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Strategies under development to decrease *Streptococcus pneumoniae* related infections

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Abstract

Streptococcus pneumoniae is a bacterium that resides as a commensal in the human nasopharynx. Nonetheless, *S. pneumoniae* can cause both invasive and noninvasive disease, which result in almost half a million deaths among children world-wide. There are currently licensed vaccines against pneumococcal disease and these are effective against preventing invasive pneumococcal disease. However, these vaccines are starting to work less effectively due to the large amount of genetic variation in the pneumococcus, specifically in the capsule locus. This genetic variability is in large part due to the natural competence of the pneumococcus and allows for rapid exchange of genetic material between strains. This has also led to less successful treatment with antibiotics because of widespread antibiotic resistance and multi-drug resistance. Therefore new methods to decrease or prevent *S. pneumoniae* related deaths need to be developed. This has been under development for several years and numerous studies have tested several potential vaccine candidates. While several candidates have been proposed and tested there are also short comings. In this paper the main advantages and disadvantages of several vaccine strategies and novel treatments under development are being discussed.

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Introduction

Streptococcus pneumoniae is a Gram-positive bacteria that colonizes the human nasopharynx. *S. pneumoniae* naturally resides as a human commensal, but pneumococcal infections do occur. Infection with *S. pneumoniae* can cause pneumonia, otitis media, meningitis and several other diseases. Infection with *S. pneumoniae* is among the leading causes of death among young children, elderly persons and the immunocompromised[16,18]. The majority of deaths among children occur in developing countries in Africa and Asia[18,25]. For instance, in 2011 an estimated 411.000 children younger than five years died of *S. pneumoniae* related pneumonia[25]. In addition, it is possible to have decreased lung capacity after recovering from pneumonia[25]. Also, people who survive meningitis, could develop long term sequelae including hearing loss and could suffer from neurological deficits[3]. Therefore it is necessary to develop strategies to prevent infection with *S. pneumoniae*.

Strains of *S. pneumoniae* can be divided into 90 different capsular serotypes, which are distinguished by the type and arrangement of the repetitive polysaccharides units. The capsule completely encloses the cell and is one of the most important virulence factors of *S. pneumoniae*.[12] In turn, the serotype is a determinant of the invasiveness of the cell. This is due to the fact that the capsule prevents the host's immune system from interacting with the bacterial cell by hindering complement deposition. A thick capsule shields the bacterial cell more than cells with a thin capsule[13]. Therefore, strains expressing a thick capsule are more infectious than strains with a thin capsule. At the same time, the capsule shields cell wall associated proteins of the bacterial cells, which can be targets for antibodies[7]. These cell wall proteins are involved in binding to the respiratory epithelial surface of the nasopharynx and promote colonization and having a thick capsule is now a disadvantage. To overcome this problem *S. pneumoniae* can shift between two phase variants. The opaque variant has a thick capsule and is more virulent, while the transparent variant has a thin capsule and promotes

colonization[7]. However, new serotypes are emerging that have not been associated with invasive disease, but are now causing infections[10].

Infection with *S. pneumoniae* is normally treated with antibiotics. However, the frequent and unnecessary use of antibiotics has led to resistance among many bacterial species including *S. pneumoniae.* Once again infection with *S. pneumoniae* is a threat[16,20]. Another way to prevent pneumococcal infection is through vaccination. The first vaccine created against *S. pneumoniae* was a pneumococcal polysaccharide vaccine (PPV). The first PPV, PPV23, protects against the 23 most prevalent serotypes. This vaccine was a success, but is ineffective in children younger than 2 years old and after a while different virulent serotypes not covered by PPV23 emerged[21]. Therefore another vaccine was created for use in small children. This was a pneumococcal conjugate vaccine (PCV). The first PCV, PCV7, protects against 7 different serotypes[9]. After a while different virulent serotypes not covered by PCV7 emerged, this is called serotype replacement[3]. Therefore PCV13 was created and protects against 13 different serotypes, which include the serotypes that became prevalent after introduction of PCV7[10]. Currently this vaccine is starting to be less effective for the same reason that PCV7 was less effective, i.e. serotype replacement. Figure 1 and 2 show the rates of invasive pneumococcal disease in children younger than 5 years old and adults between 1998 and 2015 in the United States respectively. A vaccine based on capsular polysaccharides is not a long term solution due to serotype replacement. To keep people protected from infection with new invasive serotypes a new vaccine must be created or another way of treatment has to be developed. A lot of effort is put into the development of new strategies to prevent and treat pneumococcal related disease once and for all. This paper describes some of the most promising strategies and point out the main advantages and disadvantages of these methods.

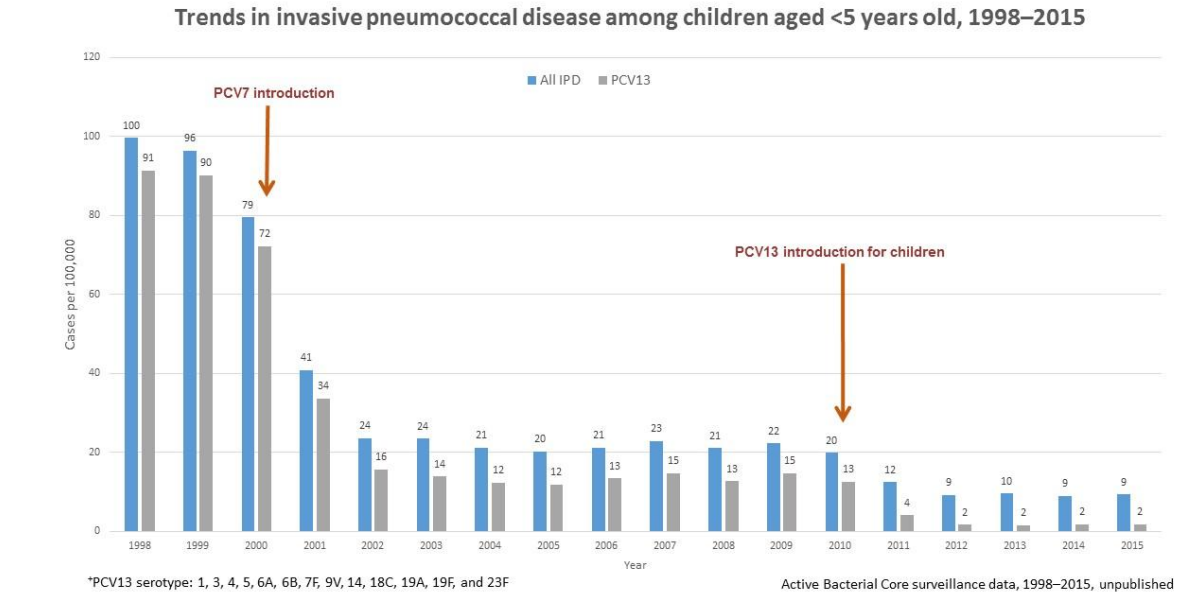

Figure 1: trends in invasive pneumococcal disease (IPD) among children younger than 5 years old between 1998 and 2015 in the United States[1].

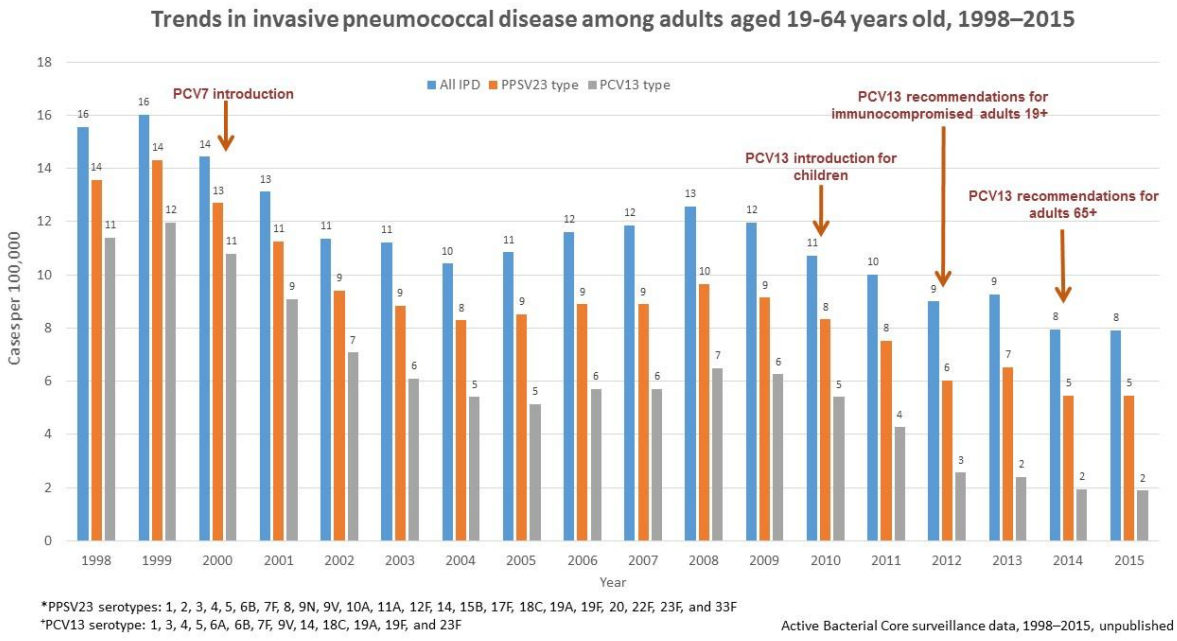

Figure 2: Trends in invasive pneumococcal disease (IPD) among adults aged 19-64 years old between 1998 and 2015 in the United States. PPSV23 is the pneumococcal polysaccharide vaccine (PPV23)[1].

Infection

For a better understanding of the possible strategies of protection against infection with *S. pneumoniae* it is necessary to understand the mechanism of colonization, infection and the immune response of the human body.

Before infection the bacteria has to colonize the nasopharynx. This process occurs through spread from human contact and transmission through fomites. This process happens more often in crowded areas and for this reason children in daycare centers are more susceptible to colonization with *S. pneumoniae*[3]. For successful colonization the bacteria must reach and attach to the epithelial surface of the nasopharynx, but when entering the nasopharynx, the cells encounter mucus secretion. A thick capsule prevents the cell from getting stuck in the mucus, because most of the capsular polysaccharides are negatively charged and repel the polysaccharides found in the mucus[7]. *S. pneumoniae* also produces neuraminidases which cleave N-acetylneuramic acid found in the mucus. By cleaving N-acetylneuramic acid the viscosity of the mucus is decreased and the adherence to the epithelial surface is increased[3].

After encountering the epithelial surface, the bacterial cell has to attach itself to the epithelial cells. Bacterial cells can do this via cell wall proteins, which can bind to sugars on epithelial cells[3]. Some cells express a pilus like structure which also may help the binding of *S pneumoniae* to the epithelial cells of the nasopharynx[7]. After adherence *S. pneumoniae* forms surface associated communities and lives as a commensal.

Infection occurs when there is a local generation of inflammatory factors such as interleukin-1 and tumor necrosis factor (TNF)[3]. Most of the time this happens shortly after a viral infection, including an influenza infection[12]. The neuramindase activity of *S. pneumoniae* is thought to aid in viral infection[3]. In response to these inflammatory factors upregulation of some epithelial receptors can change the epithelial surface. For instance the

platelet activation factor receptor (rPAF) is increased during pneumococcal infection[3]. Cell wall proteins of *S. pneumoniae* can then bind to these upregulated receptors. ChoP is a cell wall associated protein that can bind to the upregulated rPAF[7].

After binding to inflamed tissue, the cells must evade the immune response of the host. The immune response of the host consists of opsonization and phagocytosis of the pneumococcus to clear an infection. Opsonization factors, such as complement and antibodies bind to *S. pneumoniae*, which can then be effectively phagocytosed[7]. *S. pneumoniae* has several ways to avoid phagocytosis, one way is to cleave immunoglobulin A (IgA_1). By cleaving IgA_1 the host has a reduced ability to recognize the bacteria and the clearance of the cells does not start until other opsonins attach to the bacteria[7]. The most important and effective way of evading the immune response by stopping opsonization is having a capsule. The capsule shields the bacterial cell wall proteins from opsonins. If a bacterial cell is not opsonized, than the chances of phagocytosis decreases and the chance of infection increases[14,20]. Thus when *S. pneumoniae* are expressing a thick capsule, they are more virulent than when expressing a thin capsule, due to reduced phagocytosis. After binding to inflamed tissue the bacterial cells can progress further into the sterile sites of the human body. Here it causes the previously mentioned diseases.

Treatment

Currently infection with *S. pneumoniae* is treated with antibiotics, but as mentioned earlier, the treatment with common antibiotics is not sufficient anymore. This is due to rising resistance among pneumococcal isolates. The cause of this resistance is the inappropriate use of antibiotics. This includes overuse for minor infections and misuse by using antibiotics when there is not a bacterial infection. This problem does not only apply to infection with *S. pneumoniae,* but emerging resistance is found in many more bacterial species, which cause life threatening infections. Worldwide efforts need to be made to reduce the rise of antibiotic

resistance among bacteria. Furthermore, antibiotics are expensive and the development of new antibiotics is especially costly. Therefore countries with the highest number of *S. pneumoniae* related deaths, such as developing countries in Africa and southeast Asia, cannot afford this treatment[18,25]. Overall it is much more convenient and cost effective to develop a way to prevent infection.

To prevent infection it is possible to vaccinate with the previously mentioned PPV and PCV vaccines, but these vaccines are starting to become less efficient[19]. Also, creating conjugate vaccines is much more costly than creating protein based vaccines. Thus new PPVs and PCVs protecting against more serotypes can be developed, but will be unavailable in developing countries due to cost. Because of this, new ways of vaccination independent of capsular polysaccharides need to be developed. A new way of vaccination is to target proteins found in all serotypes or to vaccinate against proteins found only in virulent serotypes. Another promising strategy is to prevent colonization of the nasopharynx. There are also strategies that are not thoroughly being investigated but might be useful as well. These strategies include the prevention of capsular polysaccharide production, a whole cell vaccine and new drug delivery methods. In the following sections these strategies will be discussed.

PCVs and PPVs

In 1983 the first pneumococcal vaccine was created, PPV23, which contains 23 different capsular polysaccharides. This vaccine was very effective, however this vaccine did not work in young children. This is because young children do not have a fully matured immune system. To protect young children too, a new vaccine was created in 2000. This vaccine was PCV7. PCV7 contains seven capsular polysaccharides conjugated to the diphtheria toxoid CRM_{197}, which is a highly immunogenic and non toxic material. Therefore PCV7 induces a stronger immune response and can be processed by the immature immune

system of young children[21]. Currently the effectiveness of PCV7 is decreasing. Thus PCV13 was developed in 2010. This vaccine works in the same way PCV7 works, but PCV13 contains six additional capsular polysaccharides conjugates not contained within PCV7. PCV13 has proven very effective in reducing invasive pneumococcal disease. However, it is likely that a shift in serotypes causing invasive pneumococcal disease will be seen, as happened after the introduction of PCV7. It is possible to develop a vaccine that contains emerging serotypes and protects against these new emerging virulent serotypes, but then again it is likely that this new vaccine will start to be less effective over time. Therefore it is not reasonable to develop new capsular polysaccharide based vaccines.

Another way to keep vaccinating with PCVs or PPVs is to create a vaccine against all possible serotypes. Due to the high number of different serotypes this will be very expensive, less effective, and there are also pneumococci without a capsule. Although a capsule increases the chances of colonization, it is not a necessary factor to colonize and to infect. Nonencapsulated *S. pneumoniae* can also colonize the nasopharynx and cause disease[11]. By using vaccines that target the capsule, nonencapsulated pneumococci can replace the encapsulated serotypes in the nasopharynx. Therefore the use of PCVs and PPVs will increase the chances of infection with serotypes not targeted by the vaccine[11]. Thus decreasing the effectiveness of the PCVs and PPVs over time.

New vaccine strategies

Virulence factors are molecules which are found in virulent serotypes and aid in causing disease. This includes factors involved in colonization, the evasion of the immune system, suppression of the immune system and invasion to sterile sites of the host[20]. The virulence factors of *S. pneumoniae* include the capsule, pneumolysin, the pilus and several cell wall proteins. It is possible to develop vaccines which target virulence factors. For example, PPVs and PCVs target the capsule. A lot of effort is put into the development of

vaccines based on virulence factors, specifically protein based vaccines, common to all virulent serotypes. In this section a couple of promising factors will be discussed.

Pneumolysin (Ply) is a cholesterol dependent toxin of 52 kDa and forms a pore in the membrane of the target cell. This pore contains about 40 Ply monomers[7]. Ply promotes colonization by inhibiting the movement of cilia in the respiratory epithelium, promotes evading of the immune system by inhibiting phagocyte activation and also causes damage to the epithelium by disruption of tight junctions'[19]. Ply is actively involved in virulence, therefore it is an interesting target for the development of a new vaccine. However, Ply is secreted by *S. pneumoniae* and is not bound to the bacterial cell. Therefore a vaccine targeting Ply is not clearing the cells from the nasopharynx, but the vaccine is only clearing Ply[23].

Pneumococcal surface protein A (PspA) is a choline binding protein and it attached to the cell wall of *S. pneumoniae*. It promotes colonization by binding to sugars located on the respiratory epithelial surface[3]. PspA promotes the evasion of the immune system by inhibition of opsonization with complement factor C3 and thus decreasing phagocytosis. PspA also promotes the suppression of the immune system by inhibition of lactoferrin[4].

Choline binding protein A (CbpA), also known as PspC and SpsA is another cell wall protein which promotes colonization by binding to sugars on the respiratory epithelial surface[19]. CbpA promotes invasion to the sterile sites of the host by binding to the human secretory component of the polymeric immunoglobulin receptor (pIgR)[7]. Normally pIgR translocates IgA and IgM across the epithelial cells. By binding to this receptor *S. pneumoniae* may use the pIgR to cross the epithelial membrane through retrograde translocation and thus invading the host[14]. CbpA also promotes evasion of the immune system by binding to IgA and complement factors H . IgA is abundant in the mucus and helps with the clearance of bacterial cells. By binding to IgA *S. pneumoniae* decreases this clearance.

Also, binding to complement factor H decreases the effectiveness of the alternative complement pathway and therefore the effectiveness of the immune response[4].

Pneumococcal surface antigen A (PsaA) is a part of an ABC transporter which transports manganese across the bacterial membrane. Manganese is necessary for the normal growth of *S. pneumoniae*. Thus by interfering with PsaA and therefore disrupting the ABC transporter, the growth and colonization of *S. pneumoniae* is inhibited[7]. PsaA plays also a role in the adherence to the epithelial surface of the nasopharynx. The precise mechanism of adherence is not clear, but PsaA promotes colonization through this action[4, 15].

Pneumococcal iron acquisition A (PiaA) and pneumococcal iron uptake A (PiuA) are both proteins which are part of an ABC transporter. This ABC transporter translocates iron across the bacterial membrane, which is necessary for normal growth. The normal growth and colonization of *S. pneumoniae* is inhibited by immunization with PiaA and PiuA[7, 15].

The involvement of pneumococcal histidine triad proteins (Pht) in virulence is not completely clear, but it is likely that Pht-proteins promote colonization. It also may be involved in decreasing opsonization by interfering with the complement deposition on the bacterial surface[4]. The most promising vaccine candidate of the Pht proteins is PhtD, because this protein is found in all strains[8].

The above mentioned virulence factors are among the most promising and most investigated factors as part of the development of new vaccination strategies. There are many other virulence factors which vary in their efficacy as vaccine candidates for various reasons. These factors include LytB, LytC, StkP, neuraminidases and many others. LytB and LytC are both choline binding proteins and StkP is involved in the metabolism of the cell wall and iron uptake. Neuraminidases help to colonize the nasopharynx by decreasing the viscosity of the mucus[4, 15].

A problem with the development of the above mentioned protein based vaccines is the variation in the amino acid sequence. All serotypes produce these proteins, but the sequence can differ from strain to strain thus decreasing the effectiveness of a vaccine. These variations in the gene sequence produces antigenically distinct proteins hindering broad coverage. Also, the thick capsule of invasive pneumococcal isolates limits the exposed epitopes of virulence factors. A solution for this problem may be the development of vaccines with multiple targets. It has been shown that a combination of PspA and pneumolysin increases protection against invasive disease. The same protection from invasive disease is also observed for a combination of CbpA and pneumolysin. Unfortunately this does not apply to all combinations of virulence factors. For example the combination of Pht-proteins with PspA showed a decrease in protection against invasive disease[19].

The pilus of *S. pneumoniae* consist of three structural proteins named RrgA, RrgB and RrgC and the pilus is involved in adherence to the epithelial surface and thus colonization of the nasopharynx[2]. Therefore the pilus may be called a virulence factor, although it isn't directly involved in disease. A major problem with the immunization against pilus proteins is the low distribution of pili, with only one third of *S. pneumoniae* expressing a pilus[4].

Another strategy is the development of a whole cell vaccine, which is made of dead unencapsulated cells. The main advantages of this approach are that all the surface associated proteins are presented to the immune system. Also costs of using a whole cell vaccine are usually lower compared to the above mentioned vaccines. However, effective immunization with whole cell vaccines is usually a bit more complicated. Whole cell vaccines tend to have a lower immune response due to the numerous antigens present on the surface. Thus immunization with a whole cell vaccine requires multiple shots. Nonetheless a whole cell vaccine is currently in phase 1 trails[15].

Preventing colonization

Colonization is always the first step in the development of *S. pneumoniae* related disease, but colonization does not mean that the bacteria will cause disease. In most cases of *S. pneumoniae* colonization the bacteria is harmless and it lives as a commensal[3]. The current opinion is that disease will be prevented if colonization can be prevented. Most of the virulence factors that are being investigated for the development of a new vaccine are promoting adherence to the epithelial surface and therefore promoting colonization of the nasopharynx. Therefore the development of a vaccine targeting those virulence factors seems the best strategy. However, this strategy may have major disadvantages. One of them is the replacement of other, more pathogenic, species.

S. pneumoniae resides in the human nasopharynx, but it is not the only species that colonizes the nasopharynx. The microbiome consists also of bacterial species including *Staphylococcus aureus, Haemophilus influenzae* and *Neisseria meningitis*. These species normally live in a natural balance as harmless commensals together with *S. pneumoniae*. However, just as *S. pneumoniae*, these species can also become pathogenic[22].

The eradication of a species from a niche gives other species the opportunity to occupy this niche. An example of such a process is the appearance of new serotypes of *S. pneumoniae* after the introduction of PPVs and PCVs. Eradication of all *S. pneumoniae* serotypes results in an open niche. This niche can be colonized by other pathogenic bacteria like *S. aureus* and *H. influenzae*[5]. At first this may not be a problem, because *S. aureus* and *H. influenzae* are normal, mostly commensal inhabitants of the human nasopharynx. However, the problem may be the disturbance of a natural balance between the co-colonizing species. Also the chance of infection increases if there are more cells of a certain pathogen[5].

Preventing the colonization of *S. pneumoniae* via a new vaccine, may give rise to a whole new set of problems. At the end not much is known about indirect side effects after the

introduction of new vaccines, because most of the research involves the target species. Therefore it is a good idea to investigate the effects on the whole nasopharyngeal microbiome before introduction of a new form of prevention.

At the same time eradication of a virulent serotype results in less co-colonization between different serotypes. This results in less horizontal gene transfer between different serotypes. Horizontal gene transfer by *S. pneumoniae* happens when cells take up DNA from their surroundings. The new DNA may have genes which contribute to the survival of the cells and can contain virulence factors. This horizontal gene transfer is a major way of evolving and becoming virulent for *S. pneumoniae*. Less horizontal gene transfer leads to less uptake of virulence factors by non-virulent serotypes and therefore results in less virulence[24].

Novel vaccine strategies

New ways of vaccination are the most investigated strategy to prevent *S. pneumoniae* infection. However, there may be other ways to prevent infection, such as stopping specific cellular processes. These are not as thoroughly under investigation as the above mentioned methods, but nonetheless it may be important for future use to investigate these novel prevention methods. Several of these strategies will be discussed in this section.

When a serotype has a thick capsule, it is more likely to be virulent than when expressing a thin capsule. Therefore it may be useful to look into the formation of the capsule. Once the formation of the capsule is blocked or locked in a thin state, than the strain will become less virulent. Since the capsule is made of polysaccharides, a promising strategy for interfering with the formation of the capsule is to block the production of these capsular polysaccharides (CPS). The amount of CPS can be determined by measuring the transcription level of the *cps* locus[26]. The *cps* locus has a promoter and transcription factors bind to this promoter and regulate the transcription of the *cps* locus. Therefore the transcription factors can regulate the production of CPS and maybe regulating virulence among *S. pneumoniae*.

CpsR is a CPS production regulator and this regulator negatively controls the production of CPS. Increased expression of CpsR leads to a decrease in resistance to killing. CpsR was the first regulator of the *cps* locus discovered[27]. Further investigation of these regulators may lead to new ways to interfere with polysaccharide production and therefore new ways of interfering with virulence. Unfortunately there has been an emergence in virulent nonencapsulated strains[11], but nevertheless investigating CPS production may be useful in the future.

Future research

The current strategies for treatment or prevention of pneumococcal infection are mainly vaccine or antibiotic based. Antibiotic based methods can have toxic effects on healthy tissue and the medicine does not arrive in concentrated doses. These problems can be overcome by new emerging drug delivery methods like bioengineered bacteria and nanocarriers that deliver drugs to the specific targets sites inside the body.

A problem with drug delivery with bioengineered bacteria is the immune system. The immune system recognizes bacterial cells and will start removing the cells from the body, just as it does with every other foreign cell. This problem may be overcome by decreasing the toxicity of the bacterial cell via genetic modification. However, changing the genetics of bacterial cells may also affect therapeutic use and even after the removal of factors involved in toxicity there is still residual toxicity reported. The remaining toxicity could be problematic for the immunocompromised, which is a group in which *S. pneumoniae* related infection can lead to death[6].

Nanocarriers are molecules that can carry medicine to specific parts of the body and with the rise of stimuli-responsive nanocarriers it is possible to be even more accurate. Stimuli-responsive nanocarriers are molecules that carry inactive medicine. If the medicine reaches a certain stimulus, it will undergo a reaction in which it becomes active. In theory this

stimulus will only take place at the site of the infection. Therefore the medicine will only be active at the site of infection. These stimuli can be exogenous and endogenous. Exogenous stimuli are variations in temperature, changes in the magnetic field and exposure to light. These exogenous stimuli can be applied by a physician at the area of interest. Endogenous stimuli are changes in pH, variations in the expression of protein or changes in the concentration of other molecules. The main advantage of endogenous stimuli are that they occur at the place of infection and therefore the medicine will be mainly active at the place of infection. A major problem in the development is designing stimuli-responsiveness at the site of infection. The molecules have to react in a precise manner when encountering a certain stimulus. Also, the molecules have to be non-toxic and easy to handle. Further research is necessary to overcome these and other hurdles[17].

Final remarks

S. pneumoniae related disease poses still a major threat. Every year nearly a million children die from pneumococcal diseases including pneumonia and meningitis. Most of these children live in developing countries. The current treatment consists of antibiotics, but *S. pneumoniae* and many other bacteria with it, are becoming resistant against the most commonly used antibiotics. Another form of reducing pneumococcal disease is the prevention of infection by vaccinating against *S. pneumoniae*. The current vaccines: PPV23, PCV7 and PCV13 worked very well, but in recent years they have been less effective due to serotype replacement. This is due to the fact that above mentioned vaccines target the capsule. Thus the current vaccination strategy does not work, since there are over 90 different serotypes of *S. pneumoniae*, which all have antigenically distinct capsules. A lot of effort is put into the development of new vaccines that target specific virulence factors or factors common to all serotypes. Some of these factors look very promising and are already in different phases of human trials. However, vaccination which leads to eradication of all *S. pneumoniae* may

eventually have negative consequences. Overall the cure for *S. pneumoniae* related disease may seem far away, but with the development of new forms of treatment and with the worldwide effort put into understanding the role of *S. pneumoniae* pathogenesis it is certainly possible to get rid of those diseases once and for all.

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