DanCARD

MID-TERM REPORT

2010-2013

The Danish Centre for Antibiotic Research and Development

STATENS SERUM INSTITUT

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| CONTACTS

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| PREFACE

The DanCARD project has now been running for three plus years. All PhD students planned have been appointed and have started or - for some early projects - are in their final write-up process. As viewed from the literature list including abstracts and master theses the project is successful in producing published science. Many antimicrobial compounds – mostly peptides or related molecules – are being tested both in vitro and in vivo, and some products appear very interesting in their ability to kill Gram negative pathogens, the group of bacteria with the most urgent need for novel treatment moeties. We still hope, that the project will succeed in finding one or two compounds, which can move further towards preclinical testing. Further, the work on improving "old" antibiotics also moves ahead with first expanded characterisation of the PKPD of these molecules, which have not been tested previously. Many stumbling stones have been detected such as difficulties in accessing compound material for testing (fosfomycin) or difficulties in solubilizing the compound and measuring plasma concentrations or molding with nanomedicine (nitrofurantoin), but most of this has been overcome and interesting results are under way.

The project has succeeded in excellent collaboration among the partners involved, which has been nurtured by the spring- and fall meetings in the DanCARD group with high and fruitful attendance by the partners for all meetings.

We are looking forward to the many more results, not the least from externally funded projects, which have been inspired by the DanCARD project.

Project Head
Professor Niels Frimodt-Møller

| SCIENTIFIC REPORTS FROM COLLABORATORS

Karen A. Krogfelt
Department of Microbiology and Infection Control, Statens Serum Institut (SSI)

The maggot defensin Lucifensin:
The structure of the defensin, Lucifensin, produced by *Luciliasericata* (blowfly) maggots, which is used for wound treatments, was characterized by NMR. The peptide was recombinantly produced and purified by SSI and Novozymes A/S in collaboration. The structure was analysed at AAU. This work resulted in a publication and two masters projects.

Bacteria in wounds:
Investigating the bacterial colonization of wounds: By comparing results obtained using three different sampling methods, it was found that biopsy sampling is not needed in order to identify the bacterial species present in a wound. Data were published and well received by the community; the study was commented by John G. Bartlett, Professor of Medicine, Johns Hopkins University as follows: "I am writing about your magnificent paper on wound sampling for cultures. The reason is that I plan to report it at the annual meeting of the American College of physicians as one of the best publications in the field of infectious diseases. I like it because the bacteriology was so well done, and the issue addressed is such a common source of confusion in clinical care…" The nasal cavity has been suggested to be the source for contamination of wounds with *Staphylococcus aureus*. PFGE analysis revealed that patients harbouring *S. aureus* both in the nasal cavity and the leg ulcer had the same type both places. This study was recently published.

Cytotoxicity and antimicrobial activity:
A range of *in vitro* cytotoxicity assays has been established. Antimicrobial peptoids, discovered by DanCARD partners, are currently being investigated for effects on apoptosis, cell viability and proliferation.

The antibacterial activity of a lysophospholipid is under investigation. The compound is effective against *Staphylococcus aureus* strains (including methicillin resistant strains) and enhances the antibiotic activity of ampicillin against *Pseudomonas aeruginosa*. Also the cytotoxicity of this compound was tested. A master student is enrolled.

Microbial resistance to ionic silver: and natural compounds
Clinically relevant bacterial isolates have been screened for the occurrence of silver resistant isolates. No resistant isolates were identified; however, future monitoring is necessary to secure safe further use of ionic silver as an antimicrobial additive.
Results were presented at an international conference and published.

The anti-adhesive capacity of extracts from tea, mushrooms and fish was tested. *Helicobacter pylori* was used as a model organism. Publication is being prepared

Luca Guardabassi,
Department of Veterinary Disease Biology, HEALTH, University of Copenhagen (UCPH-HEALTH)

At the Department of Veterinary Disease Biology, the two aims of the project are to i) rationalize use of existing antibiotics in veterinary medicine and ii) develop non-antibiotic alternatives for prevention and treatment of bacterial infections in animals. The project has progressed without major deviations from the original plan. Additional funding by an EU project has allowed extension of the original plan and inclusion of new research activities that were not described in the application. The results achieved in the first part of the project can be summarized as follow:

1) Based on in vitro activity against *Clostridium difficile* and *Clostridium perfringens*, five commercial probiotic strains belonging to Lactobacillus (n=4) and Bifidobacterium (n=1) were selected for further in vitro and in vivo testing. Four of them were shown to reduce *C. perfringens* beta2 toxin expression by 9-10-fold and one additionally reduced expression of alpha toxin. A probiotic prototype for equine use containing a mixture of these strains is being manufactured in collaboration with the industrial partner Clerici-Sacco Group. In 2013, the probiotic prototype will be tested by a randomized placebo controlled clinical trial in neonatal foals, to determine whether probiotic administration may reduce the incidence of diarrhea and antibiotic use in horse farms.

2) In vitro and in vivo pharmacokinetic /pharmacodynamics (PKPD) data of tetracyclines, nitrofurantoin and phenicols were generated to optimize treatment regimens in dogs. The results show that some of these antibiotics are promising candidates for treatment of canine infections caused by multidrug-resistant (MDR) bacteria. As these older drugs have narrower spectrum compared to antibiotics commonly used in small animal practice, this research is expected to impact both animal health (by providing new therapeutic options) and public health (by reducing veterinary use of critically important antibiotics in human medicine). As a spin-off of the study, a new veterinary breakpoint for doxycycline is going to be approved by the Clinical Laboratory Standards Institute (CLSI).

3) The in vitro activity of 10 new peptoid compounds synthesized at (UCPH-HEALTH) was tested against the main pathogens causing skin infection and otitis in dogs. Two leads displaying the best antimicrobial activity and the lowest toxicity on red blod cells were selected for further characterization. Time killing studies and cytotoxicity assays indicated rapid bactericidal activity and strong selective toxicity, respectively. A pilot mice skin infection experiment failed to provide evidence of therapeutic effect, probably due to the low concentration of peptoid used or to an inhibitory effect of the gel formulation used. The next steps will be to optimise dosage and formulation.

4) Seven lytic and six lysogenic phages able to lyse MDR *Staphylococcus pseudintermedius* were isolated and characterized as members of the Syphoviridae family by electron microscopy and genome sequencing. Variable lysis patterns were observed among methicillin-susceptible strains, indicating that the isolated phages have selective bactericidal effect against MDR strains. This is a very promising result as these phages could enable treatment of infection without harming the commensal staphylococcal flora.

5) A pilot study on the possible use of helper drugs to inhibit plasmid transfer during antibiotic therapy was started in October 2012. To follow in real-time how transfer of plasmids carrying ESBL genes changes in response to cefotaxime, promoters of the genes responsible for plasmid transfer (tra operons) were fused with a gfp-marker. Preliminary results show that for some plasmids expression of transfer genes is enhanced by exposure to cefotaxime. This observation is very interesting as it suggests that cephalosporins may favour spread of ESBLs not only by antibiotic selective pressure but also by enhancing plasmid transfer. As the next steps, these preliminary results will be confirmed by testing more plasmids and cephalosporins, and the genes that are involved in transfer regulation will be identified.

6) A new porcine intestinal model was developed in collaboration with another project funded by the Strategic Research Council (Miniresist). The model involves surgical attachment of the caecum to the abdominal wall, allowing easy access to caecal content by needle insertion through the skin. This model is a useful tool for studying horizontal gene transfer and antimicrobial effects in the gut microbiome.

Paul Robert Hansen
Department of Natural Sciences and Environment, HEALTH, University of Copenhagen (UCPH-HEALTH)

The primary aim of our work is to identify anoplin analogs which selectively target Gram negative species such as *E. coli*. To this end, we have synthesized and purified 65 anoplin analogs and subsequently characterized them with regard to their activity against five microorganisms in cooperation with SSI. Furthermore, their hemolytic activities have been determined. Several peptides with unprecedented activity and specificity towards *E. coli* relative to other microorganisms as well as human red blood cells have been identified.

In collaboration with Dr. Wimmer´s group at University of Aalborg (AAU), we have shown that the process of optimization of anoplin analog properties should abide certain rules; the balance between charged and hydrophobic residues is important, and while the size of the hydrophobic residues governs membrane affinity, the polar amino acid composition determines antimicrobial specificity.
Applying statistical design and analysis strategies to the biological data obtained from the anoplin analogs has revealed that mathematical relations exist between amino acid sequence and biological activity. We have shown that biological activity measurements from a designed subset of amino acid combinations can be used to reliably predict the biological activities of other combinations. This makes synthesis of all amino acid combinations unnecessary, thus shortcutting to the peptide with desired properties, saving time and other precious resources.
We have found that very discrete modifications of a central amino acid can dramatically affect biological activity. This has been investigated in cooperation with University of Aarhus using molecular dynamics simulations in a system of water, ions and lipid bilayers resembling either the bacterial membrane or the red blood cell membrane. A clear effect of the subtle modifications is evident, observable as varying mechanisms of peptide attack on the membranes. A relation between the rotational behavior of the varied central amino acid side chain and the hemolytic activity has been identified, thus possibly providing atomic-level explanation of the hemolytic properties of peptides in general.
In collaboration with Dr. Peter Damborg and Professor Luca Guardabassi, 10 peptide-peptoid hybrids have been synthesized and tested against *E. coli*, *S. aureus* and *S. pseudintermedius* as potential veterinary antibiotics. Two of these compounds were tested against 50 *S. pseudintermedius* and *Pseudomonas aeruginosa* isolates. Time kill curves showed that the peptide-peptoid hybrid killed the bacteria at 4 x MIC within 30 and 120 min respectively. These compounds are now being developed into veterinary antibiotics in the project FP7-PEOPLE-2011-ITN TRAIN-ASAP ("Training Research AImed at Novel Antibacterial Strategies in Animals and People") an interdisciplinary collaboration between 7 research groups from various institutions and Pfizer. The ITN is headed by Professor Luca Guardabassi (UCPH-HEALTH).

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Hanne Mørck Nielsen & Henrik Franzyk
Department of Pharmacy & Department of Drug Design and Pharmacology, HEALTH, University of Copenhagen (UCPH-HEALTH)

Department of Pharmacy (previously Department of Pharmaceutics and Analytical Chemistry) at UCPH-HEALTH mainly address the aims 1) to develop new antibiotics and 2) to enhance the activity of present antibiotics by improving targeting to (intracellularly residing) bacteria as well as improving the selective effect on bacteria. Activities are related to WP2a (incorporation of antimicrobial compounds in nanoparticles) and WP2b (improvement of drug delivery at the target site). Within the centre, this is done in collaboration with Henrik Franzyk at Department of Drug Design and Pharmacology and Anne Sandberg-Schaal, Hvidovre Hospital (previously SSI). Additional collaboration with national and international scientists has been initiated. At department of Pharmacy, UCPH-HEALTH, two PhD students have been employed; their PhD projects are currently ongoing. Further, two master student have been involved in the project (one complete, on ongoing).

New types of antibiotics with improved delivery to the target site may be achieved by optimized design of e.g. peptides with antimicrobial effect. Thus, a series of different analogs of the cell-penetrating peptide (CPP), penetratin, have been synthesized and their antimicrobial effect evaluated towards Gram positive and Gram negative bacteria and related to their eukaryotic cell membrane-penetrating efficacy and cellular toxicity. In addition, approaches to evaluate how peptide sequences and structure correlated to the selective prokaryote effect was investigated (Bahnsen et al., BBA biomembranes, 2012). Likewise, another selection of antimicrobial peptides (AMPs) and peptidomimetics with well-known antimicrobial effect have been selected, synthesized and tested for their effect on bacteria and eukaryote cells; initial studies demonstrate efficient uptake of some of these into eukaryotic cells in vitro. Studies on their uptake mechanisms are ongoing. The most interesting peptides/peptidomimetics will be evaluated with regards to their potential in killing intracellularly residing *S. aureus* by use of an intracellular infection model of infected airway cells, which has currently been established in the laboratory. This model has been used for evaluating the killing effect of the previously mentioned CPPs on intracellular bacteria. Overall, by mechanistically distinguishing the CPP effect from the AMP effect of selected peptides/peptidomimetics, we expect to be able to aid the identification of peptides/peptidomimetics with optimal properties for targeting intracellular bacteria.

Another approach of improving efficient delivery to target involves formulation of novel as well as well-known antibiotics into nanoparticles. Thus, poly-lactide-co-glycolic acid-based (PLGA) nanoparticles (NPs) have been loaded with different antibiotics (ciprofloxacin, fosfomycin and vancomycin) for treatment of urinary tract infections (*E. coli*). Formulation efficacy will initially be assessed (by Hvidovre/SSI) using an in vitro intracellular infection model, where *E. coli* is opsonized into bladder epithelial cell monolayers and subsequently treated with the formulation, and then efficacy will be compared to free drug. Formulation of a well-known antimicrobial peptide in PLGA NPs have shown to slightly increase the bacterial killing in the airway infection model described above; additional mechanistic experiments are currently ongoing. Preparation and characterization of liposome formulations of antibiotics, as well as of polymer-based NPs (by microfluidics) has recently been initiated. Further, testing of metal oxide NPs as antibiotics is planned in near future.

Department of Drug Design and Pharmacology (previously Department of Medicinal Chemsitry) at UCPH-HEALTH addresses the aim of developing new antibiotic peptidomimetics, and thus our activities are mainly related to WP1 (Development of novel antibiotics). The microbiological evaluation of the synthesized compounds is performed via collaboration with SSI and Hvidovre Hospital (Anne Sandberg-Schaal and Niels Frimodt-Møller). In addition the peptidomimetics are examined for their cytotoxicity in collaboration with Hanne Mørck Nielsen at Department of Pharmacy. At Department of Drug Design and Pharmacology, UCPH-HEALTH, one PhD student has been employed, and this PhD project is currently ongoing.

In this PhD project we have focused on performing the first comparative study of several different peptidomimetic backbones displaying the same sequence of side chains (Jahnsen et al.; J. Med. Chem. 2012). This involved preparation of several suitably protected building blocks, which subsequently were utilized in the microwave-assisted solid-phase synthesis of an array of peptidomimetics corresponding to a reference α-peptide sequence. In addition, the all-D form of this reference peptide was included in the study as well in order to estimate the effect of partial enzymatic degradation of the natural peptide. Both homomeric peptidomimetics and hybrids consisting of alternating natural alfa-amino

acids and unnatural residues were included in the array consisting of eleven compounds. These were examined with respect to antimicrobial activity, hemolysis, and cytotoxicity. In addition CD-spectroscopic studies were performed in buffer with and without the presence of liposomes mimicking either human membranes or bacterial membranes. In contrast to the inactive peptides, most peptidomimetic compounds exhibited antibacterial activity with minimal inhibitory concentrations (MIC) of 2-16 μM towards the Gram-negative *E. coli* (including ESBL and NDM-1 strains), while low or no activity was found for *Klebsiella pneumoniae* and the Gram-positive *Staphylococcus aureus* (methicillin-resistant strain: MRSA). The degree of secondary structure as indicated by the CD-spectra correlated well with the cytotoxicity towards HeLa cells, whereas the trend was much weaker for hemolysis and antibacterial effects.

Currently, another library of twelve compounds, displaying chain lengths of 10-14 residues, are undergoing evaluation of their antibacterial (six multidrug resistant bacteria) and cytotoxicity towards both cancer-related (immortalized) and benign cells (6-7 cell lines) in order to determine the chain length that gives rise to the optimal therapeutic index. Yet another library is being prepared with the purpose of examining the effect of end-group modification of the N-terminus, and initial test of some members indicate that the selectivity between Gram-negative and Gram-positive bacteria may be interchanged depending of the type of moiety introduced. Finally, a solid-phase methodology enabling gram-scale synthesis of such peptidomimetics is under development.

In collaboration with Department of Microbiology and Immunology at UBC (Visiting scholar R. Jahnsen), peptidomimetics are examined regarding (i) immunomodulatory activity, (ii) antibiofilm activity and (iii) direct antibacterial activity.

Anders Løbner Olesen
Department of Biology, Functional genomics, BIO, University of Copenhagen (UCPH-BIO)

Our research is focused on identification of small circular peptides interfering with the protein-protein interaction in the replication machinery of *S. aureus*. We believe that such compounds will inhibit replication and thus result in cessation of bacteria growth. We have identified several putative targets in the bacterial replisome. A reverse bacterial two hybrid screen based on 5-FOA selection of pyrF- cells was used to screen two of our intracellular libraries of small cyclic peptides. We identified several peptides that prevented interaction of DnaN-DnaN (the beta clamp) and DnaB-DnaB (the helicase loader). The structure of the purified peptides was verified by mass spectrometry. Exposure *S. aureus* to purified peptides results in cessation of DNA synthesis, growth attenuation and enlargement the cells. The MIC of the most potent peptides is approximately 20-50 microgram/ml against *S. aureus*. The peptides also inhibit growth of two other gram positive organisms, *S. epidermidis* and *B. subtilis*, but did not have any effect *E. coli*. In collaboration with Paul Robert Hansen at UCPH-HEALTH we have synthesized some of the identified peptides although in small amounts. We are currently optimizing the procedure.

Reinhard Wimmer
Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University (AAU)

Structure-activity relationships of new antibiotics:
A series of anoplin and anoplin derivatives were synthesized in collaboration with Paul Robert Hansen's group and tested both for antimicrobial and hemolytic activity. The high-resolution structures of a number of candidates were solved and their interaction with lipid mimics were elucidated. A trend linking lipid interaction with antimicrobial activity was discovered. Based on this trend, new, potentially improved anoplin derivatives have been designed, synthesized and are currently being tested for activity.

Structures of peptoids:
Peptoid derivatives of anoplin were synthesized in collaboration with Paul R. Hansen (UCPH-HEALTH) and NMR studies on the peptoids showed that the variants suffered from the presence of multiple conformers, presumable cis-trans isomerism around the peptide bond leading to the N-substituted glycine. Therefore, a different set of peptoids were designed and are currently being investigated. The structures show interesting features of these novel type of compounds, where no structural information has ever been reported in the literature before.

Structure of the insect defensin lucifensin:
Lucifensin, a 40 amino acid defensing from *Lucilia sericata*, had previously been identified as a key player in fighting off infections during maggot debridement therapy. We have solved the 3D structure of lucifensin. The structure follows the classical defensing motif. Analysis of the structure revealed an orientation of amino acids resembling the homologue sapecin, which has been shown to permeabilize bacterial membranes. However, as lucifensin had previously been shown to act by binding lipid II, the question remains whether lucifensin has two modes of action. The studies were made in collaboration with Anders S. Andersen and Karen A. Krogfelt (Novozymes/SSI).

Metabolic profiling of pathogenic bacteria:
Pathogenic bacteria grown in different media (Müller-Hinton broth, synthetic urine) were subjected to metabolic fingerprinting. A series of pathogens could easily be detected from fingerprinting of the biomass and the medium. This can potentially be developed into a rapid urine test for pathogen identification

Søren Molin
Center for Systems Microbiology, Technical University of Denmark (DTU)

Bacterial small regulatory RNAs (sRNAs) function in post-transcriptional control of gene expression and control a variety of processes including metabolic reactions, stress responses and pathogenesis in response to environmental signals or cell stress, such as antibiotics. We have used RNA sequencing (RNA-seq) to identify novel transcripts in *Pseudomonas aeruginosa* involving a combination of three different sequencing libraries. The RNA sequenced was harvested under different stress conditions, including the most used antibiotics in clinical treatments. Using our approach, we could identify over 500 novel intergenic sRNAs and around 250 antisense sRNAs. We anticipate that the data will be useful for the study of regulatory sRNAs in bacteria and that the approach described here may be applied to identify sRNAs in any bacterium under different growth and stress conditions.

Currently, our efforts are directed to characterize 5 sRNAs that might be involved in antibiotic tolerance. Two of these sRNAs are intergenic and found to be highly expressed during fluoroquinolone treatment and oxidative stress. They are suspected to control the expression of pyocins. Pyocins are inducible bacteriocins produced by *P.aeruginosa*, which are released by bacterial lysis, specifically attach to the cell surface of susceptible bacteria (belonging to the same species) and provoke their death. The other 3 sRNAs under study are antisense to already-annotated genes known to affect antibiotic susceptibility and virulence: the chromosomal replication initiator protein *dnaA*, the transcriptional regulator gene *lasR,* and the penicillin-binding protein 1A gene *ponA*.

Niels Frimodt-Møller & Klaus Skovbo Jensen
Department of Clinical Microbiology, Copenhagen University Hospital, Hvidovre
Department of Biomedical Sciences, HEALTH, The University of Copenhagen (UCPH-HEALTH)

Improvement of dosing of antibiotics in patients Cefuroxime:
A pharmacokinetic study of administration of intravenous cefuroxime in 2 different doses has been performed in 20 volunteers. Each volunteer received 2 doses with en interval of 14 days. Ten plasma samples were taken after injection of each dose within a time frame of 8 hours. Cefuroxime concentrations were determined by bioassay. Plasma protein binding was determined by ultrafiltration. Mathematical analysis is ongoing. Optimal dosing regimens of patients will be estimated by Monte-Carlo simulation. *Phenoxymethyl penicillin*: A similar study as above has been performed with 2 different oral doses of penicillinV in 20 volunteers (2 doses each). Penicillin concentrations have been determined by LCMS at UCPH-HEALTH. Similar mathematical modeling will be performed as above.
Further clinical pharmacokinetics and -dynamics (PKPD) studies are planned for dicloxacillin and penicillinG.

Therapeutic drug monitoring:
Deflected form the human PKPD studies above and combined with the relocation of the research–group to Hvidovre Hospital, preliminary studies on introducing therapeutic drug monitoring of antibiotic treatments in patients at Hvidovre Hospital has been initiated.
Today the recommended antibiotic dosing regimens are based on PK data obtained in healthy volunteers as those described above or less severely ill patients. Therefore, there is a great risk of treatment failure in patients with more complex PK profiles e.g. the critically ill.
Assays for the estimations of serum concentrations are expected to be validated during spring 2013 and the run of the first actual patient samples during summer 2013. Initially, the study will include all bacteremia patients admitted to Hvidovre Hospital and the infection course and outcome of these patients will be assessed from the following parameters and compared to historically data obtained at Hvidovre Hospital since 2006: a) 30-day mortality b) length of hospitalisation c) length of antibiotic treatment d) total use of antibiotics e) number of patients with *C. difficile* associated diarrhoea

PKPD studies of older antibiotics in experimental animal models:
The following older antibiotics with excellent effect against multidrug resistant (e.g. ESBL) Enterobacteriaceae have not been studied for their optimal PKPD, i.e. to estimate the optimal dosing regimens in treatment of urinary tract infections: Temocillin, mecillinam, nitrofurantoin, fosfomycin, ciprofloxacin. An in vivo bladder cell model as well as the mouse urinary tract infection model and the mouse peritonitis model are used for these studies. All 5 antibiotics are under scrutiny at the present. For each antibiotic a scientific paper will be submitted. Temocillin was the subject of a master thesis by Anja Sigl defended in July 2012.

Impact of low-level fluoroquinolone resistance genes qnrA1, qnrB19 and qnrS1 on ciprofloxacin treatment of Escherichia coli urinary tract infection:
From a wild-type (wt) *Escherichia coli* UTI isolate, three isogenic strains were constructed carrying low-level ciprofloxacin resistance genes qnrA1, qnrB19 or qnrS1 (ciprofloxacin MIC range: 0.19-0.38 mg/L). Time-kill studies were performed for all four isogenic strains at the following concentrations: 1×, 2×, 4×, 8× and 16× MIC. In the murine UTI model, mice infected with each of the isogenic qnr strains or the wt strain were treated with ciprofloxacin corresponding to a standard human dose (0.2 mg/mouse) or saline (only the *E. coli* wt) subcutaneously four times daily for 3 days starting 24 h after bacterial inoculation. In vitro, the strains responded to ciprofloxacin concentrations of 4-16× MIC by several log(10) reductions. In vivo, despite ciprofloxacin reaching urine concentrations far exceeding the MICs for the strains (500 mg/L), ciprofloxacin was significantly less efficient at reducing the urine and bladder bacterial counts of qnrA1-, qnrB19- and qnrS1-positive strains compared with the ciprofloxacin-treated wt strain ($P < 0.05$). The results were published in J Antimicrob Chemother 2012.

Testing novel peptides with antimicrobial activity in vitro and in vivo in animal models:
Peptides produced by collaborators at UCPH-HEALTH are tested for their antibacterial activity against selected human pathogens by microtiter assay and time-kill curves. The most interesting compounds are further tested in the mouse peritonitis model or the mouse skin infection model. These investigations are ongoing.
Additionally, an assay to detect the hemolysis activity of antimicrobial peptides has been established as a

part of a selection process for the in vivo studies. So far, 40 potential antimicrobial peptides form the laboratory of Henrik Franzyk (UCPH-HEALTH), have been analysed and studies are still in progress.

Optimal extraction of information from in vitro time-kill experiments:
It has become increasingly clear that the traditional MIC concept as well as PK/PD indices such as AUC and time above MIC are inadequate to fully capture the observed relations between exposure (drug dosage regimens) and effect (cumulated bacterial growth/kill). In this study, we aim at constructing a prototype or framework of mathematical model that will allow us to link together the various concepts/indices and thereby facilitating the interpretation of time-kill data and evaluation of the efficacy of antibiotics.
So far, in the DanCARD project, time-kill data for *E. coli* exposed to cefuroxime or cefotaxime have been utilized to propose a first version of a mechanism-based mathematical model for the observed microbial growth kinetics. Additional time-kill experiments will be conducted e.g. to gain information on various dynamical phenomena related to a sudden decrease in external drug concentration.

ESBL gene determination in E. coli isolates and selection abilities of clinically important antibiotics:
In collaboration with Dennis Schrøder Hansen, Hvidovre Hospital, several extended spectrum β-lactamase (ESBL) producing *E. coli* isolates have been investigated for the occurrence of certain ESBL genes. The presence of ESBL genes was investigated by polymerase chain reaction (PCR) and further sequenced to determine the specific ESBL gene. The results have shown that the CTX-M-15 genes are the most dominant group of ESBL genes amongst *E. coli* isolates. Further studies regarding this project are being conducted at Hvidovre Hospital.
An intestinal colonisation mouse model is being used to study the selective properties of commonly used antibiotics such as ampicillin, dicloxacillin, cefuroxime, cefotaxime, ciprofloxacin, clindamycin, gentamicin and metronidazole. Apart from the change in *E.coli* flora , anaerobes and gram-positive organism are also screened in order to evaluate the effect of these antibiotics on the intestinal flora. A cooperation with Dr. Kluytmans in the Netherlands has been initiated with respect to studying the microbiome of the mouse intestinal flora with a new easy PCR-method. Interesting results have already been presented at several conferences and a scientific publication Is pending.

Mechanisms of fluoroquinolone resistance in E. coli:
The objective of this study was to investigate the occurrence of ciprofloxacin resistance mechanisms, phenotypic co-resistance and if ciprofloxacin resistance was due to clonal spread or to individual mutational. Of the 77 *E. coli* isolates investigated, the majority was resistant to ciprofloxacin (91%) and multiresistant (90%). A significant, positive correlation was found regarding MIC for ciprofloxacin and the number of target mutations and efflux was found as a resistance mechanism in 77% of isolates tested (n=60). No overall clonal relationship among isolates was found according to PFGE and target modification was the dominating fluoroquinolone resistance mechanism. Increasing ciprofloxacin resistance in *E. coli* is mainly due to mutational events and not to spread of clones. Results have been accepted for publication in Microbial Drug Resistance, February 2011.
Furthermore, an experimental urinary tract infection in mice was used to show the clinical efficacy of ciprofloxacin on infections caused by *E.coli* carrying the newly detected *qnr*-genes conferring low-level resistance toewrds ciprofloxacin. The study showed, that in spite of high concentrations of ciprofloxacin in the urine and tissues, the low-level resistant E. coli were not removed from the urinary tract. The results were published in Journal of Antimicrobial Chemotherapy in 2012.

In vitro-antagonism between β-lactams and macrolides.
A master thesis has been conducted investigating the potential antagonism between β-lactams and macrolides when used against *Streptococcus pneumoniae*. Pneumonia is normally treated empirically with a regimen of a β-lactam and a macrolide to cove both typical and atypical etiologies. However antagonism between the two types of antibiotics is suspected and could have a negative impact on treatment outcome. The in vitro studies showed that antagonism occurred but only when certain conditions were meet e.g. high concentrations of the β-lactams combined with low concentrations of the macrolide close to the MIC value of the causing pathogen which gives food for thoughts when considering the dosing regimens of the two drugs. The in vitro study also showed that the risk of antagonism increased when the macrolide were applied 2 hrs before the β-lactam. The studies also revealed that the occurrence of antagonism depended on the specific drug combination and the bacterial strain.

The importance of human antimicrobial peptides in the urinary tract for defence against infection (UTI) in the bladder:
In a clinical study 50 women with *E. coli* UTI and 50 women, who had never had UTI, were screened for the presence of *E. coli* clones in their rectal flora, which serves as the origin for bladder infection. Urine was sampled as well and the human antimicrobial peptide cathelicidin was measured in urine and compared between infected and control individuals. We found a lower cathelicidin concentration in the infected women after infection than in controls indicating, that low production of this peptide may cause increased tendency for UTI. The faecal and UTI strains have been full-genome sequenced at the Broad Institute in Boston, and bioinfomatic evaluation is ongoing to evaluate differences in virulence and other genetic factors in *E. coli* causing infection or not, as well as studies of genes conferring resistance towards cathelicidin. These studies will hopefully allow us to understand the pathogenesis of *E. coli* UTI including the ability of the host to defend itself against infection, which again may be used to enhance the natural defence in order to avoid antibiotic treatment.

Investigation of silver resistance in clinical and non-clinical isolates:
The aim of this study was to investigate the occurrence of silver resistance in bacterial isolates from a collection of 349 clinical isolates, including historical isolates. Isolates with varying antimicrobial susceptibility, i.e. ranging from fully susceptible to multidrug resistant, were chosen from each group of isolates. Minimal inhibitory concentrations (MICs) for silver nitrat were determined (2–128 mg/L) for all isolates by agar dilution assay. The MIC values for all isolates, regardless of species, origin or time of sampling, ranged between 6 mg/L and 16 mg/L and were interpreted in the silver-susceptible category. Interestingly, none of the isolates exposed to silver exhibited resistance. The results were published in Int J Antimicrob Agents 2011.

Evaluation of the association between E. coli from production animals, meat with E. coli causing urinary tract infections (UTI) in humans:
A total of 964 geographically and temporally matched *E. coli* isolates from UTI patients, community-dwelling humans, Danish and imported broiler chicken meat, healthy Danish broiler chickens, Danish and imported pork and healthy Danish pigs were investigated for phenotypic antimicrobial resistance, for phylogroups (A, B1, B2, D), ExPEC-related virulence genes, virulence in a murine model of ascending UTI Clonal group A (CgA) status, and clonal relationship. Results showed that food animals and fresh retail meat are sources of B2 and D isolates (strongly associated with UTI) including invasive human Clonal group A isolates. Meat and animal isolates are similar to human isolates with respect to resistance and virulence genes. Further, animal and meat isolates can cause infection in bladder and kidneys in a murine model providing evidence that UTI can be a zoonosis. Finally a PFGE typing showed a clonal link between E. coli of veterinary origin with E. coli from healthy humans and a patient with UTI. Results have been reported in Foodborne Pathog Dis 2010, Int J Food Microbiol 2010, J Clin Microbiol 2010, App Environ Microbiol 2010, J Med Microbiol 2011, Eur J Clin Microbiol Infect Dis 2012.

| COMMUNICATION

Peer-review articles:

1. **Bahnsen, J.S., Franzyk, H., Sandberg-Schaal, A., Nielsen, H.M.**
   Antimicrobial and cell-penetrating properties of penetratin analogs: Effect of sequence and secondary structure.
   BBA Biomembranes (2013) 1828: 223-232.

2. **Bojer, M.S., Jakobsen, H., Struve, C., Krogfelt, K.A., and Løbner-Olesen, A.**
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