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Impact of molecular methods in the analysis of the invasiveness of *Streptococcus pneumoniae*

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DISCUSSION

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This doctoral thesis proves the necessity of applying molecular techniques in direct sample for a more accurate study of the Invasive Disease Potential (IDP) of *Streptococcus pneumoniae* and for its epidemiological surveillance. These methods allow a better detection and serotype identification of the pneumococcal population in both Invasive Pneumococcal Disease (IPD) and nasopharyngeal carriers. However, to better understand the behavior of the different serotypes a complete analysis of the clonal types of pneumococci (from disease and carriage) should be also performed.

In the first two studies the IDP of pneumococcal serotypes found in the pediatric population of Catalonia (Spain) was estimated. In the first study, traditional methods based only in culture were used for both the detection of *Streptococcus pneumoniae* and the identification of the capsular type; while in the second study, the same estimation was performed but this time adding molecular techniques in direct sample for both the detection and serotyping.

When comparing the results of both studies, the addition of molecular techniques in direct sample doubled the number of pneumococci identified causing IPD and in the nasopharynx of healthy children. This detection improvement due to the ability to detect the pathogen in culture-negative specimens has been previously well described for the diagnose of cases of meningitis, septicemia and parapneumonic empyema (96-98). Like in our results, in these studies the great increase in the pneumococcus detection revealed a different distribution of the serotypes circulating in the population of the area, when compared with the data that had been obtained only by culture, which was translated in a huge impact in the IDP of these serotypes.

For example, when analyzing the invasive population an important changed was found in serotype 3. This serotype ranked 5th in the first study but ranked 3rd in the second one, with a 5-fold increase detection with respect to the culture. The underestimation of this serotype in children in our geographical area, caused by difficulties in recovering the strain in culture, was already reported by a recent publication of our group (48). As well, serotype 1, although ranking in the first position in both studies, experienced an almost doubled rate of detection which showed the real magnitude of the serotype in our area. In England, a study by Eltringham G et al. also showed an increased detection of penicillin-sensitive serotype 1 in empyema fluids obtained from children with suspicion of *Streptococcus pneumoniae* infection when molecular methods were applied (99). A recent study performed by our group analyzing strains of serotype 1 from children of Barcelona in a 20-year period reported that all the strains were susceptible to penicillin and cefotaxime, which enhances the need to add molecular techniques to the pneumococcus diagnose for when antibiotics have been previously administered (26).

On the other hand, in the carrier's population an important proportion in the increase of serotypes that were able to be identified was due to the detection of multiple colonies in one sample, with up to four different serotypes distinguished in the same sample. Previous studies have highlighted the need for the development of more sensitive assays than culture to detect simultaneous carriage of multiple serotypes. Some of them have also proved the better results that could be obtained using molecular techniques (70, 82, 100-101). One of the most surprising observations was the detection of the serotype grouping 10FC/33C. This serotype grouping was not detected in the first study but ranked 2nd in the second study. Failed detection of these serotypes by culture could be explained by the increase possibility to detect multiple colonization with molecular techniques in direct sample, since this serotype grouping appeared to be significantly

associated with co-colonization in our results. Equally interesting was the detection of serotype 1 in the nasopharynx. It has been demonstrated that this serotype is rarely found in carriers, even in geographical areas where it is one of the main causes of IPD (102). However, a study by Nunes S et al. reported an emergence of serotype 1 among healthy Portuguese children after PCV7 associated with a single pneumococcal lineage (103). Although the two cases of serotype 1 in carriage were detected in both studies, the analysis in direct sample revealed that both cases corresponded to co-colonization. In fact, some studies have suggested that the use of techniques able to detect multiple colonization could improve the identification of this serotype in carriage (104-105).

The better estimation of the ranking order of the most common serotypes in IPD and in the nasopharynx also allowed a more accurate analysis of the effects of PCV7 in the study time period. As reported in other European countries, the results from the second study showed a low rate of serotypes covered by PCV7 with serotypes 1, 19A, 3 and 7FA being the main causes of IPD, all serotypes included in PCV13 (106-108). The United States also experienced a decrease of PCV7 serotypes and an emergence of serotypes 19A, 7F and 3, but the incidence of serotype 1 between pre- and post-vaccine period seemed to remained stable (12, 109). The high levels of serotype 19A were of concern since a high proportion of the multidrug resistance strains were found expressing this serotype in the first study.

This improve detection by molecular methods significantly changed the results obtained in the estimation of the IDP of pneumococcal serotypes in the second study. Until now, and as far as we know, calculation of the invasiveness of serotypes has always been performed in pneumococcal strains and the Quellung reaction has been the serotyping method normally used (7-8, 89, 110-114). Comparing the results obtained from our two studies, differences in the invasive disease potential of serotypes were found because of

the change in methodology. When molecular methods where applied in direct sample in our study populations there was an important changed in the ranked of the more invasive serotypes. Although serotypes 1 and 7FA maintained its significant association with high invasiveness when comparing with the first study, serotype 5 and 19A lost this association. And what it is more important, serotype 3, which in the first study did not reach significance, presented a significantly association with high invasiveness in the second study. A substantial changed was also experienced in the IDP of serotypes commonly found in carriage due to the improved detection of serotypes that were poorly found or not found at all in the first study. Serotypes that by culture only showed a tendency to be associated with carriage (like serotypes 19FBC, 6A, 15BC, 21, 23B, 35B or 34) reached a significant value when analyzed in the second study. And most remarkable, serotypes that were not even detected in the first study showed a significant association with carriage like serotype 10FC/33C.

Of special interest, was the high invasiveness found in the pediatric population of our area for serotype 3 in both studies. The invasiveness of this serotype is very controversial. Since the early 20th century the double behavior of serotype 3, being associated to mortality in some individuals while harmlessly carried by other individuals, has been recognized (115-116). For example, invasiveness studies performed in Oxford and Switzerland (7, 112) found serotype 3 more associated with colonization whereas studies from Stockholm and Portugal (89, 113) found this serotype associated with invasive disease.

The results from the third study provided additional information about the invasive disease potential of serotypes causing IPD in our area due to the clonal study of the invasive strains. In addition, it provides information about the evolution of serotypes causing disease after the introduction of PCV13 in 2010, in both children and adults.

Although the most common serotypes found in the study were still PCV13 serotypes (probably due to the low vaccine coverage in our area), a significant decrease was observed, especially for serotypes 19A and 7F, followed by a significant increase of the non-PCV13 serotypes. The change in serotypes causing IPD after PCV13 introduction has been observed in other countries in both children and adults (117-121), and also some studies have already showed an effect of the vaccine on nasopharyngeal colonization (122-123).

But most importantly was the data obtained in the serotypes analysis of their clonal composition. The analysis of the clonal types being expressed by the pneumococcal serotypes could explain why some capsular types that were found usually asymptomatically carried have experienced a change of invasiveness and have emerged as main causes of IPD, since this type of analysis supplies genetic data about recombination events and population structure (124). Yildirim I et al. in a recent publication tried to define a new measure for describing the invasive disease probability of serotypes other than the OR calculation. They observed that differences of serotypes specific IDP between studies may be due to differences in invasiveness between clones of the same serotype (125). Other authors have also argued that virulence differences in pneumococcal strains might be caused by both differences in the capsular structure and in the genetic background (126-128). Despite the capsule is the most important factor in virulence, strains expressing the same serotype but belonging to different clonal types has been found to present different invasiveness (89, 110). Certain clonal types may present some characteristics that are likely to be advantageous for invasiveness, like antibiotic resistance (129).

One example, as published elsewhere, could be serotype 19A that has been proved to be enough well-adapted to the nasopharynx to exist as an asymptomatic colonizer (7, 111). Despite this evidence, the results of our first two studies showed the emergence of serotype 19A as one of the main serotypes in invasive disease after PCV7 introduction in our area. The analysis of the evolution of serotypes after PCV13 revealed that this serotype was still one of the most common, although a clear decreased was found during the study period. Serotype 19A descended from the 1st position in 2010 to not even appear as one of the main serotypes causing IPD in 2013, achieving the 5th position. The high burden of disease caused by this serotype after PCV7 could be explained by the important presence of the multiresistant clone ST320. Studies from the United States suggested that the effects of PCV7 facilitated the spread of serotype 19A, but data published in Korea found an increase of this serotype before vaccine introduction (130-131). Therefore, the association between serotype 19A and ST320 has been proposed as the main contributor to the invasiveness increase experienced by this serotype in the last years (132-133).

On the other hand, and in contrast with the significant decreased of vaccine serotypes, serotype 1 and 3 continued to be two of the main serotypes responsible for the main burden of the disease in our population. The reduced effect of the vaccine on these serotypes could be explained by the low vaccine coverage in our geographical area, maybe needing more time to cause a more significant reduction. Despite of this fact, it is important to note that with respect serotype 3 several studies analyzing the effects of PCV13 in other countries have already reported a low impact of the vaccine suggesting a possible failure (117-118, 120, 134).

With respect serotype 1, its clonal analysis revealed a low index of genetic diversity with a high association with ST306. This data is consistent with information published

that revealed an unusually high attack rate, being rarely found in carriers, and a marked geographic distribution of the few clonal types associated with this serotype (135). ST306 was identified as the most prevalent clone in continental Europe while the main type found in United States was ST227 (109, 136). These clonal type differences could be a plausible explanation for the more stable numbers of serotype 1 that were found in EEUU between pre- and post-PCV7 period when compared with other countries (137), where this serotype became one of the most important causes of IPD. However, it has been also well reported that serotype 1 is more common in Africa, Asia and Europe than in other parts of the world (138).

As for serotype 3, likewise in the two previous studies of this thesis, it was also found being responsible of a high burden of the disease in our area, even after PCV13 introduction. As for the genetic diversity, although a quite high rate was obtained, the serotype was mainly found associated to CC180. As well as with the invasiveness values for this serotype, the analyses of its genetic diversity have also been contradictory worldwide. Studies from United States, Canada and the Netherlands detected low clonal diversity (139-142), whereas studies from Singapore and Scotland found high levels of diversity for serotype 3 (143-144). One possible explanation for the higher invasiveness found in some countries, where in others a low IDP was detected, could be the association with a certain clonal type. Likewise the clonal studies from the United States, in our results a high association was found between serotype 3 in IPD and ST180. However, these results differ from the ones previously obtained from Brueggemann et al. in England where serotype 3 associated with ST180 was only found in carriage (7). A study from Isozumi et al. in Japan described a mucoid phenotype for ST180 serotype 3 that could be forming mucosal biofilms favoring the exchange of DNA with other strains (145). Thus, the mucoid capsule could be facilitating the

formation of clonal types. Another explanation for the different geographical behavior might be the epidemic trends previously reported for serotype 3. Several publications have indicated fluctuations of serotype 1 and 3 IPD during the 20th century even without vaccine selection pressure (146-147). In a recent study, Harboe et al. connected these cyclical trends to the little effect that PCV13 has had on the incidence of serotypes 1 and 3 in IPD in Denmark (32). A hypothesis that could also explained our results for these two serotypes.

Serotypes that were already included in PCV7 maintained a low proportion along the period of study, except serotype 14. In children less than 2 years old and in adults older than 65, serotype 14 was the third more frequent. This surprising emergence was especially evident in 2013, where it ranked within the more common serotypes found causing IPD. Although this serotype was included in PCV7 because of its high IDP (8,89) and this resurgence is most certainly associated to the low vaccine coverage rate of our area, another factor must be in play since it was the only PCV7 serotype showing this behavior in the study. One possible explanation could be the important association found with the clonal type ST156. In a study from Finland conducted by Hanage W.P. et al. analyzing the invasiveness of serotypes and clones among children in Finland, the OR of this ST was revealed as the highest in the study and, in particular, ST156 serotype 14 isolates appeared to have a marked predilection to cause IPD (110). Moreover, it has been found that genes for the expression of the pilus are well conserved among the isolates of this clonal type. In fact, in a recent study published by our group all ST156 serotype 14 isolates obtained from a pediatric collection between 2004 and 2010 were expressing pilus-1 (148). This observation is noteworthy because Barocchi M.A. et al. showed in a murine model that a piliated clinical isolate of ST156 was more virulent and evoked an elevated immune response than its nonpiliated mutant

(149). Likewise, Sjöström K. et al. suggested that the expression of pili provides a competitive advantage to this clonal type that has contributed to its successful spread (150). A study from Poland in 2010, another country with low vaccine coverage, also reported a quick dissemination of ST156 mainly associated with serotype 14 (151). And in Brazil the combination of ST156 and serotype 14 has been also highly detected in the last years, with a recent study reporting no apparent effect of PCV10 on this clonal type one year after its introduction in this area (152). Regev-Yochay G et al. observed a preferential increase of piliated clones among non-vaccine serotypes after several years of PCV7 in USA (153). Although they hypothesize an intrinsic advantage conferred by the pilus, they were not able to find clear reasons for this emergence. In an analysis of capsular switching, Wyres et al. hypothesized that there could be some synergism between a serotype and a specific clonal type that provides an advantage to certain combinations over others (154). Further studies would be needed to clarify why serotype 14 continues to be so prevalent in our population.

On the other hand, as previously commented a significant increase in the proportion of non-PCV13 serotypes was observed which could be indicating serotype replacement. In particular, our results showed an important emergence of serotypes 12F and 24F, two serotypes considered with high IDP (155). Both increases seemed to be age related, since serotype 24F appeared highly related with IPD in children less than 2 years old whereas serotype 12F highest numbers where in the age group between 18 and 65. Other publications analyzing the effects of PCV13 in different countries have reported the emergence of these two serotypes (118-121). Like in our results, serotype 24F increase was focused in younger children supporting our hypothesis to be age related. The possible replacement with serotype 12F is of especially concern. This serotype was classified as “epidemic” or with high invasive disease potential because of its

association with outbreaks of invasive disease. The most recent cases have been detected after pneumococcal vaccines introduction, in both Alaska and Canada (156-157). The outbreak in Canada was especially worrisome because it was the first time it was caused by a macrolide resistance strain of this serotype. It is considered a hyper invasive serotype because it has been almost exclusively detected in disease patients while rarely found in healthy carriers, which is consistent with the highly clonal association that has been published elsewhere and with the low index of diversity found in our study (152, 158). Like in our results, these studies reported a more frequent detection in adults. Our data showed an association of serotype 12F with ST989. So far outbreaks seemed to be related to ST218, however serotype 12F expressing ST989 has been reported to have a propensity to cause meningitis (159).

As previously comment, also interesting was the increase of serotype 24F. Although our data showed a high level of genetic diversity, it appeared mainly associated to two clonal types: ST230 and ST4677. A multiple drug-resistant serotype 24F strain expressing ST230 was found responsible for three cases of meningitis in Italy before PCV7 introduction (160). Studies in both Italy and Spain found an increase of this serotype associated with the expansion of this multidrug-resistant ST230 clone during the PCV7 era (161-162). In the Spain analysis, this serotype-genotype association was only detected in the Catalonia region. In Bulgaria and Portugal the increase experienced by serotype 19A was related to the increase of this resistant clone (163-164).

Other non-PCV13 serotypes of interest because of their high presence in the study collection were serotype 22F and 8. Emergence of serotype 22F after PCV13 has been also reported in USA, Germany, Norway and even Uruguay (117-118, 120, 165). Our data showed low genetic diversity for this serotype which suggests a high invasive disease potential. Besides, an important association with ST433 was revealed and the

significant expansion of this clonal type has been related to the rise of serotype 22F in the post-PCV13 era (134, 166-167). In contrast, serotype 8 presented a higher clonal diversity. However, an important relatedness with ST53 was found in our results, which is a well-recognized virulent clone (168-169). A significant increase of this serotype has been described in other settings where ST53 also appeared as its main clonal type (120, 152).