# Novel Approaches To Fight *Streptococcus pneumoniae*

Beatriz Maestro* and Jesús M. Sanz

*Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Av. Universidad, s/n. 03202 Elche (Alicante), Spain*

*Received: July 24, 2007; Accepted: August 15, 2007; Revised: September 10, 2007*

**Abstract:** *Streptococcus pneumoniae* affects millions of people worldwide. It is responsible for a wide spectrum of serious illnesses such as pneumonia, meningitis and bacteraemia. The highest rate of pneumococcal disease (and the highest mortality) occurs in young children, as well as in the elderly and the immunocompromised patients. Identification of *S. pneumoniae* in diagnostic procedures may significantly improve thanks to the descripion of new PCR-derived techniques. Vaccination based on the polysaccharidic capsule, together with benzylpenicillin-derived drugs, constitute the current choices to tackle pneumococcal diseases. However, the wide serotype diversity of *S. pneumoniae* and the emergence of antibiotic-resistant strains is fostering the development of new methods to fight this microorganism. In this sense, patents documenting the use of novel antibiotics of the fluoroquinolone or tetracycline families have recently been described. Moreover, surface-associated proteins are receiving an increasingly special attention, as they are synthesized by most pneumococcal strains and play an important role in virulence. New patented protein-based vaccines take into consideration these polypeptides. In this article we present the main relevant characteristics of this pathogen and review the most recent methods that have been patented for the prevention, diagnostic and treatment of the pneumococcal diseases.

**Keywords:** *Streptococcus pneumoniae*, pneumococcus, pneumonia, otitis, meningitis, keratitis, vaccines, polysaccharidic capsule, diagnosis, antibiotic resistance, choline-binding proteins, enzybiotics.

## INTRODUCTION

*Streptococcus pneumoniae* (pneumococcus) is a Gram-positive encapsulated diplococcus bacterium [1]. It is a major cause of invasive infections (meningitis, bacteraemia) and diseases affecting the upper (otitis media and sinusitis) and lower (pneumonia) respiratory tracts [2]. Pneumococcal disease occurs worldwide and it is estimated to kill 1.6 million people annually, most of these in developing countries [3]. Children under two and the elderly constitute the main target group [3]. Moreover, HIV infection and conditions that compromise the immune system are associated with increased risk of pneumococcal illness [4].

*Streptococcus pneumoniae* is typically found in the naso-pharynx of healthy individuals. It has been estimated that about half of children are colonized with this bacterium [5]. Pneumococci are transmitted by direct contact with respiratory secretions from patients and healthy carriers and attach to epithelial cells via bacterial surface adhesins. Eventually, the infection takes place after movement of the patogen from mucosal surfaces in the nasopharynx to other parts of the body [6].

Among the wide panoply of diseases caused by *S. pneumoniae*, meningitis, pneumonia and otitis media should be regarded as the most important not only in medical but also in the economic terms associated to healthcare costs [2]. Meningitis is the most life-threatening pneumococcal illness. It causes inflammation of the layers surrounding the brain and spinal cord, with important neurological after-effects in survivors such as deafness, blindness, paralysis and mental impairment. It is estimated that *S. pneumoniae* has become the most common microorganism causing bacterial meningitis in the United States (US) [7], and may also account for about 30% of cases in developing countries [8]. The mortality associated to pneumococcal meningitis may reach values around 25%, especially in the elderly [3,9]. On the other hand, community-acquired pneumonia (CAP), of which *S. pneumoniae* is the leading causing agent, is the main cause of death due to infectious diseases in developed countries [10], and one of the major causes of mortality worldwide [11,12]. Complications of pneumonia that may occur include empyema, pleural effusion, bacteraemia and meningitis. In Europe and US the incidence of this invasive disease may reach levels of more than 100 cases per 100,000 population [13]. With respect to acute otitis media (OMA), this appears as a painful, suppurative and purulent infection of the middle ear, with an estimated incidence of more than 70% in early childhood [14 and references therein], and pneumococcus has been pointed as the most frequent cause [15]. OMA is one of the most frequent childhood diseases, the primary reason for paediatrician visit under three in developed countries [16] and the most common indication for the prescription of antibiotics [17]. One-third of patients eventually develop new episodes, leading to chronic otitis media in 5-10% of cases [18]. Complications arising from otitis media comprise tympanic perforation and meningitis, among others [16,19].

Finally, *S. pneumoniae* also plays a role in the development of other illnesses such as bacteraemia [20], keratitis [21], sinusitis [22], osteomyelitis and arthritis [23].

## DIAGNOSIS

Diagnosis of pneumococcal infections is carried out by evaluation of clinical history, signs, symptoms and (in the case of pneumonia) chest radiography. The course and duration of the disease will be established, to a great extent, by a rapid and accurate determination of the causal agent and its antibiotic susceptibility. At this point a specific therapy is defined, leaving aside the initial, empiric treatment. In the case of CAP, a correct diagnosis is important since many diseases affecting the respiratory tract are of viral origin, making unnecessary the use of antibiotics.

A culture assay is today the Gold Standard for detection of pneumococcus, and conventional microbiological tests are therefore used involving bacteria and colony appearance, α-haemolysis, susceptibility to optochin and bile or deoxycholate solubility [24]. Clinical material such as cerebrospinal fluid, sputum, lung aspirates or blood samples can be cultured if handled with care. These probes are time consuming, and many times do not finally define the etiology of the illness due to low culture yield, mostly because antibiotics have been previously administrated, or to colonization of lower airways in some patient groups. In case of purulent sputum a sample can be sent to the laboratory for Gram stain and culture, in spite of that the value of the result depends directly on the quality of the sample and the experience of the clinical microbiologist. On the other hand, serological assays identify antibodies to the bacteria,

*Address correspondence to this author at the Instituto de Biología Molecular y Celular, Universidad Miguel Hernández, Av. Universidad, s/n. 03202 Elche (Alicante), Spain; Tel: +34-966-658-460; Fax: +34-966-658-758; E-mail: bmaestro@umh.es

1574-891X/07 $100.00+.00 

but sometimes they do not appear in blood in high enough levels (infection too recent or immunocompromised patients) or they are residuals from a previous infection.

With all this in mind, currently there are not good definitive routine guidelines for the rapid and unequivocal detection of the causative agent, and in half of the cases no ethiological agent is defined. Considerable effort is being made in developing new diagnostic kits to allow immediate results for most patients. The test should be quick, easy, cost-effective and accurate. Besides, it should detect all the 91 pneumococcal serotypes. In this sense, new advances are continuously being implemented as complementary assays.

A rapid urinary antigen assay (Binax NOW® *S. pneumoniae* urinary antigen test; Binax, Portland, Maine) has been approved by the U.S. Food and Drug Administration for helping in the diagnosis of pneumococcal illness. It is a rapid immunochromatographic assay for the detection of the C-polysaccharide cell wall antigen common to all *S. pneumoniae* strains [25]. The test is quick, simple to perform, and it appears to be highly specific and sensitive in adults [26,27]. However, the performance of the test in children remains controversial because of the widespread nasopharyngeal carriage of pneumococci, especially in developing countries [28]. The Binax NOW test has also been used in cerebrospinal fluid (CSF) samples for the diagnosis of meningitis [29].

Many rapid diagnostic kits have been published and/or patented based on the polymerase chain reaction (PCR) [30]. The aim is to detect genes that are unique to the *S. pneumoniae* species. Oligonucleotide primers may be labeled with fluorescent or chromophore probes, although the amplification product may also be visualized by usual agarose gel electrophoresis. This kind of tests have been proved to be rapid, highly sensitive and specific. Moreover, continuous real-time PCR monitoring allows the determination of the samples in a high-throughput format [31]. Besides, previous administration of antibiotics do not affect the sensitivity of the assay. However, despite its great potential, these assays are still not regularly used. Although the technique is widely accepted for sterile samples, contamination in non-sterile ones may compromise the interpretation of the results, and in some cases, cross-reaction with highly related species has been documented [32]. As a consequence, it has been suggested the need of performing the test with three different samples in order to enhance the sensitivity [33]. Nevertheless, due to its potential, it is expected that current and future refinements will finally lead to the widespread use of this diagnostic technique in a routinary basis.

The *S. pneumoniae ply* gene encodes the pneumococcal cytolytic protein pneumolysin, that is present in all the relevant serotypes [34]. Several studies have demonstrated the great potential of the PCR technique with the *ply* probe, with a sensitivity between 90-100% while Gram staining and microbiological culture only account for 62% and 36% respectively, and displaying limited cross-reaction with other bacterial, virus and human genes [35,36]. Furthermore, this method can be tested with clinical samples from cerebrospinal fluid, plasma, serum, lung aspirates and whole blood, and it is possible to employ specific primers for the main three species causing meningitis (*Neisseria meningitidis, Haemophilus influenziae type B* and *S. pneumoniae*) with no cross-reactions between them, thus allowing the identification of the infectious microorganism with a single experiment [35]. However, other studies describe the possibility of cross-reaction with pneumococcus-like species of the viridans type [32].

Another probe that has been evaluated for PCR-based diagnosis is the *psaA* gene. It codes for the pneumococcal surface adhesin PsaA, a 37-kDa lipidated protein present in the surface of all serotypes of *S. pneumoniae* [37,38]. Although *S. mitis* and *S. oralis* possess a gene that is 95% similar to the *psaA* gene, a PCR with primers derived from the pneumococcal *psaA* gene does not amplify a fragment of the target size in these heterologous pathogens [39,40] so that *psaA* PCR-based diagnosis could be set if detection is made by gel electrophoresis. A kit for the specific diagnosis of pneumococcal disease by *psaA* PCR has already been patented [41].

The *lytA* gene has also been reported in numerous studies as target for pneumococcal PCR diagnosis [42-44]. The *lytA* gene codes for the N-acetylmuramoyl-L-alanine amidase and has been extensively studied [45]. Comparative studies with the three different PCR target genes most commonly used (*i.e. ply*, *lytA* and *psaA*) demonstrated that *lytA* PCR is the most specific in discriminating true pneumococci from atypical streptococci [32,46]. Finally, in a recent study, Llull *et al.* [47] found that the nucleotide sequences of the *lytA* gene from atypical alleles present a 6-bp deletion at the 3' end of the sequence, together with other mutations that confer them distinctive signatures. Based of these features, they propose a *lytA* PCR diagnostic method combined with restriction analysis to unambiguously identify between typical and atypical *lytA* alleles, therefore improving the specificity of the diagnostic tool.

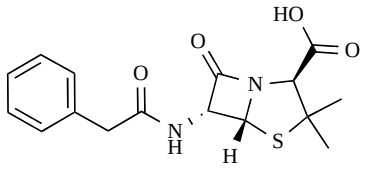
Other diagnostic tests include the penicillin binding proteins [48,49], the superoxide dismutase gen *sodA* [50], and specific 16S ribosomal RNA sequences (ACCUPROBE®, GenProbe, San Diego, California).

## ANTIBIOTIC THERAPY

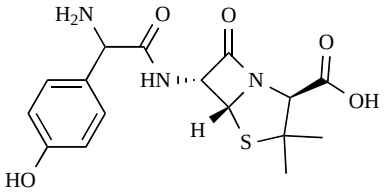
Given the high variety and complexity of pneumococcal diseases, a comprehensive review of the practical guidelines for their management is out of the scope of this article. Doses and class of antibiotic strongly depend on parameters such as the type and severity of the disease, patient history and physical state, age, antibiotic resistance, etc. [51-54]. In many cases the first treatment is often taken in the emergency department, and it is usually decided empirically. As a general rule, β-lactam antibiotics are usually the first choice (Fig. **1**), being penicillin G (benzyl-penicillin) the preferred agent for penicillin-sensitive strains, sometimes in combination with amoxicillin or a macrolide [52]. Current guidelines also include the use of fluoroquinolones (Fig. **2**) such as gemifloxacin or garenoxacin as an alternative first treatment option, especially in the elderly and other risk-patients, or when a β-lactam allergy is suspected [55-57].

For the treatment of pneumococcal meningitis, cephalosporins (cefotaxime and ceftriaxone, Fig. **1**) are used in combination with vancomycin [58], although some data suggest that in certain situations the addition of rifampicin (rifampin) to ceftriaxone may be a better choice [59]. Besides, therapeutic possibilities against CAP are very diverse. Among β-lactams, administration of amoxicillin in combination with clavulanic acid is very common, as it is the use of cephalosporins (cefdinir, ceftriaxone, cefuroxime) [13,52] and recently tested carbapenems (ertapenem, [60]) (Fig. **1**). To overcome allergy problems, alternatives include fluoroquinolones (levofloxacin, moxifloxacin, gemifloxacin) [13,52] (Fig. **2**). In this sense, it has been described that garenoxacin displays a high efficiency against virtually all pneumococcal strains and is independent of resistant types [61]. Other posibility is the use of azithromycin [62] (Fig. **3**) or macrolides such as doxycycline, although the studies on the effects of these compounds on CAP are scarce and therefore their administration in combination with β-lactams is recommended [63].

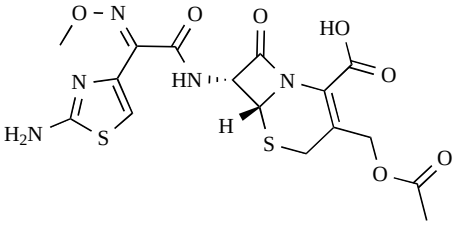
Treatment of OMA is mainly empirical, fundamentally with prescription of amoxicillin and ceftriaxone [64,65] (Fig. **1**). Recently, carbapenems such as tebipenem pivoxil (Fig. **1**) are receiving increasing attention [66]. However, many studies question the convenience of antibiotic therapy for this disease: only 13% of children benefit from antibiotic treatment, while about 80% of cases evolve spontaneously [67,68].



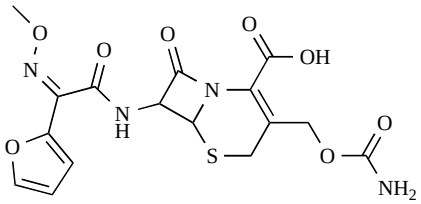
Benzylpenicillin (Penicillin G)



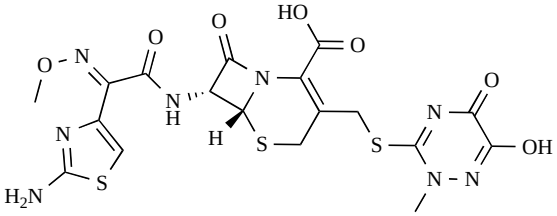
Amoxicillin



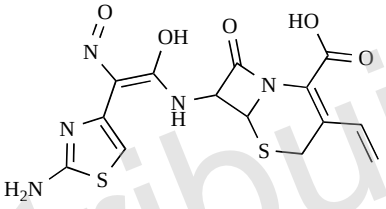
Cefotaxime



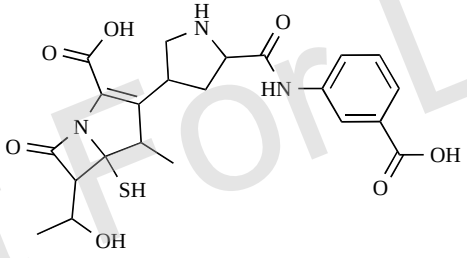
Cefuroxime



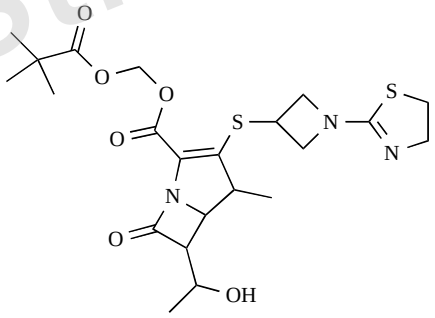
Ceftriaxone



Cefdinir



Ertapenem



Tebipenem pivoxil

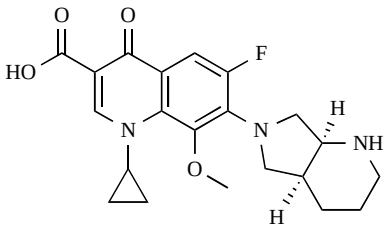


**Fig. (1).** Structures of several β-lactam antibiotics.

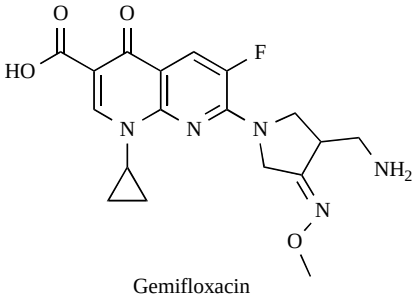
Finally, topical benzylpenicillin (penicillin G), imipenem or ciprofloxacin have historically been used for the treatment of pneumococcal keratitis [20, 69-71], although recently cholesterol has been shown to be very useful as an alternative [72].

**Antibiotic Resistance**

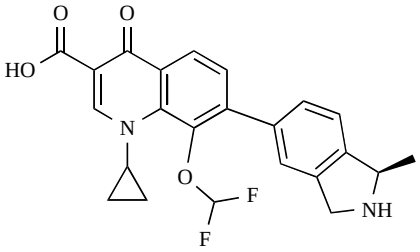
The increased prevalence of drug-resistant pneumococci is a matter of extreme interest [73]. The treatment of patients infected with these organisms requires expensive alternative antimicrobial agents and may result in prolonged hospital stays. Resistance to β-lactams is mostly recognized. The incidence of invasive *S. pneumoniae* isolates insensitive to penicillin has been reported to be around 34% in 2000 in the US [74]. Remarkably, penicillin resistance in Southern Europe skyrocketed from about 5% to nearly 50% in only a decade, whereas, on the contrary, resistance levels in Northern Europe (where the use of antibiotics is more restricted) are contained below 5% ([75] and references therein). One of the solutions to tackle this problem makes use of formulations combining amoxicillin and a salt of clavulanic acid [76,77]. The mechanism of penicillin resistance is based on the production of low-affinity penicillin-binding proteins (PBPs) [78], the normal function of which is the catalysis of the latest phases of the biosynthesis of cell wall peptidoglycan [79]. Nevertheless, other genes not directly involved with the PBPs have been shown to participate in the resistance process. This is the case of proteins like MurM and MurN that catalyze the addition of the first amino acid residue of the short dipeptide branches to the muropeptide units of the pneumococcal peptidoglycan [80]. Inactivation of *murMN* operon causes not only alterations in the peptidic cross-bridges of the cell-wall peptidoglycan, but also a complete loss of penicillin resistance [81]. These observations have allowed the development of methods for treating penicillin-resistant *S. pneumoniae* infections by inhibiting MurM and/or MurN proteins, which would result in losing the penicillin resistance [82].



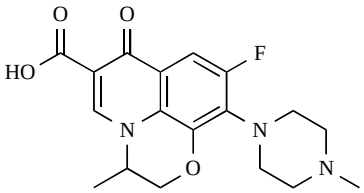
Moxifloxacin



Gemifloxacin

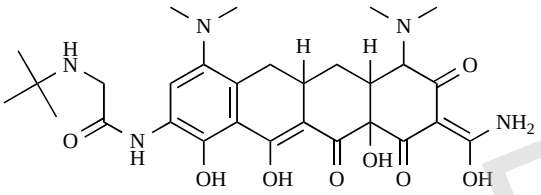


Garenoxacin

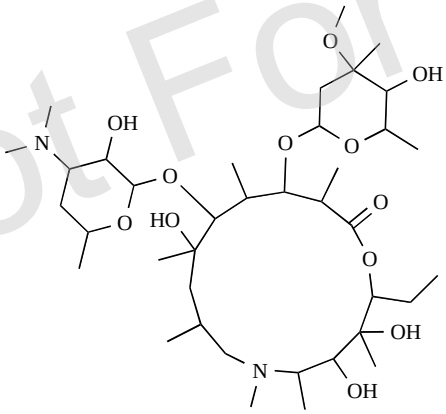


Levofloxacin

**Fig. (2).** Structures of several fluoroquinolones.



Tigecycline



Azithromycin

**Fig. (3)**. Structures of other representative antipneumococcal antibiotics.

Worryingly enough, *S. pneumoniae* not only displays resistance against β-lactam antibiotics, but also to fluoroquinolones and macrolides [83] among others. In fact, the prevalence of macrolide-resistant strains is even higher than that of penicillin-resistant ones in many countries [84]. Moreover, there seems to be a strong correlation between penicillin and fluoroquinolone [85] and macrolide [86] resistances. The appearance of multi-drug-resistant strains is certainly a major concern in the fight against pneumococcus, making necessary the continuous development of new drugs. In this sense, new fluoroquinolones such as levofloxacin, moxifloxacin and gemifloxacin (Fig. **2**) are related with a current resistance lower than 3%. Furthermore, tigecycline is a glycylcycline (a derivative of tetracyclines) that may also be promising against penicillin-resistant pneumococci [87,88] (Fig. **3**).

## VACCINES

### Vaccines Based in Cell Capsule

The polysaccharidic capsule is a major virulence factor of *S. pneumoniae*, and is essential for invasive disease. It is antigenic and constitutes the major candidate for the development of vaccines. Moreover, it represents the basis for classifying all the pneumococcal serotypes. There are at least 91 different serotypes, based on differences in the bacterial polysaccharide or 'sugar coat'. The major pneumococcal diseases are associated with a small number of serotypes, which may vary by region. Globally, only 13 serotypes cause at least 75% of invasive disease in children [3].

Protective immunity against pneumococcal disease is currently dependent upon type-specific anticapsular antibodies. The adult vaccine is based on polysaccharides from the 23 more prevalent serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, and 33F) (PNEUMOVAX®, Merck Research Laboratories). However, its main drawback is that its non-conjugated polysaccharide antigens are T-cell independent and cannot be presented to the MHC molecules to interact with T-cells. These capsular polysaccharides can only activate mature B cells, which may be the reason why infants respond poorly to such vaccine [2]. Moreover, the 23-valent vaccine does not demonstrate protection against pneumococcal pneumonia and otitis media diseases [89] and some adverse side effects of vaccination may be produced in the elderly.

An improved polysaccharide-based vaccine for the prevention of pnemococcal infection (particularly pneumonia) of the elderly (and/or infants and toddlers) has been presented by Laferriere and Poolman [90]. The invention uses at least one *S. pneumoniae* polysaccharide antigen (conjugated or not) and at least one pneumococcal protein antigen selected from: PolyHistidine Triad family (Pht; e.g. PhtA, PhtB, PhtD or PhtE), Lyt family (e.g. LytA, LytB, or LytC), SpsA (PspC, CbpA), Sp128, Sp130, Sp125 (ZmpB), Sp101 and Sp133, optionally with a Th1 adjuvant. The proteins can be processed or presented to MHC molecules to

interact with T-cells, whereas the polysaccharides can stimulate the immune system through B-cells. With the simultaneous stimulation of T-cell and humoral immune system a synergy is achieved for the prevention and/or treatment of pneumonia in the elderly. The presence of the adjuvant in the vaccine formulation enhances the synergy between both branches of the immune system.

Other authors have explored the idea of combining antigens from different bacteria to fight a unique disease. Combining antigens into a single dose is attractive, although it often presents difficulties due to antigen interference or competition. However, in the case of the meningitis, O´Hagan [91] has presented a composition for mucosal delivery comprising an antigen from *S. pneumoniae*, *Haemophilus influenza*e and *Neisseria meningitidis*. All antigens are of capsular saccharide type and are preferably conjugated to a carrier protein. This combination of polysaccharide protein conjugate vaccine for intranasal inmunization has turned out effective in the antibody response to either antigen.

The 7-valent pneumococcal conjugate vaccine, known as PCV-7 and first licensed in the US in 2000 as PREVNAR® (Wyeth) is the main vaccine to help prevent invasive pneumococcal disease in infants and toddlers [3]. It contains saccharides derived from the capsular antigens of seven *S. pneumoniae* serotypes (4, 6B, 9V, 14, 18C, 19F and 23F), conjugated to a nontoxic variant of the highly immunogenic diphtheria toxin. The serotypes included are the most commonly involved in invasive disease in children younger than 6 years in the US [92]. The vaccine has been licensed in many countries around the world and its effectiveness has been extensively assessed [93,94]. However, this vaccine is far from being a definitive solution. The protective efficacy of the PCV-7 against infections of the respiratory tract is currently questioned [3,89]. Moreover, the vaccine serotypes are now being simply replaced by nonvaccine ones, taking their place in causing disease [95,96], and what is more, new polysaccharide types can be acquired by natural transformation [97]. For all these reasons, more efforts are directed to improve pneumococcal vaccine compositions and to make them more widely available.

Currently, 9-, 10-, 11- and 13-valent vaccines are under the last trial phases [98]. A 13-valent pneumococcal conjugate vaccine (13vPnC) has been described as comprising the seven serotypes in the PCV-7 vaccine plus six additional ones (1, 3, 5, 6A, 7F and 19A) [99]. Serotypes 6A and 19A, which are associated with antibiotic resistance, play an important role in otitis media [100], and provide a better coverage to children under 2 years of age.

As mentioned before, *S. pneumoniae* is a pathogen which enters the body through the respiratory mucosa. In this sense, a patent [101] relates to the potential of mucosal immunization with pneumococcal polysaccharide vaccines, either conjugated or not. It has been shown that intranasal immunization with pneumococcal polysaccharides from serotypes 1 and 3 conjugated to a carrier polypeptide and mixed with RhinoVax® as adjuvant was able to protect mice against infection after intranasal challenge with the respective pneumococcal serotypes and that the protection was related to the levels of type specific serum IgG and IgA antibodies [101].

### Vaccines Based on Pneumococcal Proteins

The investigation of several immunogenic pneumococcal proteins as candidates for the development of alternative vaccines is currently receiving an increasing attention. A family of these proteins are the so-called choline-binding proteins (CBPs). These proteins are present in all pneumococcal isolates, have several important physiologic roles, and are related to virulence [102,103]. They contain a functional module linked to a choline-binding module that is responsible for the anchoring of the protein to the choline-containing teichoic acids in the cell wall. Pneumococcal peptidoglycan hydrolases (LytA, LytB, LytC), the surface protein PspA, and adhesins such as PspC (CbpA) and PcpA, are members of this family. One major advantage of these vaccines would be that, since CBPs are common to all strains, they would overcome in principle the insufficient coverage of all serotypes displayed by the polysaccharidic vaccines. Moreover, the costs associated to these vaccines are certainly lower and this should allow the adequate availability by developing countries [104]. Pneumococcal surface protein A (PspA) is a well-known virulence factor that is expressed by all pneumococcal types [105,106]. It is a surface polypeptide that leads to a significant cross-reactivity between the different families of the protein [105,107]. Therefore, it might constitute a good candidate for a vaccine and a patent has been issued accordingly [108]. In fact, immunization against PspA, as well as the adhesin PspC (CbpA), has demonstrated some success in reducing otitis media infections in rats [109], and a patent has taken this into account [110]. The same approach was also useful for treatment of pulmonary infection and sepsis in mice [111]. Furthermore, human antibodies raised against PspA have been found to protect mice challenged intraperitoneally with pneumococci [112]. An interesting variation is the conjugation of PspA with a capsular polysaccharide from *N. meningiditis* in search of a meningitis conjugate vaccine [113]. Patent US2005196405 also takes into account the immunological potential of the adhesin PspC [114]. Other patents involving CBPs as vaccine components have also been issued recently [115-117].

Besides CBPs, other surface proteins like the pneumococcal surface adhesin A (PsaA) may also be useful. It has been shown that oral vaccination of mice with PsaA induces a significant protection against colonization, pneumonia and septicaemia [118]. Nevertheless, despite these promising results, allelic variation within individual proteins seems to prevent species-wide pneumococcal protection. Therefore, an efficient protein-based vaccine will probably have to take into account a combination of different proteins like the cited above [119], or all allelic variants of the same protein [104].

## NEW THERAPIES

### Choline-Binding Proteins as New Targets

As mentioned above, the antibiotic resistance problem, together with the limited efficacy of vaccines and the fact that these may be economically non-viable for developing countries [120], make necessary the search for novel, alternative ways to fight pneumococcus. In any case, the most effective and selective treatments should take into account virulence factors common to all pneumococcal strains. In this sense, much attention has been focused on the surface exposed proteins like the CBPs described above, since they are involved in important functions for the cell such as adhesion to the host cell, bacterial sepsis, etc.

Inhibition of the biological role of CBPs might constitute a promising way for new therapies. As all the CBPs share the ability of recognizing choline in the cell wall, in an ideal case, the same drug should inhibit all of them, therefore difficulting the appearance of antimicrobial resistances. In fact, addition of choline to pneumococcal cultures inhibits daughter cell separation and induces the formation of long chains [121]. Exogenously added choline competes with the choline residues present in the cell-wall, and interferes with the normal binding of CBPs. It has been reported that ofloxacin-type quinolones may act as choline analogs and are able to inhibit the activity of some CBPs [122]. In a recent study [123], we have shown that other analogs of choline, *i.e.* esters of bicyclic amines (atropine and ipratropium), are much more effective inhibitors of CBPs than choline itself, and they also arrest cell growth and induce the formation of cells of abnormal shape. This suggests that these compounds bind to proteins essential for the pneumococcal life cycle, and opens the possibility of a new selective therapy to tackle pneumococcal diseases.

In some cases, it may be interesting to activate the function of some CBPs rather than to inhibit it, especially if the CBP is a lytic enzyme. Miltefosine, currently licensed as an oral antileishmanial agent, has been found to activate the release of the LytA amidase [124], therefore promoting the uncontrolled lysis of pneumococcal cultures.

It should be highlighted that not only the CBPs but also the choline *per se* is also involved in virulence. Kharat and Tomasz [125] have recently demonstrated that, whereas the need for choline in growth can be bypassed, its requirement for pneumococcal virulence is unquestionable. Choline utilization mutants were isolated that showed a severe inhibition to adhere to human nasopharyngeal cells, thus been unable to invade them, and these mutants were also avirulent in intraperitoneal and intravenous models of mouse infection. This means that in the absence of choline in the cell-wall the bacteria would be taken away from their *in vivo* habitat, and this might constitute a new target for action against pneumococcus. In this sense, it has been found that the genes in the *lic* locus of *H. influenzae* encode enzymes that participate in the addition of choline to the cell wall. Moreover, one of the products of this locus, the LicA choline kinase, is similar in sequence to a choline kinase described for *S. pneumoniae*. Therefore, it might be possible to find drugs inhibiting these enzymes (or their biosynthesis), preventing the incorporation of choline into the cell wall [126]. Moreover, since the human choline kinase is considered an oncogene, and enzyme inhibitors with antitumoral properties have been patented [127,128], the possibility exists that such compounds may also be at the basis for the development of inhibitors of the pneumococcal protein.

### Enzybiotics

Lytic enzymes are produced not only by the pneumococcus but also by pneumococcal bacteriophages. They hydrolyze the cell wall at the end of their replicative cycle, releasing the phage progeny [103]. It follows then that such bacteriophages, or their encoded peptidoglycan hydrolases, are attractive candidates for the specific lysis of *S. pneumoniae.* Fischetti and coworkers demonstrated that purifed pneumococcal bacteriophage cell wall hydrolases were able to kill most serotypes of pneumococci both *in vivo* and *in vitro*, and first coined the term *enzybiotic* for designating these enzymes [129]. Pal and Cpl-1 enzymes from Dp-1 and Cp-1 phages respectively are also effective in the eradication of the nasopharyngeal/otic colonization, where the pneumococcus has its reservoir [130,131], as well as in a murine sepsis model [132]. Patent US2006159671 [133] discloses a method for treating or preventing pneumococcal infections by synergistic combination of these two lytic enzymes and a suitable carrier. It includes infections that are resistant to treatment with antibiotics such as penicillin. Moreover the Cpl-1 lysozyme has proved 100% effective in preventing OMA [134]. As these enzymes are only active on microorganisms containing choline in their cell wall, they have the advantage of interfering neither with the normal microbiota, nor with the mammalian tissues.

## CURRENT AND FUTURE DEVELOPMENTS

*S. pneumoniae* is one of the major pathogens, as well as a very elusive one due to its ability for serotype-shifting and the rise of antibiotic resistance. Therefore, the strategies to fight this microorganism need to be multiple, varied and imaginative. Fig. (**4**) summarizes the current and developing approaches aimed to stop pneumococcal infection. Besides the usual investigation in antibiotics and capsule-based vaccines, new advances are being incorporated that involve other targets such as pneumococcal surface proteins and lytic enzymes. Research should be carried out to understand (and prevent) colonization from nasopharyngeal cells. Diagnostic success is boosting thanks to PCR techniques, that are

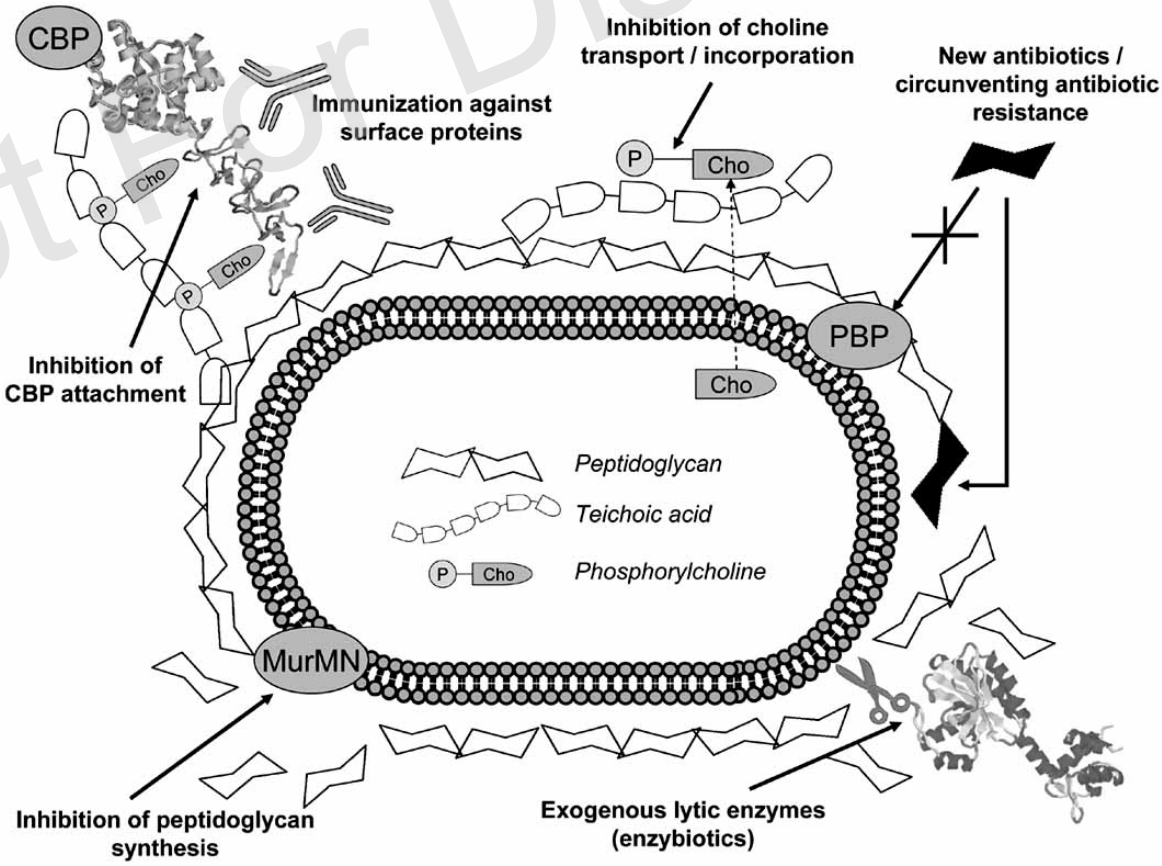



**Fig. (4).** A summary of different antipneumococcal approaches.

continuously improving to avoid cross-reactions with related species. Moreover, in the development of new vaccines, special attention must be paid to the virulent serotypes in developing countries, which often differ from those included in current formulations. Efforts should also be made for the vaccines to be affordable for developing countries. Besides, antibiotic resistance is an important threat that must be combated, above all, with a reasonable and controlled administration of antibiotics to the population. Furthermore, new targets such as pneumococcal surface proteins represent a promising source of both novel protein-based vaccines as well as a new generation of antimicrobials that may be common to all serotypes. Finally, the use of lytic enzymes as "magic bullets" against pneumococcus has an enormous potential that require an intense research to constitute a valid alternative to the current procedures.

## ACKNOWLEDGEMENTS

We thank the anonymous referee #2 for his/her very helpful comments and significant improvement of the manuscript. We acknowledge the Escuela Valenciana de Estudios para la Salud (Generalidad Valenciana, Spain, Grant 95⁄2005) and the Fundación Salvat Inquifarma (Spain) for financial support of some of our experiments described in the text.

## REFERENCES

[1] Tuomanen EI, Mitchell TJ, Morrison DA, Spratt BG Eds. The Pneumococcus. ASM Press, Washington, D.C. 2004; 136-47.

[2] Tan TQ. Pneumococcal infections in children. Pediatr Ann 2002; 31: 241-47.

[3] WHO. Pneumococcal conjugate vaccine for childhood immunization - WHO position paper. Weekly epidemiological record 2007; 82: 93-104.

[4] Klugman KP, Madhi SA, Feldman C. HIV and pneumococcal disease. Curr Opin Infect Dis 2007; 20: 11-15.

[5] Revai K, McCormick DP, Patel J, *et al*. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. Pediatrics 2006; 117: 1823-29.

[6] Weiser JN. In: The Pneumococcus. Tuomanen EI, Mitchell TJ, Morrison DA, Spratt BG Eds. ASM Press, Washington, D.C. 2004; 169-82.

[7] Short WR, Tunkel AR. Changing epidemiology of bacterial meningitis in the United States. Curr Infect Dis Rep 2000; 2: 327-31

[8] Molyneux E, Walsh A, Phiri A, Molyneux A. Acute bacterial meningitis in children admitted to the Queen Elizabeth Central Hospital, Blantyre, Malawi in 1996-1997. Trop Med Int Health 1998; 3: 610-18.

[9] Kastenbauer S, Pfister H-W. Pneumococcal meningitis in adults: spectrum of complications and prognostic factors in a series of 87 cases. Brain 2003; 126: 1015-25.

[10] Garibaldi RA. Epidemiology of communit-acquired respiratory tract infections in adults: incidence, aetiology and impact. Am J Med 1985; 78: 32S-38S.

[11] Williams BG, Gouws E, Boschi-Pinto C, Bryce J, Dye C. Estimates of world-wide distribution of child deaths from acute respiratory infections. Lancet Infect Dis 2002; 2: 25-32.

[12] Fadson DS, Scott J. The burden of pneumococcal disease among adults in developed and developing countries: what is and is not known. Vaccine 1999; 17: s11-18.

[13] Garau J. Role of β-lactam agents in the treatment of community-acquired pneumonia. Eur J Clin Microbiol Infect Dis 2005; 24: 83-99.

[14] Alho OP, Koivu M, Sorri M, Rantakallio P. The occurrence of acute otitis media in infants: a life-table analysis. Int J Pediatr Otorhinolaryngol 1991; 21: 7-14.

[15] Mufson MA. In: Mandell GL, Douglas R, Benett JE Eds. Principle and Practice of Infectious Diseases. Churchill Levinstone, N.Y. 1990; 1539-50.

[16] Cripps AW, Otczyk DC, Kyd JM. Bacterial otitis media: a vaccine preventable disease?. Vaccine 2005; 23: 2304-310.

[17] Gonzales R, Malone DC, Maselli JH, Sande MA. Excessive antibiotic use for acute respiratory infections in the United States. Clin Infect Dis 2001; 33: 757-62.

[18] Giebink G. Otitis media: the chinchilla model. Microb Drug Resist 1999; 5: 57-72.

[19] Berman S. Otitis media in developing countries. Pediatrics 1995; 96:126-31.

[20] Yu VL, Chiou CC, Feldman C, *et al*. An international prospective study of pneumococcal bacteraemia: correlation with *in vitro* resistance, antibiotics administered, and clinical outcome. Clin Infect Dis 2003; 37: 230-37.

[21] Parmar P, Salman A, Kalavathy CM, Jesudasan CA, Thomas PA. Pneumococcal keratitis: a clinical profile. Clin Experiment Ophthalmol 2003; 31: 44-47.

[22] Brook I. Bacteriology of acute and chronic ethmoid sinusitis. J Clin Microbiol 2005; 43: 3479-80.

[23] Bradley JS, Kaplan SL, Tan TQ, *et al*. Pediatric pneumococcal bone and joint infections. Pediatrics 1998; 102: 1376-82.

[24] Lund E, Henrichsen J. Laboratory diagnosis, serology and epidemiology of *Streptococcus pneumoniae*. Methods Microbiol 1978; 12: 241-62.

[25] Henney JE. Quick test for pneumonia. JAMA 1999; 282: 1218.

[26] Murdoch DR, Laing R, Mills GD, *et al*. Evaluation of a rapid immono-chromatographic test for detection of *Streptococcus pneumoniae* antigen in urine samples from adults with community-acquired pneumonia. J Clin Microbiol 2001; 39: 3495-98.

[27] Domínguez J, Galí S, Blanco S, *et al*. Detection of *Streptococcus pneumoniae* antigen by a rapid immunochromatographic assay in urine samples. Chest 2001; 119: 243-49.

[28] Navarro D, García-Maset L, Gimeno C, *et al*. Performance of the Binax NOW *Streptococcus pneumoniae* urinary antigen assay for diagnosis of pneumonia in children with underlying pulmonary diseases in the absence of acute pneumococcal infection. J Clin Microbiol 2004; 42: 4853-55.

[29] Marcos MA, Martínez E, Almela M, Mensa J, Jiménez de Anta MT. New rapid antigen test for diagnosis of pneumococcal meningitis. Lancet 2001; 357: 1499-500.

[30] Millar BC, Moore JE. Molecular diagnostics: current options. Methods Mol Biol 2004; 266: 139-66.

[31] Saunders NA. Real-time PCR. Methods Mol Biol 2004; 266: 191-211.

[32] Carvalho MGS, Tondella ML, McCaustland K, *et al*. Evaluation and improvement of real-time PCR detection assays to *lytA*, *ply* and *psaA* genes for detection of pneumococcal DNA. J Clin Microbiol 2007 doi:10.1128/ JCM.02498-06, in press.

[33] Toikka P, Nikkari S, Ruuskanen O, Leinonen M, Mertsola J. Pneumolysin PCR-based diagnostics of invasive pneumococcal infection in children. J Clin Microbiol 1999; 37: 633-37.

[34] Kanclerski K, Mollby R. Production and purification of *Streptococcus pneumoniae* hemolysin (pneumolysin). J Clin Microbiol 1987; 25: 222-25.

[35] Corless CE, Guiver M, Borrow R, Edwards-Jones V, Fox AJ, Kaczmarski EB. Simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae* and *Streptococcus pneumoniae* in suspected cases of meningitis and septicemia using real-time PCR. J Clin Microbiol 2001; 39: 1553-58.

[36] van Gastel EV, Bruyneels P, Verstrepen W, Mertens A. Evaluation of a real-time polymerase chain reaction assay for the diagnosis of pneumococcal and meningococcal meningitis in a tertiary care hospital. Eur J Clin Microbiol Infect Dis 2007, doi 10.1007/s10096-007-0350-0, in press.

[37] Russell H, Tharpe JA, Wells DE, White EG, Johnson JE. Monoclonal antibody recognizing a species-specific protein from *Streptococcus pneumoniae*. J Clin Microbiol 1990; 28: 2191-95.

[38] Crook J, Tharpe JA, Johnson SE, *et al*. Immunoreactivity of five monoclonal antibodies against the 37-kilodalton common cell wall protein (PsaA) of *Streptococcus pneumoniae*. Clin Diagn Lab Immunol 1998; 5: 205-10.

[39] Jado I, Fenoll A, Casal J, Pérez A. Identification of the *psaA* gene, coding for pneumococcal surface adhesin A, in *viridans* group streptococci other than *Streptococcus pneumoniae*. Clin Diagn Lab Immunol 2001; 8: 895-98.

[40] Morrison KE, Lake DL, Crook J, *et al*. Confirmation of psaA in all 90 serotypes of *Streptococcus pneumoniae* by PCR and potential of this assay for identification in diagnosis. J Clin Microbiol 2000; 38: 434-37.

[41] Sampson, J.S., Morrison, K.E., Whitmon, J.C., Ades, E., Carlone, G.M.: US20056869767 (**2005**).

[42] McAvin JC, Reilly, PA, Roudabush RM, *et al*. Sensitive and specific method for rapid identification of *Streptococcus pneumoniae* using real-time fluorescence PCR. J Clin Microbiol 2001; 39: 3446-51.

[43] Sheppard CL, Harrison TG, Morris R, Hogan A, George RC. Autolysin-targeted Light Cycler assay including internal process control for detection of *Streptococcus pneumoniae* DNA in clinical samples. J Med Microbiol 2004; 53: 189-95.

[44] Seki, M., Tsuda, H.: WO06043368 (**2006**).

[45] López R, García E. Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes and bacteriophage. FEMS Microbiol Rev 2004; 28: 553-80.

[46] Messmer TO, Sampson JS, Stinson A, Wong B, Carlone GM, Facklam RR. Comparison of four polymerase chain reaction assays for specificity in the identification of *Streptococcus pneumoniae*. Diagn Microbiol Infect Dis 2004; 49: 249-54.

[47] Llull D, López R, García E. Characteristic signatures of the *lytA* gene provide a basis for rapid and reliable diagnosis of *Streptococcus pneumoniae* infections. J Clin Microbiol 2006; 44: 1250-56.

[48] O'Neill AM, Gillespie SH, Whiting GC. Detection of penicillin susceptibility in *Streptococcus pneumoniae* by *pbp2b* PCR-restriction fragment length polymorphism analysis. J Clin Microbiol 1999; 37: 157-60.
[49] Hakenbeck, R.: US2004146886 (**2004**).
[50] Kawamura Y, Whiley RA, Shu SE, Ezaki T, Hardie JM. Genetic approaches to the identification of the *mitis* group within the genus *Streptococcus*. Microbiology 1999; 145: 2605-13.
[51] Sharpe BA, Flanders SA. Community-acquired pneumonia: a practical approach to management for the hospitalist. J Hosp Med 2006; 1: 177-90.
[52] Mandell LA, Barlett JG, Dowell SF, File TM, Musher DM, Whitney C. Update of practice guidelines for the management of community-acquired pneumonia in immunocompetent adults. Clin Infect Dis 2003; 37: 1405-33.
[53] Tunkel AR, Hartman BJ, Kaplan SL, *et al*. Practice guidelines for the management of bacterial meningitis. Clin Infect Dis 2004; 39: 1267-84.
[54] Powers JH. Diagnosis and treatment of acute otitis media: evaluating the evidence. Infect Dis Clin North Am 2007; 21: 409-26.
[55] Ferrara AM. New fluoroquinolones in lower respiratory tract infections and emerging patterns of pneumococcal resistance. Infection 2005; 33: 106-14.
[56] Jones RN, Sader HS, Stilwell MG, Fritsche TR. Garenoxacin activity against isolates form patients hospitalized with community-acquired pneumonia and multidrug-resistant *Streptococcus pneumoniae.* Diagn Microbiol Infect Dis 2007; 58: 1-7.
[57] Bast, D., Davidson, R.J., DeAzavedo, J., Low, D.E.: US2004147527 (**2004**)
[58] Kaplan SL, Mason EO Jr. Management of infections due to antibiotic-resistant *Streptococcus pneumoniae*. Clin Microbiol Rev 1998; 11: 628-44.
[59] Yogev R, Guzman-Cottrill J. Bacterial meningitis in children: critical review of current concepts. Drugs 2005; 65: 1097-112.
[60] Wexler HM. *In vitro* activity of ertapenem: review of recent studies. J Antimicrob Chemother. 2004; 53(Suppl S2): ii11-ii21.
[61] Jones RN, Sader HS, Stilwell MG, Fritsche TR. Garenoxacin activity against isolates from patients hospitalized with community-acquired pneumonia and multidrug-resistant *Streptococcus pneumoniae*. Diagn Microbiol Infect Dis 2007; 58: 1-7.
[62] Swainston-Harrison T, Keam SJ. Azithromycin extended release: a review of its use in the treatment of acute bacterial sinusitis and community-acquired pneumonia in the US. Drugs 2007; 67: 773-92.
[63] Sharpe BA, Flanders SA. Community-acquired pneumonia: a practical approach to management for the hospitalist. J Hosp Med 2006; 1: 177-90.
[64] American Academy of Pediatrics (AAP) and American Academy of Family Physicians (AAFP) subcommitee on Management of Acute Otitis Media. Diagnosis and management of acute otitis media. Pediatrics 2004; 113: 1451-65.
[65] Segal N, Leibovitz E, Dagan R, Leiberman A. Acute otitis media - diagnosis and treatment in the era of antibiotic resitant organisms: updated clinical practice guidelines. Int J Pediatr Otorhinolaryngol 2005; 69: 1311-19.
[66] Hotomi M, Suzumoto M, Itahashi K, *et al*. Efficacy of a novel oral carbapenem, tebipenem pivoxil (TBM-PI), against experimental otitis media caused by penicillin resistant *Streptococcus pneumoniae* in chinchilla. Vaccine 2007; 25: 2478-84.
[67] Rosenfeld RM, Vertrees JE, Carr J, *et al*. Clinical efficacy of antimicrobial drugs for acute otitis media: metaanalysis of 5400 children from thirty-three randomized trials. J Pediatr 1994; 124: 355-67.
[68] Glasziou PP, Del Mar CB, Sanders SL, Hayem M. Antibiotics for acute otitis media in children. Cochrane Database Syst Rev 2004; 1: CD000219.
[69] Wong T, Ormonde S, Gamble G, McGhee CN. Severe infective keratitis leading to hospital admission in New Zealand. Br J Ophthalmol 2003; 87: 1103-108.
[70] Liesegang TJ. Bacterial Keratitis. In: Kaufman HE, Barron BA, McDonald MB (eds), The Cornea, Butterworth-Heinemann, Boston, 1998; 159-218.
[71] Cutarelli PE, Lazarus HM, Jacobs MR, Lass JH. Antimicrobial activity and *in vitro* corneal epithelial toxicity of antimicrobial agents for gram-positive corneal pathogens. Curr Eye Res 1993; 12: 603-608.
[72] Marquart, M.E., O'Callaghan, R.J.: US2006229283 (**2006**).
[73] Whitney CG, Farley MM, Hadler J, *et al*. Increasing prevalence of multi-drug-resistant *Streptococcus pneumoniae* in the United States. N Engl J Med 2000; 343: 1917-24.
[74] Fogarty C, Torres A, Choudhri S, Herrington J, Ambler J. Efficacy of moxifloxacin for treatment of penicillin-, macrolide- and multidrug-resistant *Streptococcus pneumoniae* in community-acquired pneumonia. Int J Clin Pract 2005; 59: 1253-59.
[75] Cartwright K. Pneumococcal disease in western Europe: burden of disease, antibiotic resistance and management. Eur J Pediatr 2002; 161: 188-95.
[76] Craig WA. Overview of newer antimicrobial formulations for overcoming pneumococcal resistance. Am J Med 2004; 117(suppl 3A): 16S-22S.
[77] Bax, R.P.: US20016214359 (**2001**).
[78] Hakenbeck R, Grebe T, Zahner D, Stock JB. β-Lactam resistance in *Streptococcus pneumoniae*: penicillin-binding proteins and non-penicillin-binding proteins. Mol Microbiol 1999; 33: 673-78.
[79] Goffin C, Ghuysen JM. Multimodular penicillin-binding proteins: an enigmatic family of orthologs and paralogs. Microbiol Mol Biol Rev 1998; 62: 1079-93.
[80] Filipe SR, Pinho MG, Tomasz A. Characterization of the *murMN* operon involved in the synthesis of branched peptidoglycan peptides in *Streptococcus pneumoniae*. J Biol Chem 2000; 275: 27768-74.
[81] Filipe SR, Tomasz A. Inhibition of the expression of penicillin resistance in *Streptococcus pneumoniae* by inactivation of cell wall muropeptide branching genes. Proc Natl Acad Sci USA 2000; 97: 4891-96.
[82] Tomasz, A., Filipe, S.R.: US2005165224 (**2005**).
[83] Felmingham D, Cantón R, Jenkins SG. Regional trends in β-lactam, macrolide, fluoroquinolone and telithromycin resistance among *Streptococcus pneumoniae* isolates 2001-2004. J Infect 2007; 55: 111-118.
[84] Jacobs MR, Felmingham D, Applebaum PC, Grüneberg RN. The Alexander Project 1998-2000: susceptibility of pathogens isolated from community-acquired respiratory tract infection to commonly used antimicrobial agents. J Antimicrob Chemother 2003; 52: 229-46.
[85] McGee L, Goldsmith CE, Klugman K. Fluoroquinolone resistance among clinical isolates of *Streptococcus pneumoniae* belonging to international multiresistant clones. J Antimicrob Chemother 2002; 49: 173-76.
[86] Dias R, Louro D, Canica M. Antimicrobial susceptibility of invasive *Streptococcus pneumoniae* isolates in Portugal over an 11-year period. Antimicrob Agents Chemother 2006; 50: 2098-105.
[87] Rossi F, Andreazzi D. Overview of tigecycline and its role in the era of antibiotic resistance. Braz J Infect Dis 2006; 10: 203-16.
[88] Testa, R.T., Calhoun, J., Mader, J.T., Mader, D.: US2005148553 (**2005**).
[89] Heerbeek N, Straetemans M, Wiertsema SP, *et al*. Effect of combined pneumococcal conjugate and polysaccharide vaccination on recurrent otitis media with effusion. Pediatrics 2006; 117: 603-608.
[90] Laferriere, C.A.J., Poolman, J.: US2006051361 (**2006**).
[91] O'Hagan, D.: US2006147466 (**2006**).
[92] Hausdorff W, Feikin DR, Klugman KP. Epidemiological differences among pneumococcal serotypes. Lancet Infect Dis 2005; 5: 83-93.
[93] Eskola J, Kilpi T, Palmu A, *et al*. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. N Engl J Med 2001; 344: 403-409.
[94] Darkes MJ, Plosker GL. Pneumococcal conjugate vaccine (Prevnar; PNCRM7): a review of its use in the prevention of *Streptococcus pneumoniae* infection. Paediatr Drugs 2002; 4: 609-30.
[95] Kaplan SL, Mason EO Jr, Wald ER, *et al*. Decrease of invasive pneumococcal infections in children among 8 children's hospitals in the United States after the introduction of the 7-valent pneumococcal conjugate vaccine. Pediatrics 2004; 113: 443-49.
[96] Byington CL, Samore MH, Stoddard GJ, *et al*. Temporal trends of invasive disease due to *Streptococcus pneumoniae* among children in the inter-mountain west: emergence of nonvaccine serogroups. Clin Infect Dis 2005; 41: 21-29.
[97] Nesin M, Ramirez M, Tomasz A. Capsular transformation of multidrug-resistant *Streptococcus pneumoniae in vivo*. J Infect Dis 1998; 177: 707-713.
[98] Beutels P, Thiry N, Damme PV. Convincing or confusing? Economic evaluations of childhood pneumococcal conjugate vaccination- a review (2002-2006). Vaccine 2007; 25: 1355-67.
[99] Hausdorff, W.P., Siber, G.R., Paradiso, P.R.: WO06110381 (**2006**).
[100] Hausdorff WP, Yothers G, Dagan R, *et al*. Multinational study of pneumococcal serotypes causing acute otitis media in children. Pediatr Infect Dis J 2002; 21: 1008-16.
[101] Jonsdottir, I.: US2006140981 (**2006**).
[102] Gosink KK, Mann E, Guglielmo C, Tuomanen EI, Masure HR. Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. Infect Immun 2000; 68: 5690-95.
[103] López R, García E, García P, García JL. In: The Pneumococcus (Tuomanen EI, Mitchell TJ, Morrison D, Spratt BG eds). ASM Press, 2004; 75-88.
[104] Bogaert D, Hermans PWM, Adrian PV, Rümke HC, de Groot R. Pneumococcal vaccines: an update on current strategies. Vaccine 2004; 22: 2209-20.
[105] McDaniel LS, Sheffield JS, Delucchi P, Briles DE. PspA, a surface protein of *Streptococcus pneumoniae*, is capable of eliciting protection against pneumococci of more than one capsular type. Infect Immun 1991; 59: 222-28.
[106] Ren B, Szalai AJ, Thomas O, Hollingshead SK, Briles DE. Both family 1 and family 2 PspA proteins can inhibit complement deposition and confer virulence to a capsular serotype 3 strain of *Streptococcus pneumoniae*. Infect Immun 2003; 71: 75-85.

[107] Moore QC, Bosarge JR, Quin LR, McDaniel LS. Enhanced protective immunity against pneumococcal infection with PspA DNA and protein. Vaccine 2006, 24; 5755-61.
[108] Briles, D.E., Yother, J.L., McDaniel, L.S.: EP0622081 (**2002**).
[109] White P, Hermansson A, Svanborg C, Briles D, Prellner K. Effects of active immunization with a pneumococcal surface protein (PspA) and of locally applied antibodies in experimental otitis media. ORL J Otorhinolaryngol Relat Spec 1999; 61: 206-11.
[110] Wizemann, T.M., Koenig, S., Johnson, L.S.: WO9951266 (**1999**).
[111] Wu HY, Nahm MH, Guo Y, Rusell MW, Briles DE. Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*. J Infect Dis 1997; 175: 839-46.
[112] Briles DE, Hollingshead SK, King J, *et al*. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. J Infect Dis 2000; 182: 1694-701.
[113] D'Ambra, A.J., Arnold, F.J., Maleckar, J.R., McMaster, R.P.: US20030035806 (**2003**).
[114] Briles, D.E., Hollingshead, S.K., Brooks-Walter, A.: US2005196405 (**2005**).
[115] Masure, H. R., Rosenow, C.I., Tuomanen, E., Wizemann, T.M.: US2005048590 (**2005**).
[116] Masure, H. R., Rosenow, C.I., Tuomanen, E., Wizemann, T.M.: US20030175293 (**2003**).
[117] Wizemann, T.M., Johnson, L., Koenig, S.: NZ507717 (**2004**).
[118] Seo J-Y, Seong SY, Ahn B-Y, Kwon IC, Chung H, Jeong SY. Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin A encapsulated in microspheres. Infect Immun 2002; 70: 1143-49.
[119] Huebner, R.C., Sampson, J.S., Carlone, G.M., Ades, E., Briles, D.E.: WO000076541 (**2000**).
[120] García-Suárez MM, Vázquez F, Méndez FJ. *Streptococcus pneumoniae* virulence factors and their clinical impact: an update. Enferm Infecc Microbiol Clin 2006; 24: 512-17.
[121] Briese T, Hakenbeck R. Interaction of the pneumococcal amidase with lipoteichoic acid and choline. Eur J Biochem 1985; 146: 417-27.
[122] Fernández-Tornero C, García E, Pascual-Teresa B, López R, Giménez-Gallego G, Romero A. Ofloxacin-like antibiotics inhibit pneumococcal cell wall-degrading virulence factors. J Biol Chem 2005; 280: 19948-57.
[123] Maestro B, González A, García P, Sanz JM. Inhibition of pneumococcal choline-binding proteins and cell growth by esters of bicyclic amines. FEBS J 2007; 274: 364-76.
[124] Llull D, Rivas L, García, E. *in vitro* Bactericidal activity of the antiprotozoal drug miltefosine against *Streptococcus pneumoniae* and other pathogenic Streptococci. Antimicrob Agents Chemother 2007; 51: 1844-48.
[125] Kharat AS, Tomasz A. Drastic reduction in the virulence of *Streptococcus pneumoniae* expressing type 2 capsular polysaccharide but lacking choline residues in the cell wall. Mol Microbiol 2006; 60: 93-107.
[126] Weiser, J.N.: WO9812346 (**1998**).
[127] Espinosa, A., Lacal, J.C., Gallo, M.A., Campos, J.M.: WO9805644 (**1998**).
[128] Lacal, J.C.: ES2148092 (**2000**).
[129] Nelson D, Loomis L, Fischetti VA. Prevention and elimination of upper respiratory colonization of mice by group A streptococci by using a bacteriophage lytic enzyme. Proc Natl Acad Sci USA 2001; 98: 4107-12.
[130] Loeffler JM, Nelson D, Fischetti VA. Rapid killing of *Streptococcus pneumoniae* with a bacteriophage cell wall hydrolase. Science 2001; 294: 2170-72.
[131] Loeffler JM, Djurkovic S, Fischetti VA. Phage lytic enzyme Cpl-1 as a novel antimicrobial for pneumococcal bacteremia. Infect Immun 2003; 71: 6199-204.
[132] Jado I, López R, García E, Fenoll A, Casal J, García P. Phage lytic enzymes as therapy for antibiotic-resistant *Streptococcus pneumoniae* infection in a murine sepsis model. J Antimicrob Chemother 2003; 52: 967-73.
[133] Fischetti, V.A.: US20060159671 (**2006**).
[134] McCullers JA, Karlström Á, Iverson A, Loeffler JM, Fischetti VA. Novel strategy to prevent otitis media caused by colonizing *Streptococcus pneumoniae*. PloS Pathog 2007; 3: e28. doi:10.1371/journal.ppat.0030028.