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# Adult zebrafish model for pneumococcal pathogenesis

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ABSTRACT

*Streptococcus pneumoniae* (pneumococcus) is a leading cause of community acquired pneumonia, septicemia, and meningitis. Due to incomplete understanding of the host and bacterial factors contributing to these diseases optimal treatment and prevention methods are lacking. In the present study we examined whether the adult zebrafish (*Danio rerio*) can be used to investigate the pathophysiology of pneumococcal diseases. Here we show that both intraperitoneal and intramuscular injections of the pneumococcal strain TIGR4 cause a fulminant, dose-dependent infection in adult zebrafish, while isogenic mutant bacteria lacking the polysaccharide capsule, autolysin, or pneumolysin are attenuated in the model. Infection through the intraperitoneal route is characterized by rapid expansion of pneumococci in the bloodstream, followed by penetration of the blood–brain barrier and progression to meningitis. Using Rag1 mutant zebrafish, which are devoid of somatic recombination and thus lack adaptive immune responses, we show that clearance of pneumococci in adult zebrafish depends mainly on innate immune responses. In conclusion, this study provides evidence that the adult zebrafish can be used as a model for a pneumococcal infection, and that it can be used to study both host and bacterial factors involved in the pathogenesis. However, our results do not support the use of the zebrafish in studies on the role of adaptive immunity in pneumococcal disease or in the development of new pneumococcal vaccines.



## 1. Introduction

Despite significant advances in the development of vaccination strategies against *Streptococcus pneumoniae* (pneumococcus), the bacterium is still a major human pathogen causing over one million deaths every year, a majority among children (Lynch and Zhanel, 2010). Pneumococci are common colonizers of the human upper respiratory tract and can persist there as asymptomatic, harmless inhabitants (van der Poll and Opal, 2009). In many cases, however, pneumococci spread to the lower respiratory tract or further into the bloodstream, and cause a variety of diseases (van der Poll and Opal, 2009). These diseases range from relatively mild but very common infections, such as sinusitis or otitis media to life-threatening diseases such as pneumonia, sepsis, and meningitis (Gladstone et al., 2011).

*S. pneumoniae* is the leading cause of community acquired pneumonia worldwide (Chiavolini et al., 2008; Said et al., 2013). It has been estimated to be responsible for up to 5 million cases of pneumonia annually, with around 10% of all cases leading to death (Feldman and Anderson, 2011). Pneumococci are also one of the most important causative agents of bacterial meningitis (Brouwer et al., 2010; Randle et al., 2011). Pneumococcal meningitis is associated with a lethality of as high as 37% (Lynch and Zhanel, 2010; Mook-Kanamori et al., 2011). In addition, up to 52% of surviving patients suffer from neurological sequelae such as hearing loss, cognitive impairment, and seizures (Mook-Kanamori et al., 2011; Randle et al., 2011). Furthermore, recurrent ear infections caused by pneumococci are also the most common reason for doctors' consultations and the use of antibiotics (Boonacker et al., 2011).

Pneumococci express a range of virulence factors, such as a polysaccharide capsule, pneumolysin, autolysin, and a pilus structure, which contribute to the pathogenesis and the progression of the infection (Jedrzejas, 2001; Kadioglu et al., 2008; Orrskog et al., 2012). The polysaccharide capsule is commonly accepted as the most important virulence factor of the bacterium (Mitchell and Mitchell, 2010; Vernatter and Pirofski, 2013). The capsule is highly antiphagocytic, and un-encapsulated pneumococcal mutants are avirulent in several animal models (Nelson et al., 2007;

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Watson and Musher, 1990). The polysaccharide composition of the pneumococcal capsule is also the basis for the division of pneumococci into the so far recognized 93 serotypes (Gladstone et al., 2011). Several epidemiological studies have shown that pneumococcal capsular serotypes differ in their frequency and geographical occurrence, as well as in their tendency to cause infections in humans (Brueggemann et al., 2003; Sandgren et al., 2004; Sjöstrom et al., 2006). These studies have also shown that some pneumococcal serotypes, more often than are others, are associated with severe invasive diseases in humans. Serotypes with a high potential to cause invasive disease are for example types 1, 4 and 7F (Sandgren et al., 2005; Sjöström et al., 2006). Types 6B and 14 have a medium invasive potential, and serotype 19F a low invasive potential (Sandgren et al., 2005; Sjöström et al., 2006). However, when the different strains caused an invasive disease, serotypes belonging to the group with a high invasive potential (i.e., types 1, 4 and 7F) had a lower mortality rate than did serotypes with a lower invasive potential such as type 19F (Sjöström et al., 2006). Pneumococci of the same serotype can also vary in their pathogenicity, indicating that clonal properties other than the capsular structure also affect the ability of the bacterium to cause an invasive disease (Brueggemann et al., 2003; Sandgren et al., 2005). Although the serotype and clone specific properties are evident, the factors contributing to these differences are still poorly understood.

Two vaccine formulations against pneumococcal diseases have been developed and are in use globally: a 23-valent polysaccharide vaccine and a conjugated vaccine (Gladstone et al., 2011). These vaccines are serotype specific polysaccharide-based formulations directed against the most prevalent pneumococcal serotypes found to cause an invasive disease (Gladstone et al., 2011). However, the complex nature and structural variability of pneumococci pose challenges for the prevention and treatment of the diseases associated with this pathogen, and despite the positive impact of current vaccines pneumococci remain to be eradicated. In addition, increasing antibiotic resistance among clinical strains further complicates the control of pneumococcal spread (Lynch and Zhanel, 2010; Song et al., 2012). In order to overcome these challenges, the best possible understanding of the interactions occurring between the host and the pneumococci during the infection is required. These details are best revealed by studying animal models where both pathogenic and host specific determinants of infection can be investigated. Several rodent models have proved their feasibility and reliability in infection studies, but methodical difficulties in addition to ethical issues concerning these models complicate their use in this field (van der Sar et al., 2004). The zebrafish, *Danio rerio*, provides an attractive alternative non-mammalian vertebrate model for the study of infection biology and immunology (Lohi et al., 2013). In addition to being more ethical, zebrafish are cost-effective and easy to manipulate genetically. Importantly, the adult fish has fully developed innate and adaptive immune systems. These factors make the zebrafish an ideal infection model suitable also for large scale genetic screens (Phelps and Neely, 2005; van der Sar et al., 2004). So far, the zebrafish has been successfully used to model host-pathogen interaction in infectious diseases caused by various pathogens, including both zoonotic (e.g., *Mycobacterium marinum*) and human specific pathogens (e.g., *Salmonella typhimurium*, *Staphylococcus aureus*) (Parikka et al., 2012; Prajsnar et al., 2008; Prouty et al., 2003; van der Sar et al., 2003). These pathogens also include several streptococcal species, such as *Streptococcus iniae* and *Streptococcus agalactiae* (Neely et al., 2002; Patterson et al., 2012).

Here we introduce a new non-mammalian model to be used in the study of pathophysiological mechanisms of pneumococcal infection. We have previously shown that pneumococci can cause a fulminant infection in zebrafish embryos and that surviving embryos can combat the infection using innate immunity mechanisms (Rounioja et al., 2012). Here we show that a pneumococcal infection can also be established in adult zebrafish, which provides a more diverse model for the study of pneumococcal infection.

## 2. Materials and methods

### 2.1. Bacterial strains

The wild type pneumococcal strain TIGR4 (T4) used throughout these studies was originally isolated from a Norwegian patient suffering from a systemic pneumococcal infection (Aaberge et al., 1995). It belongs to capsular serotype 4 and a highly invasive clone ST205 and has previously been used in a zebrafish embryo model (Rounioja et al., 2012). The other pneumococcal strains used were four clinical isolates belonging to serotypes with different invasive disease potentials (serotype 1 (ST306), serotype 14 (ST124), serotype 6B (ST138) and serotype 19F (ST162)) as well as four T4 isogenic mutants deficient in either the polysaccharide capsule (T4R), autolysin (T4Δ*lytA*), pneumolysin (T4Δ*ply*), or the pilus-like structure (T4Δ*rlr*).

All the pneumococcal strains used were grown overnight on blood agar plates at 37 °C and 5.0% $CO_2$. Bacterial cells were suspended in 5 ml of Todd Hewitt broth (Becton, Dickinson and Company, New Jersey, USA) supplemented with 0.5% Todd-Hewitt yeast extract (Becton, Dickinson and Company) until they reached an $OD_{620}$ of 0.1. Bacteria were grown in the media until they reached an $OD_{620}$ of 0.4, after which the bacterial cells were harvested by centrifugation (4000 rpm, 10 min) and re-suspended in sterile 0.2 M KCl to obtain the desired concentrations. To be able to visualize the injection, 10% filtered phenol red (3 mg/ml; Sigma–Aldrich, St. Louis, Missouri, USA) was added to the injection solution. The exact bacterial numbers in inoculates were confirmed by quantitative plating before and after the experiment.

### 2.2. Zebrafish and maintenance

Wild type AB zebrafish of 5–8 months of age were used in the majority of the experiments. In addition, the mutant fish line ($Rag1^{hu1999}$) (from ZIRC), which lack active lymphocytes was used to assess the role of the adaptive immunity. All the animal studies were conducted in accordance with the regulations of the Animal Experiment Board in Finland and the fish were maintained according to standard protocols (Nusslein-Volhard and Dahm, 2002). Following infection fish were kept in an isolated stand-alone unit with a separate flow-through system.

### 2.3. Infection of adult zebrafish by intraperitoneal and intramuscular injection

Generally, fish were randomly divided into groups of 15–20 fish, each group receiving a different concentration of bacterial inoculation. Prior to injection the fish were anesthetized in water containing 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0) (Sigma–Aldrich) and gently laid on a moistened foam bed ventral side up. An Omnican 100 30G insulin needle (Braun, Melsungen, Germany) containing 5 μl of the desired injection suspension was held parallel to the fish and inserted into the midline of the abdomen, between the pectoral fins and the entire content of the needle was carefully injected into the fish peritoneum. Similarly, the fish in the control group received 5 μl 0.2 M KCl. If any leakage of the injection solution was observed at the injection site, the fish was euthanized. Successfully infected fish were transferred to a fresh water tank and their recovery was verified before moving them