogens. We can achieve this by sampling the microbiota of the gut of animals, an environment not only similar to ours, but also rich in bacteria and nutrients, with a high potential of gene sharing between bacteria. Animal stool samples are the simplest way to study their gut microbiota.

3.1.1 Purpose

The purpose of this project is to explore the environment for novel carbapenemases, to find the gene before it finds the pathogen. The project is part of a bigger framework – NoCURE - headed by Professor Joakim Larsson with the aim to discover emerging novel carbapenemases, define modes of mobility and assess the potential of these carbapenemases to spread to human pathogens, so that we might know what we will be up against before they become a real threat to us.

3.1.2 Research question

Can we find novel carbapenemases in the stool of animals never deliberately exposed to carbapenems? If yes, what characteristics would they have and what Ambler class would they belong to? What bacterial species would host these novel carbapenemases and would the genes have the capacity to transfer to known human pathogens? Which animal would be colonized with these carbapenemase-producing bacteria?

4 Material and Methods

4.1 Setting

Being an explorative study, we wanted the animal population to be as diverse as possible. We also wanted to make sure we had a somewhat controlled environment in order to reduce contamination from other species and to allow us, in case we found carbapenemase-producing bacteria, to identify what animal species was colonized by the bacteria. The obvious solution was a zoo. We contacted Nordens Ark, a zoo specializing in the preservation of endangered species 90 km north of Gothenburg. They were very supportive of the idea and agreed to get the samples we needed per our instructions.

4.2 Sampling

Out of the roughly 80 species at Nordens Ark Zoo we chose 43 species that would provide us with sufficient sample volume, a good variation and a good coverage of the mammals as well as birds and amphibians (see 13 Appendix A: Nordens ark - Species).

The 43 stool samples were collected by the animal keepers of Nordens Ark. We supplied buckets containing disposable gloves, tubes, ethanol, spoons, paper towels and instructions that stated the samples should be taken with a disinfected spoon, the feces should be fresh, samples should only contain fecal material, store the samples on ice, and that it was acceptable to pool from two or more stools from the same species to get enough material (see Appendix B for full instructions).

The samples were collected in 50 ml tubes and put on ice immediately after sampling. All samples were collected within three consecutive days and were inoculated on the fourth day.

4.3 Preparation: Inoculation and selective culturing

To promote as many different species of gram-negative bacteria as possible three different nutrient broths were used:

Tryptic soy broth (TSB)	High nutrient general purpose broth suitable for most bacteria but favors fast-growing ones.
R2A	Low nutrient broth, allow of also slow-growing bacteria
MacConkey	Promoting gram-negative and enteric bacteria like E. coli. Gram-positive bacteria are inhibited by the bile salts and crystal violet.

The following selective agents were added:

Cyclohexamide (100 mg/l) that inhibited translocation of tRNA and mRNA progression in eukaryotic cells (to inhibit growth of fungi).

Vancomycin (10 mg/l) that effectively inhibits growth of gram-positive bacteria, as some common enteric gram-positive bacteria are intrinsically carbapenem-resistant, like the *Enterococcus spp,* allowing them to grow unhindered and thereby disturbing the selective process. The gram-negative bacteria are resistant to vancomycin.

ZnSO4 (70 micromoles) provides additional zinc to promote the activity of MBLs.

Meropenem to enrich for carbapenemase-producing bacteria. The broths contained three different levels of concentration of meropenem (0, 0.25, 4 mg/l).

Approximately 1 ml feces sample was mixed with 2 ml physiological NaCl and vortexed into a solution. 100μl of the suspension was added to 5 ml broth in 10 ml tubes. The broth acts as a nutrient and a selective agent (depending on meropenem concentration) for the sample. The

resulting 387 (43 species * 3 broth types * 3 meropenem concentrations) tubes were marked with assigned animal-number and broth type and meropenem content.

The tubes were stored on a platform rocker at 30C at 150 rpm until there was visible proof of growth compared to a non-inoculated media (23 hours for the broths without meropenem supplementation and 48 to 92 hours for the supplemented ones). All samples were sorted and the tubes were marked with growth and no growth.

1 ml from each tube was pooled together within the same animal group (Mammals A, Mammals B, Birds, Amphibians, Farm animals) and meropenem concentration. All sample tubes were topped off with glycerol and put in a -80C freezer.

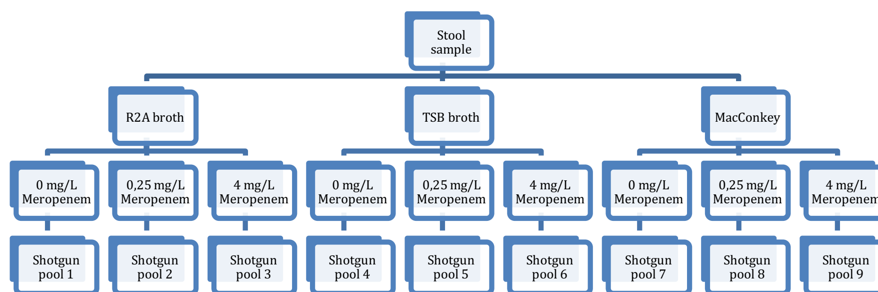


Figure 2 - Broth inoculation variants. *R2A, TSB and MacConkey with three different concentrations of meropenem.*

After the cultivating in selective broth and creating pools a number of methods were applied with the aim to identify novel carbapenemases (*Figure 3 - Methods used*).

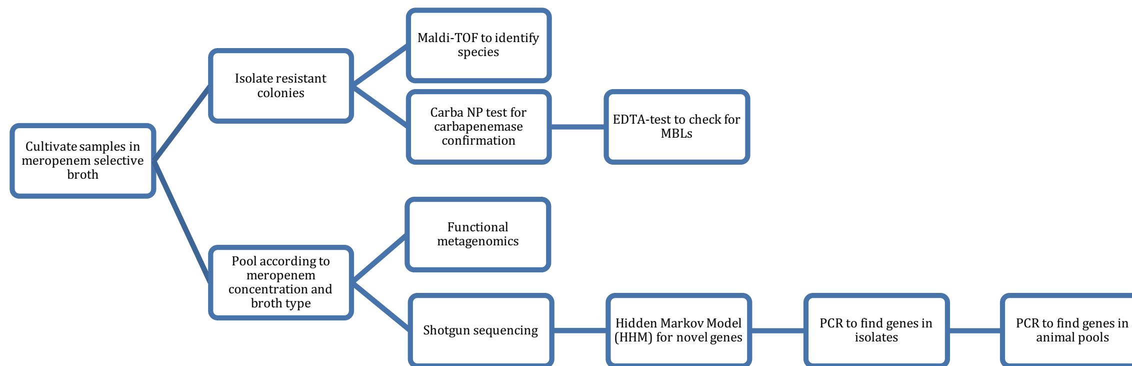


Figure 3 - Methods used. *For the subsequent methods used the source material was either resistant isolates or pooled genome.*

4.4 Isolates

A 10 µl inoculation loop of the broth tubes with added meropenem showing growth were streaked out on meropenem-supplemented agar plates of the same media type used in the broth (R2A, Tryptic Soy or MacConkey). The plates were incubated for approximately 2 days at room temperature until visible growth was seen.

4.4.1 Carba NP test on bacterial isolates

Carba NP is a simple and robust test, measuring the actual performance of the bacteria. The test takes advantage of the pH drop that occurs when carbapenemase hydrolyzes carbapenem (imipenem in this case) the drop can be visualized by using the pH-indicator phenol red.

Solution A was prepared by mixing 0.15g phenol red (Sigma, St. Louis, MO, USA) with 30 ml of dH2O and adding 249 ml of H2O and 2.7 ml of 10 mM $ZnSO_4$.

For each strain to be tested, one tube with 100 µl of Solution A (negative control) and one tube with 100 µl solution A with an Imipenem concentration of 12 mg/ml were prepared. Two

10 µl inoculation loops of bacteria were collected from Mueller-Hilton agar plates and added to 200 µl of B-PER II lysis buffer (Fisher Scientific, Rockford, IL). The mixture was vortexed for 1 min and left in room temperature for 30 min. The samples were then centrifuged for 5 min at 10 000 g before 30 µl of supernatant was added to tubes containing Solution A and Solution A + Imipenem. The tubes were incubated at 37 °C and examined after 15, 30 and 60 min. Bacterial strains expressing OXA-48, VIM-2, and KPC-2 were used as positive controls.

4.4.2 Imipenem-EDTA synergy test

MBLs need zinc in their active site to hydrolyze the β-lactams. The presence of a zinc chelator like EDTA will inhibit the action of the MBLs. To test for presence of MBLs in the collected isolates, Mueller-Hinton agar plates were inoculated evenly with the bacterial strains before a strip with a gradient of imipenem in one end and a gradient of imipenem + a fixed concentration of EDTA in the other end were placed onto the inoculated agar surfaces. The plates were incubated for 24h at 37 °C before being assessed. If bacteria-free zone on the EDTA + imipenem side was significantly larger than on the imipenem-only side the test was interpreted as positive (indicating a presence of a MBL in the isolate).

4.4.3 Maldi-TOF

Matrix-assisted laser desorption/ionization – Time of Flight mass spectrometer (Maldi-TOF) is a technique to analyze DNA, proteins, peptides, sugars, and macromolecules through laser ionization and mass spectrometry. Maldi-TOF was used to determine the species of the 31 collected isolates based on the mass spectrometry readings of the ionized substances. The readings were matched against the clinical MYLA database, and for isolates not matched, the research-oriented Saramis database was used as a complement.

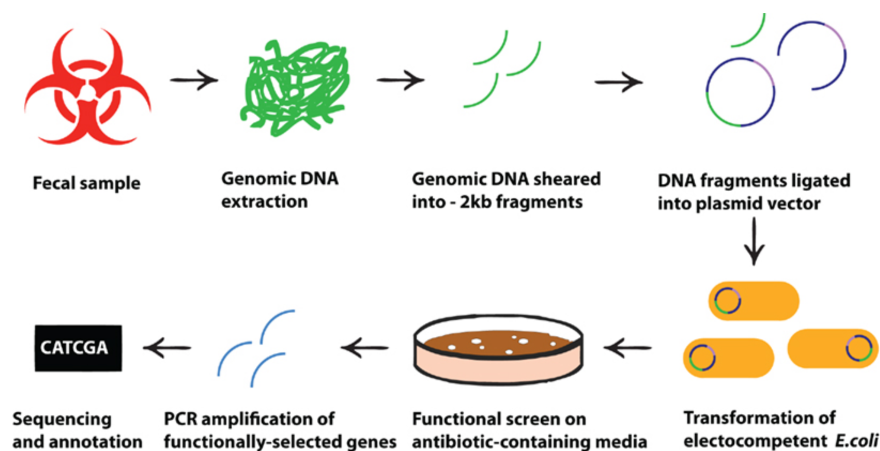
4.5 Functional metagenomics

4.5.1 DNA extraction and preparation of libraries

The inoculated tubes with broths showing growth were pooled according to the three media types and the three meropenem concentrations, nine pools in total. For the shotgun sequencing, functional metagenomics and PCR bacterial DNA was extracted from the pools. The Qiagen DNeasy standard protocol was used to prepare the bacterial DNA. The yield of the extracted DNA was measured by Qubit Fluorometer 2.0.

4.5.2 Functional metagenomics test

From the extracted DNA libraries were prepared using the pZE21-MCS vector (Expressys, Germany) following the protocol designed by Sommer, et al. [54] Through electropermeabilization the prepared metagenomic library was introduced into NEB 10-beta electrocompetent E. coli (New England Biolabs, MA, USA).



Source: Moore [55] (2011)

Figure 4 - Creating vectors in E. coli for functional metagenomics *After DNA is extracted cloning vectors are created that is then picked up through transformation by competent E. coli. E. coli with vectors can potentially express the genes of interest and through selective cultivation and PCR the necessary amount of those genes can be produced and sent for sequencing.*

In order to select for carbapenemase-producing *E. coli,* all vector-infused *E. coli* were cultivated on meropenem enriched plates of the same type as the broth (R2A, Tryptic soy,

MacConkey). Growth in presence of meropenem would then indicate cloning a functional expression of a carbapenemase gene in the *E. coli* strain. When the *E. coli* has acquired resistance and the functional screening is done, the amount of DNA to sequence is only a fraction of that of the whole sample genome. Thus, the rationale of using functional metagenomics is to filter out junk DNA to be able to focus the sequencing and assembly resources on the relevant DNA sequences. If the functional metagenomics doesn't provide any result, an alternative is to run other functional tests like Carba NP and EDTA on the isolates.

4.5.3 Metagenomic sequencing

The same DNA pools used for functional metagenomics were sent for shotgun sequencing with Illumina HiSeq at National Genomics Infrastructure (NGI) in the national center Science for Life Laboratory (scilifelab.se) in Stockholm.

4.5.4 BLAST and hidden Markov model

The HiSeq output was assembled and analyzed by Fanny Berglund, member of Erik Kristianssons research group at Chalmers Bioinformatics. SPAdes (St Petersburg genome Assembler) was utilized for the assembly and sequences were matched to the BLAST database for known genes. For detection of novel MBL B1 and B3 type genes, newly developed hidden Markov models were applied.[56] As suggested by Cornaglia et al. a gene was considered novel if it displayed less the 70% amino acid sequence identity to known MBLs.[57]

4.6 Finding novel sequences in samples through PCR

Protein sequences identified in the Hidden Markov model were aligned to a longer series of nucleotide sequences retrieved from the HiSeq sequencing. Based on the aligned sequences, we used NCBIs primer tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast) to design primers. Default settings were used except the PCR product length were set to be above 100 base pairs to get at clear separation from any primer dimers (located below 100 base pairs) and the taxa to crosscheck for alternative targets were Animalia (taxid:33208) instead of the default: Homo Sapiens. Designed PCR primers were ordered online from Eurofin.

4.6.1 PCR settings

PCR kit used was Applied biosystems AmpliTAQ (Foster City, California). No enhancer was used. For each run, a master mix was created and then primers and DNA were added separately. The reaction volume was 25,125 µl and consisted of 15 µl distilled water, 2,5 µl PCR Buffer, 2,5 µl $MgCl_2$, 2 µl dNTP, 0.125 µl of Taq polymerase, 1 µl of forward primer, 1 µl of reverse primer and 1 µl of DNA.

The thermoblock program was set to initial denaturing at 94 °C for 3 min, followed by 39 cycles of denaturing at 94 °C for 30 seconds, annealing temperature (between 56-59 °C) for 30 seconds, elongation at 72 °C for 15-35 seconds and last cycle ended with a final elongation of 72 °C for 7 minutes and the cooldown program was set to 10 °C indefinitely. The annealing temperature was decided by taking the primer's estimated melting temperatures (estimation by primer supplier Eurofin) and subtracting 3 °C. If the gel showed a lot of unspecific products, the PCR program was adjusted by increasing the annealing temperature. The elongation time in each cycle based on the length of the expected PCR product (between

100-500 base pairs) and the approximation that the polymerase should manage 1000 base pairs per minute.

4.6.2 PCR pools and isolates

To find the 19 novel genes among the 31 isolates and 43 animals samples the DNA was pooled for the first screening. The isolates were pooled according to broth media and meropenem concentration (label in parenthesis): R2A 0,25 mg/l (2), R2A 4 mg/l (3), TSB 0,25 mg/l (5), TSB 4 mg/l (6). The animal samples were pooled based on the groups: Mammal 1 (A), Mammal 2 (B), Birds (C), Amphibian (D) and Farm animals (E) (see Appendix A: Nordens ark - Species). For each novel carbapenemases gene, a total of 9 sample pools (2, 3, 5, 6, A, B, C, D, E) and positive controls and negative controls (without any DNA) were screened.

As a positive control we initially pooled all extracted DNA from one media, TSB with 0,25 mg/l meropenem (labeled +). After a couple of runs and some missing positive control bands, the remaining three combinations of meropenem concentrations (TSB 4 mg/l, R2A 0.25 mg/l, R2A 4mg/l) and broths were to create a second positive control (labeled ++).

When a positive result was retrieved from a pool, the same primer pairs and procedure were applied to find the individual isolate/isolates positive for the detected novel carbapenemase genes.

4.6.3 Agarose Gel setup

The PCR products were visualized by running them on a 1 % agarose gel stained with SYBRsafe.

7,5 µl of PCR reaction was mixed with approx. 2 µl of loading dye and then loaded into the wells. The DNA ladder 1 kb+ (Thermo-Fischer) was used as size reference. The gel was run over 40-45 minutes at 90 volts and moved to a UV-lightbox and a photo was taken.

A dominating band at the right length (and thus the same length as the positive control) in the gel was considered a positive finding. If results were ambiguous, the PCR was rerun at a couple of degrees higher annealing temperature. If still uncertain the screening was continued with the next primer pair.

5 Ethics

Animal stool samples collected from a forest setting would from an ethical point of view be quite straightforward. Samples picked from the environment with no relation to the former host animal, they could be considered environmental samples and then no ethics approval is needed.

The ethical considerations of our samples from enclosed animals are slightly more complex. On one hand, it partly follows the environmental rationale above, but to a larger extent it also affects the animal in question as collecting samples would require the keepers to enter the enclosed area possibly stressing the animal. In addition, the fecal samples can be traced back to the animal, giving it a certain ownership of the stool. The latter is important when handling human samples as stated in the Swedish law (in Etikprövningslagen) that makes a clear distinction between the aggregated fecal sampling (eg. from sewage samples) that doesn't require permission and the sampling involving biologic matter that can be traced to that person that requires an ethics permission. Out of the 3Rs commonly used in animal research (Replacement, Reduction and Refinement), only Refinement is really applicable and the way for us to refine is to lower the stress of the animal by just adding the fecal sampling to the keepers' everyday routine.[58]

In the case of the enclosed animal, we also have the owners' perspective to consider. If we find carbapenemases, will it harm their business or change how the animals are treated or viewed by the public?

After considering all the aspects and laws governing the area, we came to the conclusion that an ethical permit was not mandatory as long as we had the blessing and support of the Zoo.

The additional stress would be minimal as the fecal sampling are mainly done by the animal keepers whilst feeding the animals. The rules regarding identification as mentioned above are not really applicable to animals and any theoretical ethical dilemma diminishes compared to the benefit of being able to identify the animal as it would enable us to go back to that animal for a later study and see if carbapenemase-producing bacteria are still colonizing the gut flora.

6 Results

6.1 Collection of 31 meropenem resistant isolates of mainly opportunistic bacteria originating from animal feces

Tubes showing growth in the meropenem supplemented broth were streaked on plates, and colonies with different appearances on the plates were picked, in total 31 isolates were collected, given an ID number and frozen -80 °C in glycerol. Out of the 31 isolates, 21 could be properly identified with Maldi-TOF using the MYLA database as reference. A majority of the identified species are known opportunistic pathogens (Table 1).

Table 1: Bacterial strain identification with Maldi-TOF.

	ID	MYLA (Seramis)	Opportunistic
R2A 0.25 mg/l	21	Stenotropomonas maltophilia	Yes
	22	-	
	23	Sphingobacterium multivorum	Yes
	24	Comamonas testosteroni	Yes
	25	(Trichosporon asahii)	(Yes)
	26	Sphingobacterium multivorum	Yes
	27	Pseudomonas veronii	No
	28	Sphingobacterium multivorum	Yes
	29	-	
	210	Stenotropomonas maltophilia	Yes
	211	Empedobacter brevis	Yes
	212	Sphingobacterium multivorum	Yes
R2A 4 mg/l	30	Chryseobacterium indologenes	Yes
	31	Stenotropomonas maltophilia	Yes
	32	(Trichosporon asahii)	(Yes)
	33	(Chryseobacterium spp.)	(Yes)
	34	Leuconostoc mesenteroides	Yes
	35	Stenotropomonas maltophilia	Yes
	36	Chryseobacterium indologenes	Yes
	37	Elizabethkingia meningoseptica	Yes
TSA 0.25 mg/l	51	Streptococcus pluranimalium	No
	53	-	
	54	-	
	56	Pediococcus pentosaceus	No
	57	-	
	58	Leuconostoc mesenteroides	Yes
	59	Pseudomonas veronii	No
TSA 4 mg/l	61	-	
	62	Leuconostoc mesenteroides	Yes
	63	-	
	64	Leuconostoc mesenteroides	Yes

Bacterial species within parenthesis were not identified by MYLA, data from the Seramis database was used instead. Only species with multiple documented human infections were considered opportunistic.[59-66]

Since the isolates had managed to grow in meropenem they must have had some active resistance mechanism, either intrinsic resistance, porins, efflux pumps, modified PBPs or

carbapenemases. There was growth in some MacConkey broth but no colonies grew on the subsequent MacConkey plates.

It is worth nothing that two of the isolates (number 25 and 32) were identified as Trichosporon Ashii, a fungi that should not be able to survive the cyclohexamide in the broths. Isolate number 32 was also positive in the Carba NP and imipenem-EDTA synergy tests indicating MBL production.

6.2 Carba NP showed carbapenemase activity in collected isolates

The Carba NP test was performed on the 31 collected isolates. For some slow-growing isolates, there were difficulties getting enough colonies to do the Carba NP test. However, 17 out of the 31 isolates (that was identified with MALDI-TOF) gave rise to a color change from red to yellow in the test as a result of the hydrolyzation of imipenem, an indication that the reason they survived the meropenem plates was at least partly due to carbapenemase production (Figure 5).

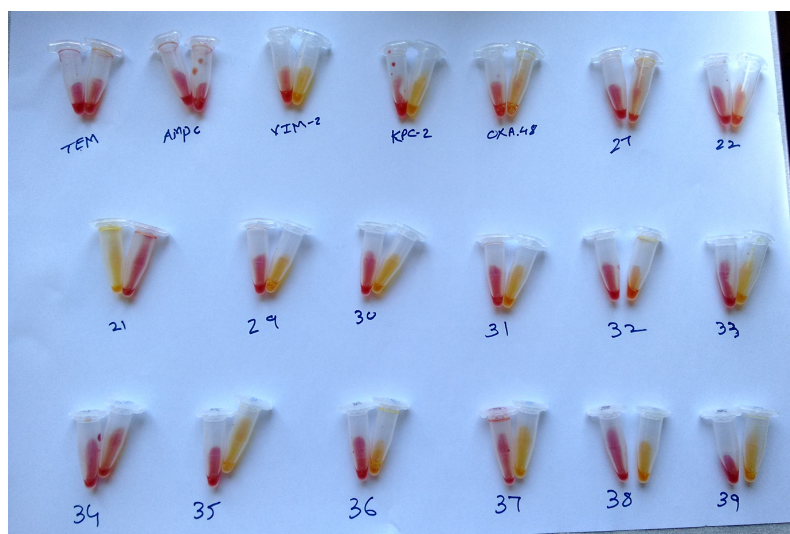


Figure 5 - Carba NP test. *The photo shows* 14 *out of the 17 positive Carba NP tests. , VIM-1, KPC-2, OXA-48 served as positive controls whereas TEM and AMpc served as negative controls*

6.3 All Carba NP positive isolates were also MBL-positive

Out of the 17 isolates that were positive with the Carba NP test all were clearly positive in the imipenem-EDTA synergy test, which suggest MBL activity (Figure 6). The three slow-growing isolates on which Carba NP could not be performed, also showed positive results in the imipenem-EDTA synergy test. In the latter cases, there was some suspicion of contamination and those findings therefore need to be confirmed by additional tests.

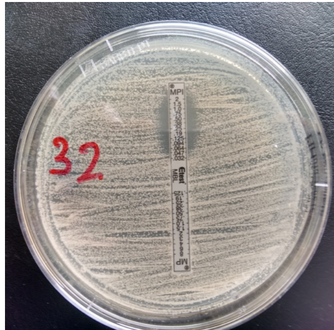


***Figure 6 – Imipenem-EDTA synergy test**. Representative photo of an isolate showing a positive result in the imipenem-EDTA synergy test. The upper part of the stick contain imipenem + EDTA whereas the lower half of the stick contains imipenem alone. The EDTA test showing growth inhibition near the EDTA+Imipenem part of the strip.*

6.4 No *E. coli* hosts survived on the selective plates during the functional metagenomics screening

For the functional metagenomics, all of the initial broths showing growth were pooled according to the three media types and the three meropenem concentrations, creating nine pools in total. Vector-infused *E. coli* were cultivated on meropenem-enriched plates of the same type as the broth. Growth would then indicate a functional expression of a carbapenemase gene, but none of the *E. coli* hosts survived the selective meropenem plates and thus there were no subjects available to send for sequencing.

6.5 Novel B1 and B3 genes found by shotgun sequencing and hidden Markov model

The same nine pools were used for the shotgun sequencing as for the functional metagenomics above. After shotgun sequencing and assembly, the hidden Markov models identified several novel MBLs in sequences longer than 200 amino acids, 13 sequences of B1 type and 6 sequences of B3 type. These corresponding nucleotide sequences were then used to design primers for PCR.

6.6 6 novel B1 genes found in the isolates

Through PCR 6 of the 13 novel B1 genes could be linked to specific isolates (Figure 7). Gene 36 and 42 were found in isolate number 29 (unidentified). Gene 2 and 31 were found in isolate 51 (*Streptococcus Pluranimalium*). Gene 41 was found in isolate 61 (unidentified) and isolate 63 (unidentified). Gene 42 and 73 were found in isolates 33 (tentatively matched as *Chryseobacterium spp.)* and 34 (*Leuconostoc Mesenteroides*).

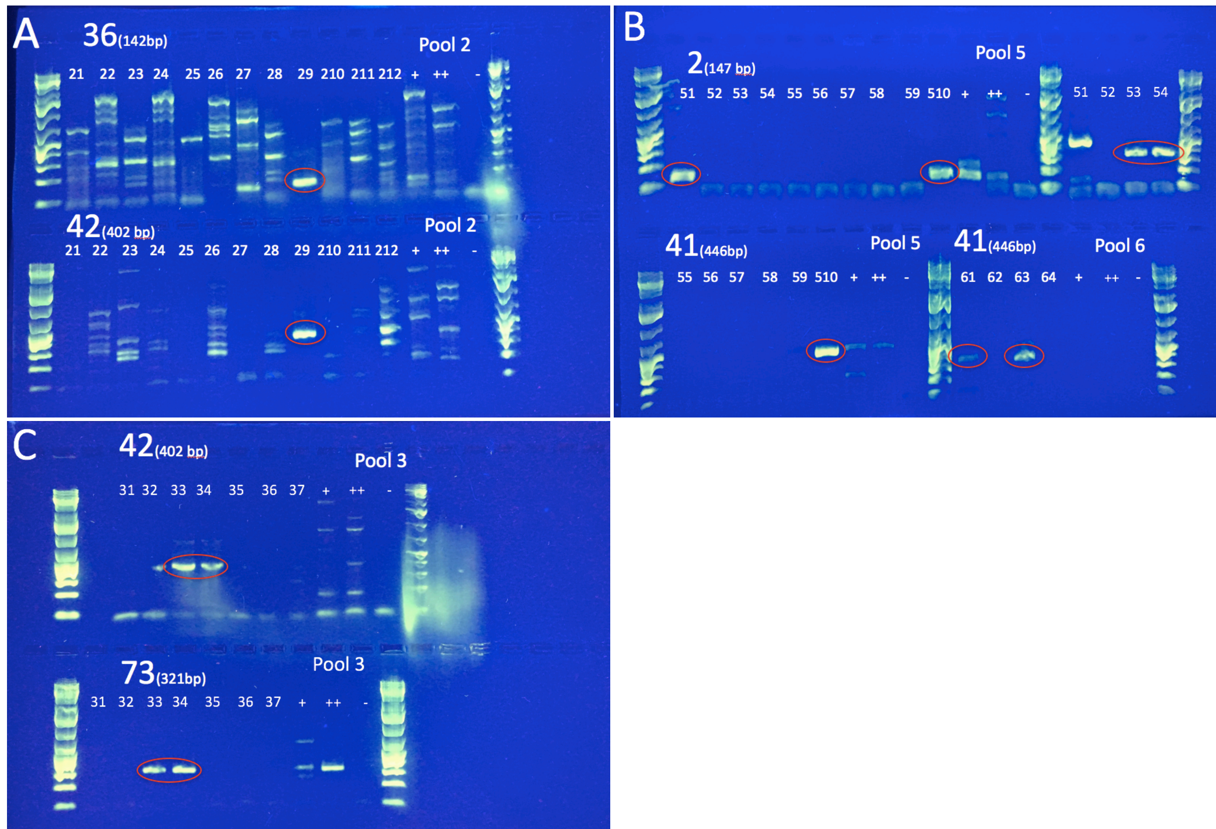


***Figure 7 – Novel MBL B1 genes detected in isolates by PCR**. A) Gene 36 & 42 found in isolate 29 B) Gene 2 & 31 found in isolate 51 and gene 41 found in isolate 61 & 63 C) B1: Gene 42 & 73 found in isolate 33 & 34*

6.7 3 novel B3 genes found in the isolates

For the novel B3 genes, 3 out of the 6 found by hidden Markov models could be detected in specific isolates (Figure 8). Gene 2 and 63 were found in isolate 27 (*Pseudomonas Veronii*). Gene 417 was found in isolate 212 (*Sphingobacterium Multivorum*). The double bands at the correct size for gene 417 in isolate 22 and 24 were ignored due to the ambiguous nature of double bands.

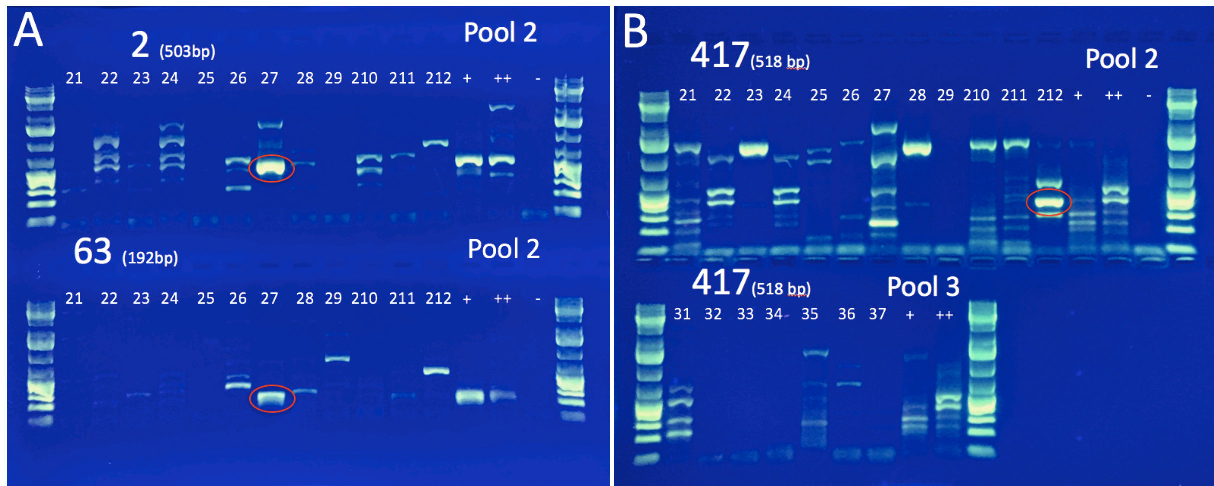


Figure 8 – Novel MBL B3 genes detected in isolates by PCR. *A) Gene 2 & 63 were found in isolate 27 B) Gene 417 was found in isolate 212*

A summary of all the findings from the collected isolates is shown in Table 2.

Table 2: Summarized findings from isolates

	ID	MYLA (Seramis)	Opportunistic	Carba NP	EDTA	Positive PCR
R2A 0.25 mg/l	21	Stenotropomonas maltophilia	Yes			
	22	-				
	23	Sphingobacterium multivorum	Yes			
	24	Comamonas testosteroni	Yes			
	25	(Trichosporon asahii)	(Yes)			
	26	Sphingobacterium multivorum	Yes			
	27	Pseudomonas veronii	No			
	28	Sphingobacterium multivorum	Yes			
	29	-				B1/B3
	210	Stenotropomonas maltophilia	Yes			
	211	Empedobacter brevis	Yes			
	212	Sphingobacterium multivorum	Yes			B3
R2A 4 mg/l	30	Chryseobacterium indologenes	Yes			
	31	Stenotropomonas maltophilia	Yes			
	32	(Trichosporon asahii)	(Yes)			
	33	(Chryseobacterium spp.)	(Yes)			B1
	34	Leuconostoc mesenteroides	Yes			B1
	35	Stenotropomonas maltophilia	Yes			
	36	Chryseobacterium indologenes	Yes			
	37	Elizabethkingia meningoseptica	Yes			
TSA 0.25 mg/l	51	Streptococcus pluranimalium	No			B1
	53	-				
	54	-				
	56	Pediococcus pentosaceus	No			
	57	-				
	58	Leuconostoc mesenteroides	Yes			B3
	59	Pseudomonas veronii	No			B1
TSA 4 mg/l	61	-				
	62	Leuconostoc mesenteroides	Yes			
	63	-				B1
	64	Leuconostoc mesenteroides	Yes			

6.8 11 novel B1 and 4 novel B3 genes found

DNA extracted from the initial meropenem-supplemented broths were also divided into five pools based on animal origin. Through PCR could the novel B1 and B3 genes be detected in all animal pools, 11 of the B1 and 4 of the B3 genes were detected at least once (Table 2). The novel genes were most common in the Amphibian pool.

Table 3: Distribution of novel genes in animal pools

Animal pool	No of species	No of B1 detected	No of B3 detected	Total	Gene/species
A Mammals 1	8	6	1	7	0,88
B Mammals 2	9	5	1	6	0,67
C Birds	7	4	2	6	0,86
D Amphibians	8	6	3	9	1,13
E Farm animals	11	3	4	7	0,64

7 Discussion

In the fecal samples collected from zoo animals, 19 novel carbapenemase genes were detected, as well as 31 mostly opportunistic meropenem-resistant bacteria, 17 of them expressing MBLs.

7.1 Conclusions

7.1.1 Could we find novel carbapenemases in the stool of animals never deliberately exposed to carbapenems?

Our study showed that novel carbapenemases are readily detectable in the gut flora of the sampled zoo animals. This is a result that is in line with previous studies in other regions of the world where carbapenemases were found in environmental and commensal bacteria.[56,67] In Walsh et al. they found plenty of the highly potent MBL NDM-1 in their Indian samples, but that is also in an environment where carbapenems are more liberally used.[49]

7.1.2 What characteristics did they have, what Ambler class would it be?

The carbapenemase genes detected in the isolates were all Ambler class B - metallo-beta-lactamases (MBLs), supporting the Rossolini et al. study that discovered multiple MBLs in predominantly environmental soil samples.[68] Were they common in our sample for having a competitive advantage in the bacteria's natural habitat? Or were they inadvertently selected for in the lab? The zinc added to the enrichment broths in this study intended to a promote MBLs, but it is unlikely that the concentration of 70 micromoles $ZnSO_4$ would have an inhibitory effect on bacteria producing other classes of carbapenemases.

7.1.3 What bacteria species did host these novel carbapenemases and did the genes have the capacity to transfer to known human pathogens?

A majority of the 31 isolates were opportunistic bacteria, and when we look at the Carba NP positive isolates 11 of 17 were opportunistic (and 3 were not identified).

The novel genes' potential to transfer to human pathogens is of course hard to predict. However, the fact that they were identified in gut bacteria from animals may reduce some barriers for transfer to human pathogens. Forsberg et al. found identical antibiotic resistance genes in the environmental bacteria, pathogens and in intestinal microbiota suggestive of interconnectivity between the three.[10] Further, whole genome sequencing of the isolates carrying the novel genes can provide information about the genetic context of these genes and thus some clues regarding their mobility potential. Some of the bacteria identified are known to have resident carbapenemase genes (e.g. *Chryseobacter spp, Stenotrophomonas maltophilia*). [68-71] But until the genome has been sequenced we cannot judge the mobility of the genes discovered in this study.

A somewhat surprising find was the two isolates identified by the Seramis database as Trichosporon Asahii, a fungus that shouldn't have survived the cycloheximide. One of the Trichosporon Asahii isolates were also positive in the Carba NP and imipenem-EDTA synergy tests, indicating MBL activity, a surprising finding in fungi.

The type of MBL can provide some hints about its mobility. In this study 13 novel B1 genes and 6 novel B3 genes were detected. B1 genes are the most important class clinically with members like IMP, NDM and VIM, and they have predominantly been seen on plasmids.[72]

B3 (and B2), on the other hand, have most often been documented in environmental bacteria as resident and is not primarily associated with mobility.[67]

7.1.4 What animal was colonized with the bacteria?

Time constraints reduced the ambition of pairing a specific gene to an animal pool instead of to an animal species. The frequency of novel carbapenemase genes were the highest in the amphibian pools but that is based on a very small number of hits and results should be interpreted carefully. The novel genes were quite evenly spread between the pools and no conclusion could be drawn.

7.2 Study strengths

By considering gene transmission barriers and using stool samples from zoo animals, our ambition was to increase the probability of finding novel carbapenemase genes that are compatible and able to transfer to human pathogens. of special interest would be found.

The inclusion of a high number of animal species and the use of three types of inoculation media allowed an explorative screening of a diverse set of animal gut bacteria. Sampling zoo animals also gives, us a possibility to investigate the microbiota further if a carbapenemase.

The hidden Markov model algorithm used to predict B1 MBLs have been validated in a previous study, 18 out of 21 detected novel carbapenemase genes in that study had a positive Carba NP test when synthesized and expressed in *E. coli*.[56]

The Carba NP and imipenem-EDTA synergy test are simple and robust functional tests. However, a potential drawback with the Carba NP test is that isolates producing OXA-48-like carbapenemases that are clinically relevant but often turn out false negative results due to their slow hydrolysis of carbapenems[73].

7.3 Study weaknesses

For most animals, except the farm animals, the only contacts with humans were through food. Nevertheless, it cannot be excluded that detected bacterial strains and genes have been transmitted from humans to animals. Farm animals that had more contact with humans didn't show a higher frequency of novel carbapenemases. Samples were collected from the ground so it is also possible that some environmental bacteria from the ground can have infiltrated the fecal material.

A key question that remains unanswered is if horizontal gene transfer is possible between the commensal bacteria we collected and a pathogen. As mentioned, whole genome sequencing of isolates carrying the novel carbapenemases might provide some clues about their mobility potential. The lack of surviving colonies in the functional metagenomics test could be a result of incompatibility between the genes and the *E.coli*. However, the lack of surviving E. coli clones on the meropenem supplemented plates during the functional metagenoms screening could also be due to the fact that E. coli are often, in addition to a functional carbapenemase, dependent on other resistance mechanisms (such as porin loss or up-regulated efflux pumps) in order to survive on meropenem-supplemented plates.[70]

The initial selection of isolates was based on perceived differences among the strains that had grown on the plates. It is possible that some isolates are in fact identical, belonging to the same strain with the same animal origin but were isolated from different enrichment broths. Whole genome sequencing of the isolates will help in identification of any duplicate strains.

The sheer number of PCR reactions required made it impossible to optimize primer design and annealing temperatures for each primer pair, which gave us some ambiguous and possibly false negative results when analyzing relevant bands on the gels.

7.4 Implications

The aim with this study was to help future decision-making. If we can find novel carbapenemases that can migrate to pathogens, they could be screened for in the clinic with whole genome sequencing when it becomes more readily available.

By looking at the flanking regions of the novel carbapenemase genes we might be able to determine the origin of the gene. If we can find the origin of resistance genes, what bacteria they come from, and understand the chain of events that transfer them from the environment to the pathogens, we might be able to break or at least slow down and intervene that process.

Knowing about whatever carbapenemase genes are present in the resistome can prevent us from designing a next generation carbapenem drug that, before it is launched, already has an abundance of resistance genes luring in resistome, just waiting to break the new drug down.

7.5 Reflections

All our carbapenemases were MBLs (Ambler class B) even though we didn't intentionally select for them. That we didn't find ambler class D carbapenemases (OXAs) was not as surprising as they are usually chromosomal. But the fact that we didn't find any of the class A carbapenemases, known to be plasmid-borne was more unexpected.

Our samples were from animals not exposed to carbapenems and yet their commensal bacteria carried an abundance of carbapenemases. In our meropenem-supplemented agar plates or broths we will not find any strict anaerobes and hardly any gram-positive bacteria or fungi (however, isolate 25 and 32 appeared to be fungi). In the Carba NP, a slow-hydrolyzing carbapenemase like the OXA-48s will go undetected. The applied hidden Markov models will only find MBL B1 and B3 genes. It is likely that we in this study heavily underestimate the number of carbapenemases, known and novel, in our samples.

It seems likely the carbapenemases fulfill an important role for the bacteria, since we find them in such abundance in an environment free of selective pressure from iatrogenic carbapenems. The role of carbapenemases could be to modulate/inhibit carbapenem-like structures produced by themselves or nearby microbes, either as a signal molecule or as a way to control the niche. Antibiotic signal molecules can modulate immune-response (clinically used in rosacea and severe acne and possibly also behind the growth promotive effect of antibiotics in animal feeds), metabolic pathways but also CNS through the glutamate transporter. [9,74] Alternatively, they perform some other critical physiological function of the bacteria (e.g. cell wall management) or are transferred bundled with other genes that whose benefit outweighs the cost for the carbapenemase gene.[31] The diversity could be explained by

carbapenemase genes being tailored over time to reduce fitness cost or to optimize the function in the microenvironment of the specific host bacteria.

7.6 Next step and future research

The isolates identified to carry carbapenemases in this study by Carba NP and/or PCR will be sent for sequencing to find out if the genes are part of mobile genetic elements or not. Sequencing can also provide clues about the origin of the genes and identify the isolates that was not identified in MALDI-TOF.

The chain of events needed to transfer carbapenemase genes between environmental/commensal bacteria and pathogens is very complex. More studies are needed to demonstrate the causality of the process.

A specific area in need of more research would be to investigate why we see such an abundance of MBLs and not any of the other carbapenemases. What makes them so frequent in our material and what function do they perform in the bacteria out in the environmental/commensal community?

8 Sammanfattning

Karbapenemer är en klass av antibiotika som är verksamt mot många olika typer av bakterier, inklusive de som många andra antibiotika inte kan ta hand om. Karbapenemer har därför en viktig roll vid behandlingen av allvarliga infektioner där de sjukdomsalstrande bakteriernas (patogenernas) känslighet för olika antibiotika är okänd. Känsligheten beror på vilka resistensmekanismer patogenerna har. I kliniken ser vi idag en ökad mängd infektioner där patogenerna inte är känsliga för karbapenemer, och den mest potenta resistensmekanismen är produktion av karbapenemaser, protein som kan bryta ner karbapenemer.

Tidigare studier har visat att i naturen finns det resistensgener, alltså gener som kan uttrycka olika typer av försvar mot antibiotika och att dessa gener kan sedan vandra till våra patogener. Frågan vi ställde oss i denna studie var: kan vi hitta tidigare okända karbapenemaser i naturen som ännu inte setts i kliniken? På så sätt kan vi förstå och eventuellt påverka hur gener vandrar mellan miljöbakterier och patogener.

För att öka sannolikheten att hitta gener som kan vandra mellan miljöbakterier och patogener ville vi titta på tarmfloran hos djur, en mikromiljö som i mångt och mycket är likt den som vi människor kan erbjuda. Ett effektivt sätt att titta på tarmfloran var att analysera spillning från 43 djur från djurparken Nordens Ark.

Proverna odlades i tre typer av buljong med olika näringsprofil samt med tre olika koncentrationer av meropenem (ett karbapenem) för att gynna de karbapenemasproducerande bakterierna. Från buljong och plattor med dessa meropenemresistenta bakterier gjordes sedan en rad olika analyser. Med ett Carba NP test kunde vi visa att 17 av de 31 isolerade bakteriestammarna på plattorna var karbapenemas-producerare och med ett annat test kunde

vi visa att alla dessa 17 tillhörde subgruppen metallo-β-laktamaser (MBL). Bakteriestammarna var opportunistiska patogener med några få undantag.

Från buljongen extraherade vi DNA från samtliga prover som visade bakteriell växt, vilket grupperades och skickade för sekvensering. Med hjälp av en nyutvecklad bioinformatisk algoritm kunde 19 tidigare okända karbapenemaser (13 av B1-typ och 6 av B3-typ) hittas i materialet. Genom PCR kunde vi hitta 6 av B1-typerna och 3 av B3-typerna bland de bakteriestammar vi isolerat. Och i de 5 huvudgrupper av djur vi hade, kunde 11 B1-typer och 4 B3-typer hittas. Mest frekvent förekom de nya generna hos groddjuren.

Vi fann oväntat många okända karbapenemaser i avföringsproverna från djuren. Att alla var av typen MBL var också förvånande. Det faktum att de nya karbapenemaserna kunde identifieras i tarmbakterier (inklusive opportunistiska patogener) från djur gör det troligare att utbyte kan ske med våra patogener då de kan överleva i liknande miljöer. För att verkligen kunna uttala oss om genernas mobilitet och ursprung kommer vi i ett senare skede sekvensera de insamlade isolaten separat.

9 Acknowledgement

First of all, I would like to thank my dedicated supervisor Carl-Fredrik "Genius" Flach and my spiritual guru Nachiket Marathe for guiding me and making sense of the complex world of microbial biology. I would also like to thank Fanny Berglund and Erik Kristiansson for all their hard work developing the hidden Markov models on which the result of this thesis is depending on. In addition, I would like to thank the rest of the lab for supporting and cheering me on.

A special thanks to Maja Genheden and Maria-Elisabeth Böhm for patient hands-on support in the lab, and to Caroline Rutgersson and Mohammad Razavi for moral support and comic relief.

Last but not least, thank you professor Joakim Larsson for support, guidance and inspiring discussions.

The study is part of NoCure, a project financed by Vetenskapsrådet.

Appendix A: Nordens ark - Species

Mammals 1

1. Amur tiger (Amurtiger)
2. Amur leopard (Amurleopard)
3. Persian leopard (Persisk leopard)
4. Snow leopard (Snöleopard)
5. Pallas cat (Pallaskatt)
6. European wildcat (Europeisk vildkatt)
7. Przewalskis wild horse (P... vildhäst)
8. Wolf (Varg)

Mammals 2

9. Southern Pudu (Sydlig Pudu)
10. Reindeer (Skogsren)
11. European ground squirrel (Sisel)
12. Maned wolf (Manvarg)
13. Otter (Utter)
14. Lesser panda (Mindre panda)
15. Tadjik markhor (Skruvhornshjort)
16. Wolverine (Järv)
17. Lynx (Lodjur)

Birds

18. White-naped crane (Glasögontrana)
19. Red-crowned crane (Japansk trana)
20. White stork (Vit stork)
21. Northern bald ibis (Eremitibis)
22. Ural owl (Slaguggla)
23. Less white fronted goose (Fjällgås)
24. Euroasian eagle owl (Bergsuv)

Amphibians

25. Mountain chicken frog (Montserrat-groda)
26. Green toad (Grönfläckig padda)
27. Long nosed horned frog (Horngroda)
28. Standings day-gecko (Standings dag-gecko)
29. Hermann's tortoise (Grekisk landsköldpadda)
30. Amazon milk frog (Mjölkgroda)
31. White lipped tree frog (Asiatisk skumbogroda)
32. Sand lizard (Sandödla)

Farm animals

33. The Gotland rabbit (Gotlandskanin)
34. The Gotland sheep (Gutefår)
35. The Blekinge duck (Blekingeanka)
36. The Gotland pony Gotlandsruss)
37. The Öland goose (Ölandsgås)
38. Mountain cattle (Fjällko)
39. Northern Swedish horse (Nordsvensk häst)
40. Orust hen (Orusthöna)
41. Old Swedish dwarf hen (Gammelsvensk dvärghöna)
42. Lapp goat (Lappget)
43. Linderöd pig (Linderödsvin)

Appendix B – Instructions for collection of stool samples

Instruction for collection of animal stool samples

There are one bucket per animal category (mammals, reptiles/amphibians, birds and farm animals). In each bucket you will find sampling tubes, rubber gloves, spoons, alcohol and tissues.

Each tube is marked with the animal species (except for reptile and amphibians, for which you can take 5-6 samples of any specie of your choice and just marked the tubes accordingly). There are a number of spare tubes if needed. Don't forget to mark the spare tubes with animal species if used.

Keep the inside of the tubes as sterile as possible.

Samples should be as fresh as possible.

Samples should only be collected during Tuesday the 12th and Wednesday the 13th of August 2014.

Preferably mix samples from different fecal piles from the same species in the same sample tube in order to increase the variation.

Samples should, if at all possible, be at least 5-10 ml for us to be able to extract DNA (tubes are graded).

Use the rubber gloves.

Samples should be collected with the included spoon that needs to be wiped thoroughly with alcohol between usages.

Store the samples dark and cold (4-8 °C). To keep samples cold during sampling please use ice or ice packs at the bottom of the bucket.

References

1. Healthcare infection society (HIS), U. Review on Antimicrobial resistance. Antimicrobial Resistance: Tackling a Crisis for the Health and Wealth of Nations. (2014).
2. WHO. Antimicrobial resistance: global report on surveillance. (2014).
3. Cars, O.*, et al.* Meeting the challenge of antibiotic resistance. *BMJ* **337**, a1438 (2008).
4. Yamamoto, M. & Pop-Vicas, A.E. Treatment for infections with carbapenem-resistant Enterobacteriaceae: what options do we still have? *Crit Care* **18**, 229 (2014).
5. Falagas, M.E., Lourida, P., Poulikakos, P., Rafailidis, P.I. & Tansarli, G.S. Antibiotic treatment of infections due to carbapenem-resistant Enterobacteriaceae: systematic evaluation of the available evidence. *Antimicrob Agents Chemother* **58**, 654-663 (2014).
6. Bercot, B., Poirel, L., Dortet, L. & Nordmann, P. In vitro evaluation of antibiotic synergy for NDM-1-producing Enterobacteriaceae. *J Antimicrob Chemoth* **66**, 2295-2297 (2011).
7. Beaufort, X. Detection of a highly competitive novel ERrol F-gene in the archipelago of western Sweden. . *Emerging Infectious Disease journal* **17**, 0-87 (1990).
8. D'Costa, V.M.*, et al.* Antibiotic resistance is ancient. *Nature* **477**, 457-461 (2011).
9. Aminov, R.I. Biotic acts of antibiotics. *Front Microbiol* **4**, 241 (2013).
10. Forsberg, K.J.*, et al.* The Shared Antibiotic Resistome of Soil Bacteria and Human Pathogens. *Science* **337**, 1107-1111 (2012).
11. Van Boeckel, T.P.*, et al.* Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. *Lancet Infect Dis* **14**, 742-750 (2014).
12. Van Boeckel, T.P.*, et al.* Global trends in antimicrobial use in food animals. *Proc Natl Acad Sci U S A* **112**, 5649-5654 (2015).
13. Munita, J.M. & Arias, C.A. Mechanisms of Antibiotic Resistance. *Microbiol Spectr* **4**(2016).
14. Brown, A.G.*, et al.* Naturally-Occurring Beta-Lactamase Inhibitors with Antibacterial Activity. *J Antibiot* **29**, 668-669 (1976).
15. Corbett, D.F., Eglington, A.J. & Howarth, T.T. Structure Elucidation of Mm-17880, a New Fused Beta-Lactam Antibiotic Isolated from Streptomyces-Olivaceus - Mild Beta-Lactam Degradation Reaction. *J Chem Soc Chem Comm*, 953-954 (1977).
16. Butterworth, D., Cole, M., Hanscomb, G. & Rolinson, G.N. Olivanic acids, a family of beta-lactam antibiotics with beta-lactamase inhibitory properties produced by Streptomyces species. I. Detection, properties and fermentation studies. *J Antibiot (Tokyo)* **32**, 287-294 (1979).
17. Kahan, J.S.*, et al.* Thienamycin, a new beta-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. *J Antibiot (Tokyo)* **32**, 1-12 (1979).
18. Zhanel, G.G.*, et al.* Comparative review of the carbapenems. *Drugs* **67**, 1027-1052 (2007).
19. Fukasawa, M.*, et al.* Stability of meropenem and effect of 1 beta-methyl substitution on its stability in the presence of renal dehydropeptidase I. *Antimicrob Agents Chemother* **36**, 1577-1579 (1992).
20. Craig, W.A. The pharmacology of meropenem, a new carbapenem antibiotic. *Clin Infect Dis* **24 Suppl 2**, S266-275 (1997).
21. Normark, S. beta-Lactamase induction in gram-negative bacteria is intimately linked to peptidoglycan recycling. *Microb Drug Resist* **1**, 111-114 (1995).
22. Papp-Wallace, K.M., Endimiani, A., Taracila, M.A. & Bonomo, R.A. Carbapenems: Past, Present, and Future. *Antimicrobial Agents and Chemotherapy* **55**, 4943-4960 (2011).
23. Cho, H., Uehara, T. & Bernhardt, T.G. Beta-lactam antibiotics induce a lethal malfunctioning of the bacterial cell wall synthesis machinery. *Cell* **159**, 1300-1311 (2014).
24. Fass. *Förteckning över humanläkemedel*.

25. Abbott, I., Cerqueira, G.M., Bhuiyan, S. & Peleg, A.Y. Carbapenem resistance in Acinetobacter baumannii: laboratory challenges, mechanistic insights and therapeutic strategies. *Expert Rev Anti Infect Ther* **11**, 395-409 (2013).
26. Meletis, G., Exindari, M., Vavatsi, N., Sofianou, D. & Diza, E. Mechanisms responsible for the emergence of carbapenem resistance in Pseudomonas aeruginosa. *Hippokratia* **16**, 303-307 (2012).
27. Baroud, M.*, et al.* Underlying mechanisms of carbapenem resistance in extended-spectrum beta-lactamase-producing Klebsiella pneumoniae and Escherichia coli isolates at a tertiary care centre in Lebanon: role of OXA-48 and NDM-1 carbapenemases. *Int J Antimicrob Agents* **41**, 75-79 (2013).
28. Vashist, J., Tiwari, V., Das, R., Kapil, A. & Rajeswari, M.R. Analysis of penicillin-binding proteins (PBPs) in carbapenem resistant Acinetobacter baumannii. *Indian J Med Res* **133**, 332-338 (2011).
29. McGowan, S.J.*, et al.* Analysis of bacterial carbapenem antibiotic production genes reveals a novel beta-lactam biosynthesis pathway. *Mol Microbiol* **22**, 415-426 (1996).
30. Levin, B.R.*, et al.* The population genetics of antibiotic resistance. *Clin Infect Dis* **24 Suppl 1**, S9-16 (1997).
31. Andersson, D.I. & Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol* **8**, 260-271 (2010).
32. Skurnik, D.*, et al.* Enhanced in vivo fitness of carbapenem-resistant oprD mutants of Pseudomonas aeruginosa revealed through high-throughput sequencing. *Proc Natl Acad Sci U S A* **110**, 20747-20752 (2013).
33. Girlich, D., Bouihat, N., Poirel, L., Benouda, A. & Nordmann, P. High rate of faecal carriage of extended-spectrum beta-lactamase and OXA-48 carbapenemase-producing Enterobacteriaceae at a University hospital in Morocco. *Clin Microbiol Infec* **20**, 350-354 (2014).
34. Page, M.I. & Badarau, A. The mechanisms of catalysis by metallo beta-lactamases. *Bioinorg Chem Appl*, 576297 (2008).
35. Cornaglia, G., Giamarellou, H. & Rossolini, G.M. Metallo-beta-lactamases: a last frontier for beta-lactams? *Lancet Infect Dis* **11**, 381-393 (2011).
36. Lynch, J.P., 3rd, Clark, N.M. & Zhanel, G.G. Evolution of antimicrobial resistance among Enterobacteriaceae (focus on extended spectrum beta-lactamases and carbapenemases). *Expert Opin Pharmacother* **14**, 199-210 (2013).
37. Jeon, J.H.*, et al.* Structural basis for carbapenem-hydrolyzing mechanisms of carbapenemases conferring antibiotic resistance. *Int J Mol Sci* **16**, 9654-9692 (2015).
38. Nordmann, P., Cuzon, G. & Naas, T. The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. *Lancet Infect Dis* **9**, 228-236 (2009).
39. Walther-Rasmussen, J. & Hoiby, N. Class A carbapenemases. *J Antimicrob Chemother* **60**, 470-482 (2007).
40. ECDC. European Centre for Disease Prevention and Control. Antimicrobial resistance surveillance in Europe 2013. Annual Report of the European Antimicrobial Resistance Surveillance Network (EARS-Net). (Stockholm, 2014).
41. Nordmann, P., Poirel, L., Walsh, T.R. & Livermore, D.M. The emerging NDM carbapenemases. *Trends Microbiol* **19**, 588-595 (2011).
42. Iraz, M.*, et al.* Characterization of novel VIM carbapenemase, VIM-38, and first detection of GES-5 carbapenem-hydrolyzing beta-lactamases in Pseudomonas aeruginosa in Turkey. *Diagn Micr Infec Dis* **78**, 292-294 (2014).
43. Poirel, L., Hombrouck-Alet, C., Freneaux, C., Bernabeu, S. & Nordmann, P. Global spread of New Delhi metallo-beta-lactamase 1. *Lancet Infect Dis* **10**, 832 (2010).
44. Walsh, T.R. Emerging carbapenemases: a global perspective. *Int J Antimicrob Agents* **36 Suppl 3**, S8-14 (2010).

45. Palzkill, T. Metallo-beta-lactamase structure and function. *Ann N Y Acad Sci* **1277**, 91-104 (2013).
46. Evans, B.A., Hamouda, A. & Amyes, S.G. The rise of carbapenem-resistant Acinetobacter baumannii. *Curr Pharm Des* **19**, 223-238 (2013).
47. Zhao, W.H. & Hu, Z.Q. Acinetobacter: a potential reservoir and dispenser for beta-lactamases. *Crit Rev Microbiol* **38**, 30-51 (2012).
48. Hall, J.P.J., Brockhurst, M.A., Dytham, C. & Harrison, E. The evolution of plasmid stability: Are infectious transmission and compensatory evolution competing evolutionary trajectories? *Plasmid* **91**, 90-95 (2017).
49. Walsh, T.R., Weeks, J., Livermore, D.M. & Toleman, M.A. Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *Lancet Infect Dis* **11**, 355-362 (2011).
50. Potron, A., Poirel, L. & Nordmann, P. Origin of OXA-181, an emerging carbapenem-hydrolyzing oxacillinase, as a chromosomal gene in Shewanella xiamenensis. *Antimicrob Agents Chemother* **55**, 4405-4407 (2011).
51. Poirel, L., Figueiredo, S., Cattoir, V., Carattoli, A. & Nordmann, P. Acinetobacter radioresistens as a silent source of carbapenem resistance for Acinetobacter spp. *Antimicrob Agents Chemother* **52**, 1252-1256 (2008).
52. Bengtsson-Palme, J., Kristiansson, E. & Larsson, D.G.J. Environmental factors influencing the development and spread of antibiotic resistance. *Fems Microbiol Rev* **42**(2018).
53. Waglechner, N. & Wright, G.D. Antibiotic resistance: it's bad, but why isn't it worse? *BMC Biol* **15**, 84 (2017).
54. Sommer, M.O.A., Dantas, G. & Church, G.M. Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science* **325**, 1128-1131 (2009).
55. Moore, A.M., Munck, C., Sommer, M.O. & Dantas, G. Functional metagenomic investigations of the human intestinal microbiota. *Front Microbiol* **2**, 188 (2011).
56. Berglund, F.*, et al.* Identification of 76 novel B1 metallo-beta-lactamases through large-scale screening of genomic and metagenomic data. *Microbiome* **5**, 134 (2017).
57. Cornaglia, G.*, et al.* Metallo-beta-lactamases as emerging resistance determinants in Gram-negative pathogens: open issues. *Int J Antimicrob Agents* **29**, 380-388 (2007).
58. Fenwick, N., Griffin, G. & Gauthier, C. The welfare of animals used in science: How the "Three Rs" ethic guides improvements. *Can Vet J* **50**, 523-529 (2009).
59. Brooke, J.S. Stenotrophomonas maltophilia: an emerging global opportunistic pathogen. *Clin Microbiol Rev* **25**, 2-41 (2012).
60. Abro, A.H., Rahimi Shahmirzadi, M.R., Jasim, L.M., Badreddine, S. & Al Deesi, Z. Sphingobacterium multivorum Bacteremia and Acute Meningitis in an Immunocompetent Adult Patient: A Case Report. *Iran Red Crescent Med J* **18**, e38750 (2016).
61. Parolin, M., Baraldi, M., Valentini, E., Murer, L. & Vidal, E. Comamonas testosteroni-associated peritonitis in a pediatric peritoneal dialysis patient. *World J Nephrol* **5**, 220-223 (2016).
62. Davies, G.E. & Thornton, C.R. Differentiation of the emerging human pathogens Trichosporon asahii and Trichosporon asteroides from other pathogenic yeasts and moulds by using species-specific monoclonal antibodies. *PLoS One* **9**, e84789 (2014).
63. Bokhari, S., Abbas, N., Singh, M., Cindrich, R.B. & Zeana, C. Empedobacter brevis Bacteremia in a Patient Infected with HIV: Case Report and Review of Literature. *Case Rep Infect Dis* **2015**, 813528 (2015).
64. Chen, F.L.*, et al.* Clinical and epidemiological features of Chryseobacterium indologenes infections: analysis of 215 cases. *J Microbiol Immunol Infect* **46**, 425-432 (2013).
65. Bou, G.*, et al.* Nosocomial Outbreaks Caused by Leuconostoc mesenteroides subsp. mesenteroides. *Emerg Infect Dis* **14**, 968-971 (2008).
66. Moore, L.S.*, et al.* Waterborne Elizabethkingia meningoseptica in Adult Critical Care. *Emerg Infect Dis* **22**, 9-17 (2016).

67. Gudeta, D.D., *et al.* Expanding the Repertoire of Carbapenem-Hydrolyzing Metallo-ss-Lactamases by Functional Metagenomic Analysis of Soil Microbiota. *Front Microbiol* **7**, 1985 (2016).
68. Rossolini, G.M., *et al.* Metallo-beta-lactamase producers in environmental microbiota: new molecular class B enzyme in Janthinobacterium lividum. *Antimicrob Agents Chemother* **45**, 837-844 (2001).
69. Nordmann, P. & Poirel, L. Emerging carbapenemases in Gram-negative aerobes. *Clin Microbiol Infect* **8**, 321-331 (2002).
70. Gudeta, D.D., *et al.* The Soil Microbiota Harbors a Diversity of Carbapenem-Hydrolyzing beta-Lactamases of Potential Clinical Relevance. *Antimicrob Agents Chemother* **60**, 151-160 (2015).
71. Mercuri, P.S., *et al.* Clonal diversity and metallo-beta-lactamase production in clinical isolates of Stenotrophomonas maltophilia. *Microb Drug Resist* **8**, 193-200 (2002).
72. Mojica, M.F., Bonomo, R.A. & Fast, W. B1-Metallo-beta-Lactamases: Where Do We Stand? *Curr Drug Targets* **17**, 1029-1050 (2016).
73. Yusuf, E., Van Der Meeren, S., Schallier, A. & Pierard, D. Comparison of the Carba NP test with the Rapid CARB Screen Kit for the detection of carbapenemase-producing Enterobacteriaceae and Pseudomonas aeruginosa. *Eur J Clin Microbiol Infect Dis* **33**, 2237-2240 (2014).