THE VIRULENCE OF PNEUMOCOCCAL STRAINS T4, ST124, ST138 AND ST306 IN ZEBRAFISH EMBRYOS

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Streptococcus pneumoniae (pneumococcus) is one of the most important human pathogens worldwide. The development of new effective protein based vaccines against the bacterium has long been goal of scientists.

The objective of this study was to gain information about the virulence of pneumococcal strains ST124, ST138 and ST306 in zebrafish embryos. These strains have known invasive disease potential in humans but the strains have not been tested in zebrafish embryos before. The T4 strain, with known survival rate in zebrafish embryos, served as a control strain to validate the results. This study gave information for future projects dealing with the development of pneumococcal vaccines and also explored further the zebrafish embryo as a model of disease.

Each bacterial strain was injected into blood circulation of zebrafish embryo. The survival of the embryos was followed daily and after 72 hours the final survival rates were calculated for each strain. The combined survival rate for ST124 was 25 %, for ST138 92% and for ST306 11 % ($P<0.0001$). The most striking result was the high survival rate of embryos infected with ST138. In humans and mice this strain and serotype (6B) is associated with high mortality.

There might be several reasons for the different behavior of the bacteria in zebrafish embryos. The somewhat lower temperature of the embryos might have an impact on pneumococcal virulence. Also, infection route was different in these experiments than in earlier mouse experiments where bacteria were inoculated into nasal cavity. Moreover, the lack of acquired immunity in zebrafish embryos might have an effect on the course of infection. It can also be that the capsule expression of ST138 is somehow altered in zebrafish embryos leading to the lower mortality observed.

As a conclusion, this study demonstrated that even though zebrafish embryos can be used as a model of disease, the differences in host environment might affect the virulence of the bacterium and must be recognized when using this model.

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INTRODUCTION

Pneumococcal diseases. *Streptococcus pneumoniae* (pneumococcus) is one of the most important human pathogens and a significant cause of death - not only in the developing world but also in the Western Countries. It causes mild infections, such as otitis media, but also severe invasive infections, for example pneumonia, meningitis and sepsis (1).

The mild infections caused by pneumococcus are very common. In Finland approximately 500 000 otitis media cases are treated every year (1). Many of these infections are of pneumococcal origin. In a French study done in 2001 pneumococcal strains were responsible for 52 % of the infections of the middle ear in children (2). It is estimated that in Finland *S. pneumoniae* causes 20-40 % of all acute otitis media (3). Hence pneumococcus greatly encumbers the public health service.

The invasive pneumococcal infections are related to high mortality rates, even though effective antibiotic treatments are available. For example pneumococcal meningitis has a death rate of 20-30 % in Finland (1). In the developing countries the situation is far worse. It is estimated that of the 2.6 million annual deaths, due to acute respiratory infection in children younger than 5 years, more than one million are caused by pneumococcal infections (4).

Streptococcus pneumoniae. *S. pneumoniae* is a gram-positive, alpha-hemolytic coccus bacterium. It is frequently found in the nasopharynx of healthy carriers. Approximately 20-40 % of children and 5-10 % of adults carry the bacterium. Pneumococcus has many virulence factors, the polysaccharide capsule being the most important. It makes the bacterium resistant to phagocytosis. Other virulence factors include e.g. protein A and pneumolysin.

Pneumococcal vaccines. Given these facts, it is no surprise that the development of vaccines against the pneumococcal diseases has long been a goal of scientist across the globe. Two kinds of vaccines already exist.

Pneumococcal polysaccharide vaccines contain capsule polysaccharides taken from different serotypes of *S. pneumoniae*. The widely used PPV23-vaccine is efficient against 23 different serotypes of *S. pneumoniae* which corresponds to approximately 90 % of all the infections caused

by the bacterium. The disadvantage of these vaccines is that they immunogenize poorly children under the age of two. It is a major drawback bearing in mind that young children (and the elderly) have the greatest risk of getting life threatening pneumococcal infection (1).

The conjugate vaccines on the contrary have better immunogenicity in children. This is achieved by attaching the capsule polysaccharides into protein molecules, for example into diphtheria toxin. In Finland the pneumococcal conjugate vaccine (PCV) was added into the Finnish National Vaccination Program in autumn 2010 (5).

These conjugate vaccines however have also their disadvantages. They are serotype specific as well, meaning that they only work against the serotypes of which they contain capsule polysaccharides from. Because the serotype profile differs in each pneumococcal disease, in every continent and age group, it is hard to develop vaccine that would work for everyone. In addition, as the serotypes that the vaccine contains become rarer, others will become more common. The conjugate vaccines are also expensive to manufacture, and so far the maximum number of serotypes in one vaccine has been just 13 in comparison to 23 in polysaccharide vaccines (1).

For these reasons, scientists have tried to develop a vaccine that would not contain capsule polysaccharides (that would not be serotype specific) but would work against the general protein structures, common to every serotype of *S. pneumoniae*. As these vaccines would possibly be more affordable they could be the answer to the devastating burden of pneumococcal diseases in the developing world.

Zebrafish as a model of disease. For approximately 20 years zebrafish (*Danio rerio*), has been used as a laboratory animal to study embryogenesis and organ development. Zebrafish is a chordate tropical fresh water fish originally native to the streams of the southeastern Himalayan region, including countries such as India, Nepal, Bangladesh

and Pakistan. There are many advantages over other vertebrate model systems that the zebrafish model possesses: The quick lifecycle, ease of breeding the fish, ex-utero development and the optical clarity of embryos.

Although anatomically different, the genome of zebrafish is remarkably similar to that of mammals. This similarity in genomes gives zebrafish model relevance over other models of disease, such as the fruit fly *Drosophila melanogaster* or worm *Caenorhabditis elegans.* These species lack the genes that are responsible for e.g. adaptive immunity. These genetic findings have made the zebrafish model increasingly more attractive for immunobiological investigations and for studying host-pathogen interactions (8).

The immune system of zebrafish. The immune system of zebrafish has many aspects common with the human immune system. The resemblance of adaptive immune system for example is a great advantage. Adult zebrafish have T- and B-lymphocytes which are known to function as in humans. The similarity between the thymus in the zebrafish and higher vertebrates has been proved by microscopic and ultrastructural analyses (8).

The immune system of zebrafish embryo however does not have the adaptive immune system. It battles solely with innate immunity against the pathogens, without the help of lymphocytes and immunoglobulins. Rounioja et al. recently published an article demonstrating that zebrafish embryos can be used to study innate immune responses as well as virulence determinants in infections caused by *S. pneumoniae* (6).

Objectives. The objective of this study was to gain information about the virulence of pneumococcal strains ST124, ST138 and ST306 in zebrafish embryos. These strains have known invasive disease potential in humans, and they have also been tested in a mouse model of pneumococcal disease (7). This study explores further the possibility of using zebrafish embryos as a model of disease for pneumococcal infection, and gives information for future projects dealing with the development of pneumococcal vaccines.

MATERIALS AND METHODS

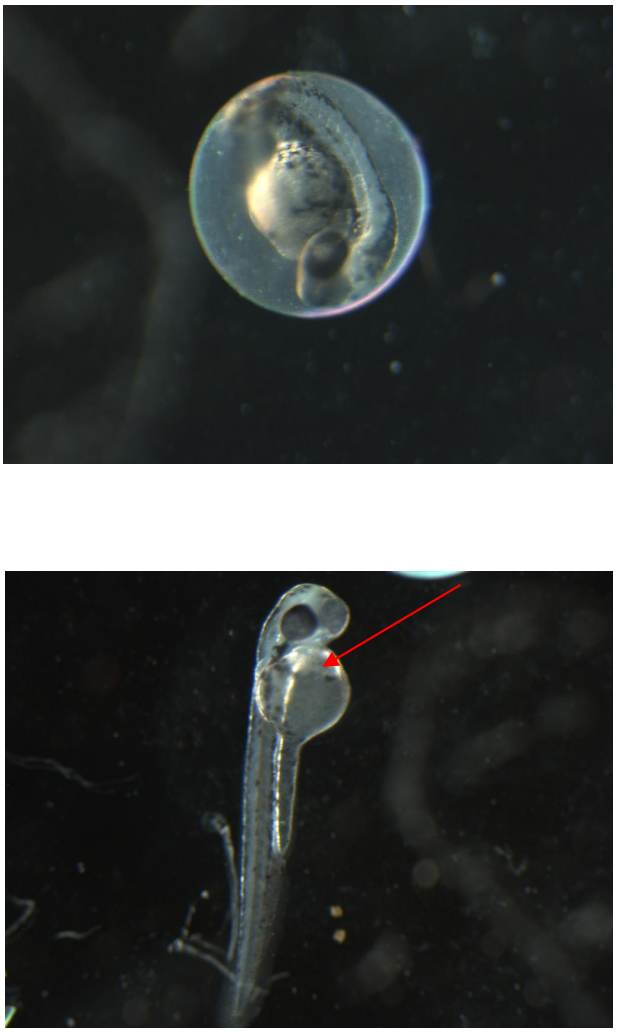
Bacterial strains. The pneumococcal strains (T4, ST124, ST138 and ST306), were received from Karolinska Institute, Department of Microbiology, Tumor and Cell Biology in Stockholm, from the research group of Birgitta Henriques-Normark and Staffan Normark. The Stockholm's group had tested ST124, ST138 and ST306 in mice but no tests had been conducted in zebrafish before. T4 served as a control strain in this study.

A day before the injections the bacterial strains were plated on blood agar plates and grown overnight (at 37°C and CO_2). The next morning the bacteria were suspended in 10 ml Todd Hewitt broth (Becton, Dickinson and Company, BD) supplemented with (0.5%) yeast extract (THY). The strains were then incubated at 37°C until they reached an OD620 of 0.4. After reaching the desired concentration, the bacteria were harvested by centrifugation (4000 rpm, 10 minutes), washed and resuspended in 0,2 M KCl-buffer.

Zebrafish embryos. The wild type AB zebrafish embryos used in this study were maintained according to standard protocols in the Zebrafish core facility of university of Tampere. During the experiments the zebrafish embryos were kept in E3-medium at temperature of 28.5°C.

Preparation of zebrafish embryos. As the bacterial strains were growing in the THY-medium, the zebrafish embryos were prepared by dechorionating them. The dechorionation was conducted under microscope using two pairs of tweezers. Before bacterial inoculation the zebrafish embryos were anaesthetized with 1 ml of buffered 0.02% tricaine (Sigma-Aldrich). Because the zebrafish used in this study were embryos no license from the Animal Experiment Board was needed.

Microinjection of zebrafish embryos. 2 µl of 70 kDa rhodamine dextran (Invitrogen/Molecular Probes) tracer was added into 10 µl of bacteria-KCl suspension. The tracer was used to facilitate the observation of injections. The microinjector was calibrated to output of 1 nl, which corresponds to 1000 cfu:s (colony forming units). The bacterial output was controlled by plating. The injections were aimed in the blood circulation valley. Each strain was injected with microcapillary needle into 24 embryos. 10 embryos at each experiment were injected with KCl-suspension that contained no bacteria. These embryos served as control.



Zebrafish embryo before and after the dechorionation. The red arrow indicates the blood circulation valley where the bacterium was injected.

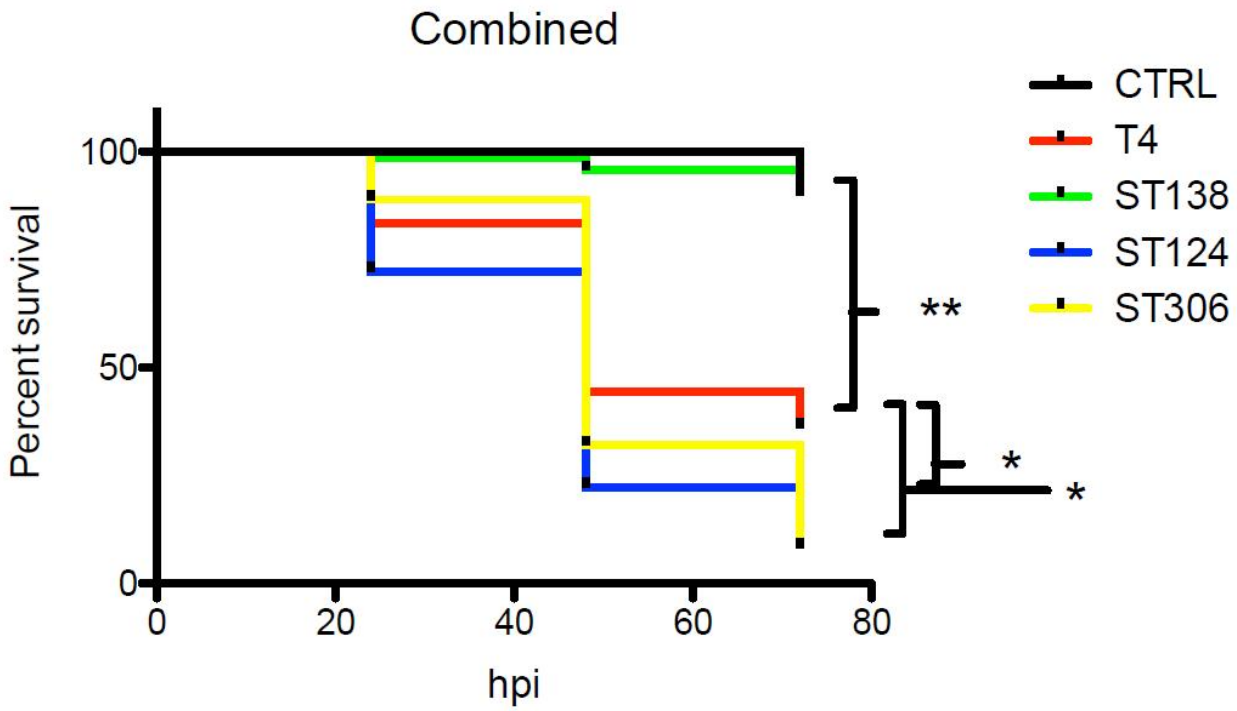
Following the mortality of infected embryos. After the injections the embryos were placed separately into 24-well plates. The condition of each embryo (106 embryos in total) was evaluated after every 24 hours, three times in total. Dead embryos were marked on the well plates. Lack of movement and cessation of heartbeat was regarded as an event of death. After 72 hours the final survival percents were calculated for each strain of Streptococcus pneumoniae.

Statistical analysis. Survival rates were analyzed using the Mantel-Cox Log Rank test. A p-value of < 0.05 was considered statistically significant. The data was analyzed using the Graph Pad Prism 5.0 software.

RESULTS

The results of different experiments are combined in the diagram below. The overall survival rate for T4 was 30 %, for ST138 92 %, for ST124 25 % and for ST306 11 % (p<0.0001). There was a statistically significant difference between mortality caused by ST138 in comparison to the reference strain T4 (p=0.002). The mortality caused by ST124 and ST138 did not differ as much when compared to T4. However the difference was still significant (p=0.01).

The amount of bacterium injected into every embryo was monitored by plating the output from every microcapillary needle onto blood agar plates after the injections. The bacterial colonies were then calculated and this data was compared with survival data.



*The diagram showing the survival rates for each strain of S. pneumoniae. ** p=0.002 compared to T4, * p=0.01 compared to T4, hpi=hour post infection.*

DISCUSSION

In this study the virulence of four different strains of *S. pneumoniae* was tested in zebrafish embryos. Besides T4, the strains had never been tested in zebrafish embryos before. After injecting the bacterial strains into embryos, the fish were followed 72 hours after which survival rates were calculated. The data from different experiments was combined to one diagram.

The T4 strain belongs to the serotype 4 which has a high invasive disease potential in humans. T4 itself is widely used as a laboratory strain in pneumococcal studies. The overall survival rate for strain T4 in our study was 30 %. This strain had previously been tested by the research group (6), and would serve as control strain to monitor the validity of the rest of the study. Rounioja et al. received similar results regarding the virulence of T4 strain. In their experiments 1000 cfu dose of T4 was associated with survival rate of 15 %. The similarity of the results indicates that the new data received in this study can be regarded reliable.

ST306 belongs to the serotype 14 which is commonly found in invasive disease and rarely found in carriers. These highly invasive serotypes tend to cause mild infections and have low mortality in humans (~ 0%) (7). In the mouse study by Sandgren et al, conducted in C57BL/6 and BALB/c mice, the strain caused no mortality in intranasal challenge but in intraperitoneal model it was associated with mortality of 65 %. This was greater than the mortality caused by ST124 even though in humans ST306 is less threatening infection. In our study the mortality for ST306 was 89 % making it the most lethal of the strains.

ST124 (serotype 14) on the other hand has an intermediate invasive disease potential. It is more often found in carriers than ST306. In humans serotype 14 is associated with intermediate mortality of 7%. In Sandgren's experiments the strain caused no mortality in intranasal challenge but in intraperitoneal model it was associated with mortality of 50 %. In our zebrafish experiment ST124 caused mortality of 75 % making it the second most lethal of the strains.

The most striking result of this study was the low lethality of the strain ST138. (Combined survival percent 92 %.) The result was unexpected because in humans this serotype (6B) has an intermediate

invasive disease potential and is associated with the high mortality of 25 %. Also in the study done in mice by Sandgren et al. ST138 was clearly the most lethal strain of all the strains investigated in this study. After intranasal challenge the ST138 strain was the third most able to cause lethal systemic disease out of the 13 strains in Sandgren's study. In intraperitoneal injections ST138 caused mortality of 100 %. What could explain this surprisingly low mortality rate in zebrafish embryos?

First one must remember that zebrafish is a different animal compared to mouse and human. The different living environments may play a role in the capability of causing disease among the different pneumococcal strains. Zebrafish are poikilothermic, meaning that their core temperature is linked to the temperature of the surroundings they live in. In our study the fish were kept at 29' C which could be less optimal for the virulence mechanisms of ST138 than the higher temperature in humans and mice.

The differences in immune systems, even though that the innate immunity is undeniably similar in all the three species, may also affect the death rates. Zebrafish embryos lack the acquired immune system, meaning that their body battles against the pathogens without the help of B- and T-lymphocytes. It could be that the high mortality associated with serotype 6B and thus ST138 in mice and humans is in some part caused by the overactivation of acquired immune system.

The capsule is the most important virulence factor of the pneumococcus. It makes *S. pneumoniae* resistant to opsonophagocytic mechanisms. Thus it might be that the capsule expression of ST138 is somehow altered in zebrafish leading to the lower mortality observed.

There was also no visible lack of growth with strain ST138 after being plated onto blood agar plated before injections. Also when the strain was reproducing itself in THY-solution it quickly reached the desired concentration. In fact, the ST138 was usually the first to reach the OD620 value of 0.4. These findings indicate that the low mortality rate observed is not due to unsatisfactory condition of our bacterial strain.

One clear difference when comparing our study to Sandgren's mice study, or to the situation in real

life pneumococcal diseases, is the route of infection. We injected the bacterium straight into the main blood circulation. In vivo *S. pneumoniae* must first adhere onto nasopharynx, then colonize the mucous membrane, after which it has the possibility to proceed into blood circulation. Oversimplifying the normal route of infection may have had an impact on the results. In Sandgren's mice study the bacterium was, in addition to intraperitoneal injection, also given intranasally which corresponds better the infection in vivo. This may be the reason why the strains behaved more as in humans in Sandgren's experiments than in our study. All these reason are probably responsible for the different behavior of the other strains as well.

Zebrafish, from an ethical point of view, can be regarded as a more preferable laboratory animal compared to mice and other mammals. Even though they are clearly less advanced animals than mice are, they are close enough to mammals to be used in studies exploring the immune system and host-pathogen interactions. In this study, the embryos were anaesthetized before potentially painful procedures to minimize stress caused to the fish. The goal was also to use as few embryos as possible to reach the desired results.

This study gave information about the usage of zebrafish embryos as a model of disease for pneumococcal infection. It demonstrated again the advantages of this model when studying host-pathogen interactions. At the moment the research group is testing different pneumococcal vaccines on adult zebrafish and in the future the goal is to create a new protein based vaccine against the pneumococcus.

In conclusion, this study demonstrated that even though zebrafish embryos clearly can be used as a model of disease, the differences in host environment affect the virulence of the bacterium and must be recognized when using this model.

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