SHORT REVIEW

Streptococcus pneumoniae: elusive mechanisms of the body's defense systems

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Key words

Streptococcus pneumoniae • Pneumonia • Capsular switch • Serotype replacement • Antibiotic resistance

Summary

Streptococcus pneumoniae is one of the most important human pathogens. It represents the most frequent cause of pneumonia, meningitis, sinusitis and otitis.
After the PCV7 vaccine introduction, a serotypic switch was noticed. This phenomenon led to the replacement of the seven serotypes contained in the vaccine with other less common ones, some of which are invasive or characterised by antibiotic-resistance. This replacement is only partially due to the vaccination. Many causes have been suggested to explain this effect: apearance of new serotypes, diffusion of minority serotypes and replacement of common serotypes due to natural secular trend.
Pneumococcus has a promiscuous "sex life", characterized by homologous recombinations within the same species and also between different species. This fact can unlock the secret of how these pathogens can develop antibiotic or vaccine-resistance.
The serotypic switch involves big loci that are responsible for capsular polysaccharide synthesis. The most important region of the genome involved in this process is near the gene tetM. The same mechanisms are also responsible for antibiotic resistance. In recent years the growth of penicillin, macrolides and clindamycine resistance has been noticed. It is also important to underline that multidrug-resistant bacteria isolation has increased.
In conclusion, to obtain more information about bacteria composition and evolution, antibiotic-resistance and vaccine response, it is fundamental to improve the epidemiological surveillance of pneumococcal infections using modern molecular diagnostic techinques.

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Streptococcus pneumoniae is one of the most important human pathogens. In fact, it represents the most frequent cause of pneumonia, meningitis, sinusitis and otitis media. The World Health Organization estimates that every year approximately 1.6 million individuals die from invasive pneumococcal diseases. Up to 1 million of these deaths are children under 5 years of age.

Pathogenesis of pneumococcal infections

Pneumonia is the most frequent infection caused by *S. pneumoniae*. Like every invasive infection it goes through different phases: adhesion and colonisation, invasion and inflammation, and in some cases, it can even give shock. In each of these phases the germ uses its virulence factors to overcome host defences: the capsule, the most important, the bacterial wall, surface proteins (about 500) and finally, the hemolysine exotoxines secreted by the Pneumococcus, of which pneumolisina is the strongest.
The first germ-host contact (adhesion) is carried out through the pili that ensure adherence to the epithelium [1]. The cellular wall, rich in residues of phosphorycholine, plays a fundamental role in this process. It is the cellular wall that guarantees adhesion because the epithelial cells are rich in specific receptors for phosphorycholine. Surface proteins are also important. Among these there is an adhesin called PsaA or Pneumococcal surface adhesin codified as the psa gene. This gene induces adhesion even indirectly, inducing the synthesis of a second adhesin, the CbpA or coline-binding-proteinA. Once adhesion to the host has occurred, the result is colonisation by that pathogenic agent. This colonisation, however, is not necessarily pathological. In fact, most patients colonised by *S. pneumoniae* remain carriers of pneumococcus, developing a serotype-specific immune response. The most well-known invasion phenomenon is pneumonia that can also represent the source of a bacteremia or sepsis. The ability of invasion is much higher for enveloped germs and it varies according to the type of capsule (chemical-physical differences) [2]. The same opsinization of the capsule by the complement is greater in some serotypes than in others. Serotypes which are poorly opsonized are phagocytized less and at the same time are less capable of inducing the production of specific antibodies [3, 4]. In general, all capsular polysaccharides interfere with phagocytosis, limiting the opsonization mediated by some complement fractions, such as the C3b. However, the capsule by itself cannot determine the resistance to the immune defences and it cannot explain the invasiveness of the germ. Germs with identical capsules can have, in fact, a different invasiveness due to the presence of other virulence factors [5]. One example is represented by the antigen CbpA that plays an essential role in the passage through the blood-brain barrier.

If the Pneumococcus manages to avoid phagocytosis, invasion begins that leads to the activation of the inflammatory cascade: the cascade of inflammatory cytokines (IL-1, IL-6, TNF), the alternative complement pathway and the coagulation cascade. The bacterial wall has a fundamental role in inducing inflammation. Its various components, through various mechanisms, trigger the inflammatory response and the production of antibodies directed against the same bacterial wall thanks to the activation of lymphocytes B activated in turn by lymphocytes T CD4+, that are sensitive to the release of proinflammatory cytokines. An enhancement of an inflammatory response is also caused by the release of pneumolysina (a hemolysin) by the Pnuemococcus. This carries out an offensive role creating pores on the inside of the double phospholipid layer of the membrane of the host cells, by exploiting the link with cholesterol residues present in human cells, inducing cell lysis. The pneumolysina is also capable of inducing the production of inflammatory cytokines and nitric oxide, inhibiting ciliary movement [6, 7]. It also induces complement activation and reduces the lymphocytic proliferative response and the bactericidal activity of the neutrophils.
Innate immunity plays a role of fundamental importance in the response against Pneumococcus and other Gram+ bacteria. In fact, the course of the illness is more severe in all those patients with an inadequate polymorphonuclear cell activity. As a consequence of the central role of the humoral response, it has recently been demonstrated how determining Toll Like Receptors (TLRs) , (membrane and cytoplasmic receptors), are in modulating the innate response. These appear to be the main receptors of lipopetide molecules, of peptidoglycans and lopoteichoic acid of the Gram+ bacteria.
TLR-4 seems to have a specific role in mediating the pro-inflammatory action of Pneumococcal pneumolysina, while TLR-2 seems to modulate the inflammation in the nervous system even during Pneumococcal infections. Finally, it has been determined that animals with TLR-9 deficiency are more susceptible to Pneumococcal infections [8, 9].

The serotypic switch phenomenon and its causes

According to the peculiar structure of the capsule, 92 serotypes of Pneumococcus with different capsular antigenic characteristics can now be identified. There are differences in the distribution of serotypes between adults and children. In the latter, the most frequent serotypes up to now appear to be 1, 19A, 3, 6A and 7F in which the antibiotic-resistance phenomenon is also frequent. The serotypic distribution also varies according to the geographical region being studied. The environment, economic and hygenic conditions, different therapeutic and preventive strategies modulate the serotypic distribution, but in any case, the most invasive types are always predominant. Moreover, some serotypes seem to be exclusive of the carriers (16, 35B, 21).

As regards invasive forms, we know that most of them are caused by a few serotypes that prove to be those that are now included in the 13-valent conjugate vaccine (PCV13): 4, 6B, 9V, 14, 18C, 19F, 23F, 1, 3, 5, 6A, 7F and 19A.
The heptavalent conjugate vaccine has been used since 2000 when it was introduced into the USA for the first time. Some years after the introduction of the PCV7, a phenomenon of serotype replacement was pointed out: the so-called serotypic switch. This complex phenomenon has determined the replacement of 7 serotypes contained in the vaccine together with other less common ones, some of which are invasive or characterised by a high antibiotic-resistance. An increase in the isolation of serotype 19A has been observed as well as non-vaccinated strains such as 1 and 7F, an increase in the rarest serotypes such as 17, 29 and 38 and non-typable strains. The replacement, however, only depends on the vaccination to a small part [10, 11]. In fact, different phenomena have been considered in order to explain this fact:

- Emergence of new serotypes on the epidemiological scene. The vaccine seems to have favoured the colonisation of nasopharyngeal carriers by new serotypes.
- Exposure of minority serotypes already present before the introduction of the vaccination. The vaccine that has determined the disappearance of the most common serotypes or at least greatly reducing their incidence, also allows for the detection of the minority strains so far “masked”.
- Serotypic switch. This phenomenon is not completely due to acquired vaccine immunity, but it depends on the intrinsic plasticity of the Pneumococcus [12]. Recently it has been possible to sequence the entire bacterial genome, containing more than 2 million bases and characterised by large regions of variability. During infection, in the carrier or also during laboratory experiments, *S. pneumoniae* is able to acquire DNA fragments from other pneumococci or even from other bacteria (i.e. Streptococci). This allows for a modification of the serotypic characteristics of the polysaccharide capsule and to acquire new qualities, among which the most dangerous is antibiotic-resistance. The micororganism becomes immune to antibiotic therapy and more virulent, capable of escaping the immune system: these recombinations give Pneumococcus an evolutionary advantage, that determines its selection [13].
- Replacement of serotypes circulating among the carriers for spontaneous secular trend [14].

The magnitude of the serotypic switch phenomenon was initially low, but over the years, it has gradually become more prevalent both in North American and Europe and in Italy, so as to partially erode the beneficial effect of PCV7. The observation of these replacement serotypes came about studying both the serotypes present in the naso-pharynx and the blood or spinal fluid samples from patients with invasive diseases.
There has been a sudden decrease in the number of infections in the invasive forms with a consequent drop

in the death rate and post-event morbidity both in the population of individuals in a vaccinal age and in newborns under three months old and, therefore, too small to be vaccinated. Unfortunately, in this case, dividing the cases on the basis of the serotypes under discussion, a small increase of non-PCV7 has been registered along with a strong decrease in vaccinal serotypes. Actually, the replacement mechanism for some serotypes, for example 19A, is not in the various case histories. The serotype switch is more correlated to the secular trend replacement and to antibiotic resistance than to the introduction of the vaccines. [15, 16]. The increase in the isolation of 19A deserves attention. Up until a few years ago it was rather rare and substantially harmless. The incidence of 19A has increased progressively (in 2005 it reached 36%). In addition, unlike many serotypes that tend to privilege some forms compared to others, the Pneumococcus 19A can be found in all possible localisations: otitis, pneumonia, meningitis, and sepsis. Another aspect that characterises it is its distinct ability to develop resistance to penicillin, but also to other antibiotics. Preliminary analyses, as well, conducted on numerous serotypes 19A islolated in children with pneumococcus invasive infections (IPD) demonstrated a capsular switch with replacement, for example, of genes which were previously present in serotype 4. Today, the introduction of the new 13-valent conjugate vaccine contains all the hopes of epidemiologists and clinicians as regards both this phenotype and others not included in the PCV7.

The capsular-switch

Bacteria have a variable "sex life" that goes from celibacy to an evident promiscuity, just like the Pneumococcus . By "sex" we mean the occurence of homologous recombinations between bacteria of the same species or of different species. It has been demonstrated how frequent recombinations can determine rapid diversifications in the different species while in those species that practice "celibacy", these come about much more slowly and they depend on the accumulation of punctiform mutations. This offers an important key to the interpretation of how these pathogens can rapidly develop resistance to antibodies and even escape vaccinal protection.
The short-term mutation rate in the various pathogenic agents had a much higher result than was expected (about 10-6 replacements per site per year) even though it remains less compared to that of RNA virus.
Recent studies conducted on 240 isolated viruses obtained from 22 different countries from 1984 to 2008 [17], have demonstrated how the *S. pneumoniae* has evolved over these 40 years and what its diffusion has been from one continent to another. Recombination is frequent in this species. In fact, 74% of the genome has undergone a recombination through replacement in at least one isolated virus. The most frequent phenomenon verified was the replacement of bases, even though deletions and insertions have been pointed out in some cases. As regards Pneumococcus, these phenomena have been underlined from among the different serotypes, but also with species of streptococci correlated with *S. pneumoniae*, among which *Streptococcus mitis*, *Streptococcus oralis* and *Streptococcus pseudopneumoniaie* should be remembered [18].
Eliminating the recombinant areas, these studies offer the opportunity to identify the presence of genetic polymorphisms that are used to rebuild the phylogenesis of the bacterial strains, in this way, allowing one to map the dynamics of the acquisition and/or loss of antibiotic resistance and the alterations in the capsular serotypes. This opportunity to acquire information about the phylogenesis of the bacterial strains that have been studied, allows a demonstration of the course of the diffusion of the bacteria in different geographical areas over time and most of all, to indicate possible serotypic switches. Switches between 19A and 19F, like between 19A. 6A, 3, 14 and 15B have, in fact, been recorded in significant amounts even after the introduction of the PCV7. The mechanisms responsible for the variation of the capsular serotype involve large loci that codify for the capsular polysaccharides. These replacements are interpreted as the consequence of the selection of the body's immunity response or more recently, of the introduction of conjugate vaccines that have a restricted number of the more than 90 capsular serotypes known as their target. In addition, the capsular switch not only provides protection from specific antibodies in the population (for example for the vaccination itself), but it modifies susceptibility at the same time. In fact, some types of capsules bind fewer antibodies than others: the type 3 capsule of the D39 serotype, in fact, binds less than half of the antibodies of type 2 of the same serotype, and in this way, increases virulence [5]. Different chromosomal areas are frequently substituted through recombinations and the area that seems to be more frequently involved is the one around the tetM gene in which the genes pspE, pspA and pspC are found. PspA and pspC are important proteins that are involved in the pathogenesis of Pneumococcus infections and they represent a potential target for vaccines as they are recognised by the antibodies produced during experimental studies on carriers. It has also been demonstrated how these genes can be very quickly removed eliminated from the chromosomes proving how the pneumococcal population is capable of responding immediately to the introduction of direct vaccines based on antigenic proteins being developed now.

The development of antibiotic resistance

The same thing is true as regards the development of the resistance to antibiotics, where a fundamental role is carried out by the selection caused by humans. In fact, the scarce selection carried out by the use of aminoglucosides and cloramphenicol has caused an occasional deletion of the loci responsible for the resistance to these antibiotics. On the contrary, resistance to penicillin has frequently been acquired during phylogenesis

which perhaps represents the most important resistance to medicines for this class of bacteria. The fulcrum of the mechanism on which the resistance to penicillin is based is represented by the penicillin binding proteins (PBP) of which six types are known. Mutations at the gene level that codify for these proteins are responsible for a low, moderate or high resistance to this class of antibiotics estimated on the basis of MIC.
The genes responsible for the synthesis of PBP are pbp1a, pbp2b, pbp2x and pbp2a. The genetics of the mechanisms that lead to the development of this resistance are very complex and regard forms of mosaicism of these genes. It is of fundamental importance to remember, since it determines a correlation between the phenomenon of the resistance to medicines and the capsular switch, that the two genes most frequently involved in determining the resistance to penicillin, i.e. Pbp2x and pbp1a, are located in the same area of the genome where the genes that determine the capsular serotype are located which are found between the dexB and aliA genes. This can be proved by the fact that among the non- vaccinal serotypes that are more frequently observed in patients and carriers, we find 19A, which is sometimes the consequence of a capsular switch between this and 19F or 4. At the same time 19A proves to be one of the serotypes that presents the strongest resistance to penicillin and often to other medicines. Other serotypes that are known for their resistance to penicillin are 23, 6, 9 and 14 [19, 20].
Through phylogenesis, the resistance to macrolides has also been acquired determined by the methylation of the targets of ribosomal RNA of the subunity 23S, in this way, facilitating the synthesis of a specific pump for the removal of these medicines called mef (macrolid efflus-type efflux pump). The same is true for another pump, the mef/mel-type efflux pump. In addition, it has been demonstrated how a strong association exists between resistance and macrolides and resistance to clindamycin, caused by alterations the ribosomal subunity 50S [21].
Finally, numerous studies have indicated considerable changes in the resistance of the Pneumococcus as regards various antibiotics, above all for non-vaccinal serotypes after the introduction of the PCV7. In the last years, in fact, we have witnessed a decrease in the resistance to Trimethoprim-Sulphamethoxazolo and to cefalosporine (such as Ceftriaxone) and cases of a high resistance to penicillin have also decreased. On the contrary, the intermediate resistance to penicillin has increased just like the resistance to macrolides and clindamicines. Finally, the isolation of multi-resistant bacterial strains (> 3 antibiotics) has also increased [22].

The role of the carrier

We must not forget what a fundamental role the carrier plays in a reality like this. The preeminent carrier of *S. pneumoniae* is represented by a young child where it is more frequently isolated in the nasopharynx.
The alterations in the distribution of bacterial serotypes is inextricably connected to the distribution of Pneumococcus in children where the colonisation rates are highest. In this way, these young patients become natural reservoirs for invasive species as well, offering an optimal environment for the evolution of this pathogenic agent. It, therefore, becomes crucial to recognise and understand the changes in the bacterial populations in these individuals who are subject to the use of an antibiotic and the universal immunisation exercised through vaccinations that have reached important levels in the pediatric populations [22].
As we know, what is called the colonisation of the mucous membrance takes place in the carrier. To carry this out, it is of utmost importance for the bacteria to adhere efficaciously to the epithelial surface. In this case, the Pneumococcus capsule represents an obstacle that impedes an optimal adhesion. In fact, it has been demonstrated that during the colonisation phase of the mucous membrane, especially for bacteria that are in close contact with the epitheliums, the pathogen loses its own capsule. This means escaping from the main defence against the Pneumococcus, represented by the anti-capsular antibodies, and at the same time, making it impossible to locate the germ using non-molecular methods [23].
In addition, where different colonies of Pneumococcus are present in a carrier, there is a risk of possible recombinations that would allow a possible capsular switch, in this way giving a greater pathogenicity and resistance to the defences of the host to one of the strains present, increasing the risk of developing an invasion and, therefore infection,
Like in invasive diseases, an important phenomenon of the serotypic switch has also been observed in the carrier that recognises the same possible causes indicated previously. In particular, after the introduction of PCV7, there has been a progressive increase in the isolation of non-vaccinal serotypes especially 19A, 35B,23A and 15B/C [22].
Today we still do not know to what extent the introduction of the conjugate vaccine can favour a serotypic and capsular switch, but it has, however, been proved that this can take place [24].

Conclusions

In conclusion, we can affirm that Pneumococcus is a very skilled pathogen that evolves over a very short time period. Frequent recombinations, as a consequence, lead to a rapid evolution of strains that are multiresistant to antibiotics and able to escape from the effects of specific vaccines against single proteinic antigens.
Therefore, it is of utmost importance to promote and optimize a continual surveillance of *S. pneumoniae* infections in all countries including those in which the antipneumococcal vaccination has not been scheduled yet. Without a doubt, molecular diagnostic techniques, such as PCR and MLST (Multi Locus Sequence Typing), are fundamental in reaching this objective and they constitute an essential approach to obtain information concerning the composition and evolution of bacterial species, offering a key for a better understanding of the pathogenic response to new antibiotics and vaccines.

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■ Received on March 11, 2012. Accepted on March 26, 2012.

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