Summary:

Use of Bacteriophages as Antimicrobials Against Avian Pathogenic Escherichia coli Infections in Poultry: from Isolation to Therapeutic Evaluation

Phage therapy, although extensively used in Russia and Georgia, has not been implemented in Western countries, notwithstanding the increasing need for therapies offering an alternative to the use of antibiotics. Antibiotic-resistant strains have to be tackled, as well as their sustained presence, inducing spread of resistance genes due to horizontal transfer. Application of phage therapy to the large food animal industry, would considerably reduce the global use of antibiotics.

The poultry industry is affected by huge economical losses due to APEC infections. Colibacillosis is namely the cause of increased mortality and morbidity, decreased productivity and carcass rejection.

For these reasons, the therapeutic use of a phage cocktail, composed of phages selected on their in vitro characteristics, has been assessed. The whole process of phage isolation until in vivo experimentation has been carried out. The thesis can be divided in three major sections, each of which has been accomplished in different laboratories.

Bacteriophages were firstly recovered from existing stocks and isolated from surface water samples to constitute a broad phage collection. This, and the subsequent phenotypic characterization, was performed at the Viral Genetics Research Group (GEVI), at the Vrije Universiteit Brussel (VUB), with Prof. Dr J.-P. Hernalsteens and Prof. Dr H. De Greve as supervisors. A total of 76 phages were firstly analyzed for their in vitro lytic capacities. The host range was determined against 31 APEC strains, recovered from colibacillosis-affected chickens in Belgian poultry houses. The strictly lytic nature of the phages was determined and revealed that all phages exhibited that compulsory characteristic. Bacteriophages with a broad (> the 13% average range cover) and complementary spectrum of infection were selected. Some of them could only grow on strains belonging to serogroup O78, including most APEC strains. Three of them could kill the challenge APEC strain CH2, used in the in vivo model of infection. Their O78-specificity was further analyzed by an antiserum agglutination test. Final confirmation of this hypothesis was obtained via site-directed mutagenesis of genes involved in the biosynthesis of the O78 antigen (De Munck, 2013). The capacity of the phages, to lyse mutants resistant against other phages, was tested. Except two phages standing out (PhAPEC2...
and PhAPEC8), the generally observed trend was that once a mutant was resistant against a phage, all other tested phages were unable to lyse that mutant. So except for PhAPEC2 and PhAPEC8, no major selection could be made on this ground. Transposon-mutagenesis identified the outer membrane protein OmpF, and putatively the outer core of the lipopolysaccharide, as the receptors of phage PhAPEC2. A total of nine phages were selected, among which four were able to grow on the challenge strain CH2 and thus later included in the cocktail. Transmission electron microscopy revealed their morphology, and gave insight into their intrinsic characteristics. Two phages displayed T4-like morphology among which PhAPEC2, included in the phage cocktail. Five phages were expected to be N4-like, among which the three O78-specific phages growing on CH2 i.e.: PhAPEC5, PhAPEC7 and PhAPEC9. Two of the selected phages showed interesting morphologies. The Myovirus PhAPEC6 displayed an outsized phage head with a rare 'hairy' tail. PhAPEC8 was worth a deeper look, as only a few phages displayed FelixO1-like morphology. None of these particular phages could lyse the challenge strain CH2.

The genomes of the selected phages underwent a further in silico analysis. This was accomplished at the Division of Gene Technology at the Katholieke Universiteit Leuven (KU Leuven), with Prof. Dr ir R. Lavigne as supervisor. The respective T4-, and N4-like nature was confirmed for each of the phages. This supports their already observed strictly lytic behavior. Moreover, because no genes encoding toxic proteins have been described in T4- or N4-like phages, their safety of use can be presumed. Nevertheless, the genomes of the four phages included in the cocktail underwent a deeper analysis in which each of the predicted proteins was examined. This analysis did not reveal similarity with any known toxin, nor the presence of conserved toxin domains. The T4-like nature of PhAPEC2 is advantageous, as T4 was extensively studied and has been chosen for the first human trials described in English literature. The phages PhAPEC5, PhAPEC7 and PhAPEC9, included in the cocktail, showed the expected typical large RNA polymerase found in N4-likes. These phages have already been found in Georgian commercialized phage mixtures destined to human application.

Two phages, although not present in the cocktail, deserved a thorough analysis because of their particular nature. Despite its FelixO1 morphology, PhAPEC8 showed only 18% and 19% similar proteins with its closest relatives, being the coliphage rV5 and the Salmonella phage PVP-SE1 respectively. This justifies the creation of a new genus within the Myoviridae. Cryo-EM analysis of the giant phage PhAPEC6, displaying the third largest sequenced genome, showed bubble formation in the phage head under increased electron dose. This phenomenon has, until now, only been reported in the unrelated phiKZ-like
phages. Moreover, reconstruction revealed an unusual triangulation number, T=28. Mass spectrometry identified 47 structural proteins, among the many hypothetical proteins with unknown function. These are intensively shared with its closest relatives, attesting for their morphological similarity.

The in silico analyzed cocktail phages were thus validated and identified as suitable for phage therapy. The therapeutic efficiency of the phage cocktail was assessed at the Division of Gene Technology at the KU Leuven, under supervision and advice of Prof. Dr B. Goddeeris. Although the cocktail phages could be re-isolated from the lung and heart of chickens that were euthanized, none of the treated groups showed a significant decrease in mortality, lesion scores or weight loss, compared to the untreated group. The efficiency of the phage cocktail used in treating CH2-infected chickens in vivo is negligible, even though in vitro, the phages in the cocktail were able to efficiently lyse the APEC strain CH2.
Abstract:

Use of Bacteriophages as Antimicrobials Against Avian Pathogenic *Escherichia coli* Infections in Poultry: from Isolation to Therapeutic Evaluation

The poultry industry is affected by huge economic losses due to APEC infections. Colibacillosis is a cause of increased mortality and morbidity, decreased productivity and carcass rejection. The application of phage therapy to the large food animal industry would considerably reduce the extensive global use of antibiotics. Because of the increasing need for therapies offering an alternative to the use of antibiotics, the therapeutic use of a phage cocktail, composed of phages selected on their in vitro characteristics, was assessed. A broad phage collection of 76 phages, isolated from surface water samples, was constituted. During the analysis of their phenotypic characteristics, strictly lytic phages with a broad and complementary spectrum of infection were selected. Some of them displayed the valuable characteristics of generating low cross-resistance and serogroup O78-specificity. A total of nine phages was selected, among which four were able to grow on the challenge strain CH2, and thus later included in the cocktail. Site-directed mutagenesis revealed that three of these recognize the O78 antigen as receptor, while the fourth showed by transposon-mutagenesis to have OmpF and putatively the outer core of the lipopolysaccharide as receptor. The genomes of the selected phages underwent a further in silico analysis, where no predicted proteins revealed similarity with any known toxin, nor the presence of conserved toxin domains. The N4- and T4-like nature of the cocktail phages confirmed their strictly lytic behavior. Moreover, a thorough genomic and proteomic analysis was performed on the phage PhAPEC6, as it possesses the third largest sequenced phage genome. The therapeutic efficiency of the in silico validated phage cocktail was further assessed by oral and intratracheal administration. Although the cocktail phages could be re-isolated from the lung and heart of chickens that were euthanized, none of the treated groups showed a significant decrease in mortality, lesion scores or weight loss, compared to the untreated group. The efficiency of the phage cocktail used in treating CH2-infected chickens in vivo is negligible, even though in vitro, the phages in the cocktail were able to efficiently lyse the APEC strain CH2.