Infection and Immunity Commentary

Title: Single nucleotide polymorphisms within the *cps* loci: another potential source of clinically important genetic variation for *Streptococcus pneumoniae*?

Re: IAI00246-21R1 Arends et al. *Examining the distribution and impact of single nucleotide polymorphisms in the capsular locus of Streptococcus pneumoniae serotype 19A*.

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Abstract:

The *Streptococcus pneumoniae* capsule is essential for disease pathogenesis, suggesting that even minor genetic changes within the *cps* locus could potentially have important consequences. Arends et al. have identified 79 different non-synonymous SNPs in the *cps* locus of 338 19A serotype strains, and shown significant variations between strains in nucleotide sugars content and capsule shedding. Further work is required to characterise whether any of these changes have important functional consequences on capsule / host interactions.

Commentary:

The virulence of the Gram positive *Streptococcus pneumoniae* pathogen is critically dependent on its polysaccharide capsule. The capsule assists initial colonisation of the nasopharynx by preventing *S. pneumoniae* being entrapped by mucus (1), and is essential for invasive infection as it inhibits both innate and adaptive immune clearance by inhibiting bacterial opsonisation with complement and antibody (2). The *S. pneumoniae* capsule consists of repeating units of oligosaccharides linked to the bacterial cell wall, and the considerable variation in the monosaccharides, their order and type of chemical bonds within the repeating unit, and the presence or absence of side chains results in multiple chemical structures which are antigenically distinct. Around 100 *S. pneumoniae* capsular serotypes have been described, and this biochemical and antigen diversity has major implications for disease. Some capsular serotypes are much more likely to cause invasive disease (eg serotypes 4, 7F, and 14), and an invasive phenotype correlates closely with resistance to complement-mediated neutrophil phagocytosis (3,4,5). Other capsular serotypes (eg 6A, 23F, and 19F) are much less invasive and are more sensitive to complement-mediated immunity, but tend to have longer duration of colonization (3). For most serotypes the genes necessary for capsule synthesis are contained within a single *cps* locus consisting of between 10 to 20 genes. As a consequence *S. pneumoniae* strains can change capsular serotype by replacing their existing *cps* locus by recombination with DNA from another *S. pneumoniae* serotype (6), which could have large effects on resistance to opsonophagocytosis. In addition, relatively minor genetic changes can alter the capsule chemical structure enough to change serotype and have alter sensitivity to complement (7). The importance of the capsule for *S. pneumoniae* biology has been further emphasized by the widespread use of vaccination of both children and adults using capsular polysaccharide as the target antigen. Vaccination of children largely eradicates the vaccine serotypes as nasopharyngeal commensals, resulting in major changes in *S. pneumoniae* ecology due to compensatory expansion in the prevalence of non-vaccine serotypes.

As well as the profound effects of capsular serotype on *S. pneumoniae* biology and interactions with the host, there are additional effects of the capsule that are independent of its chemical structure and serotype. The best described is phase variation, with opaque phase *S. pneumoniae* having a relatively thick capsule layer and an invasive phenotype whereas transparent phase *S. pneumoniae* have thinner capsule layers and are associated with colonisation and biofilm formation (8,9). In addition, sensitivity to complement mediated phagocytosis varies between *S. pneumoniae* strains expressing the same capsular serotypes (10). More recently, genetic and biochemical data have demonstrated that other commensal streptococci (mainly *Streptococcus mitis*, *S. pneumoniae*'s closest genetic relative) carry *cps* loci very similar to some *S. pneumoniae cps* loci and express serologically identical capsules (11). However, the thickness and functional effects of *S. mitis* capsules can differ compared to the same serotype expressed by *S. pneumoniae*, generally resulting in weaker protection against host innate immunity (12). These data demonstrate that serotype-independent factors can influence phenotypes associated with capsule expression, and raises the question whether genetic variation between *cps* loci of *S. pneumoniae* strains expressing the same capsular serotype can have functional consequences that are important for disease. For example, can minor genetic change in the *cps* locus alter capsule width or cause subtle changes in the capsule's biochemical structure that alter the physical properties and thereby affect interactions with the host?

The paper by Arends et al (13) starts to address this question. The authors present data on genetic variation within the *cps* loci for 338 serotype 19A strains, a serotype which increased in prevalence as a cause of invasive disease after the introduction of routine vaccination of children with Prevnar targeting seven other serotypes. The authors identify a considerable amount of genetic variation in the *cps* locus between these serotype 19A strains. This genetic variation divides the 19A strains into 8 subtypes based on a PCR method for assessing *cps* subtypes. However, the number of 19A subtypes increases substantially to 100 when *cps* loci genome data were used to divide strains into multilocus sequence types (termed cpsMLST).

In total they identified 79 different non-synonymous SNPs, mainly concentrated in three genes: *rmlB* and *rmlD*, both required for synthesis of rhamnose which is one of the three monosaccharides present in the 19A capsule repeating unit; and *wzg* which has a poorly understood function but is involved in capsule expression and perhaps shedding. The SNPs resulted in 22 different RmlB, RmlD or Wzg proteins in total, and similar *rml* gene polymorphisms tended to the cluster within each 19A subtype. Furthermore, the cpsMLST phylogenetic tree divided strains containing the SNPs into two groups consisting of SNPs1-29, 49 and SNPs30-48. Additional genetic changes in the *cps* locus identified by Arends et al include two subtypes of the *cps* locus promoter. The concentration of mutations in three out of 16 genes within the 19A *cps* locus is interesting, and could suggest potential evolutionary advantages for mutations in these genes. However, it could also reflect the opposite effect with a lack of functional effects allowing these genes to tolerate mutations more readily compared to other *cps* loci genes.

Does this genetic variation within the 19A *cps* locus have any functional consequences and therefore could be biologically important, or do the SNPs have little consequence for *S. pneumoniae* biology? The answer to this question remains unclear. The genome data indicates some clustering of the mutations to specific sites, but for *rmlB* and *rmlD* the amino acid changes were predicted to affect sites in the protein that are less likely to affect function directly. To answer whether the genetic changes affect the capsule function requires comparing suitable phenotypes between the 19A strains showing genetic differences in their *cps* loci. The authors evaluated two phenotypes that could be affected by these mutations. One was capsule shedding, which has been linked to the function of Wzg. However, capsule shedding varied markedly between strains but did not correlate with any specific *wzg* SNPs. Arends et al. also used mass spectrometry to measure levels of nucleotide sugars in the bacterial cell (13). Mass spectrometry demonstrated marked variations in the total sugar content and relative ratios of different monosaccharides between strains. This is an important observation but does require confirmation of the reproducibility of the differences between

strains over time and different culture conditions. The monosaccharides affected included those utilised in the 19A capsule such as rhamnose, and this provides a potentially link to the SNPs affecting *rmalB* and *rmalD*. However, the differences between strains in specific nucleotide sugars did not correlate to specific SNPs, although the SNPs1-29, 49 subgroup had higher levels overall of multiple monosaccharides compared to the SNPs30-48 subgroup. As the supply of substrates is thought to affect capsule quantity **(**14**),** this observation may have functional relevance that needs further investigation.

The most obvious question about the biological relevance of the SNPs that needs to be answered is do they affect the width of the capsule layer? This was not addressed by the authors as it is in fact not straight forward to measure differences in capsule width between strains, especially when investigating a large number of strains. The commonest way of measuring capsule width uses fluorescein isothiocyanate (FITC)-dextran exclusion and conventional microscopy, but this methodology lacks the sensitivity needed to identify subtle changes in capsule size (15). Perhaps the most sensitive technique is electron microscopy using capsule sparing preparation techniques, but this is a low throughput technique that can only be applied to a limited number of strains (12). Atomic force microscopy has recently been used to directly assess the physical properties of the capsule, and has the potential to identify subtle differences between strains (12). However, this technique is highly time consuming and even more low throughput than electron microscopy. Both electron and atomic force microscopy could only be used to compare a handful of strains after prior down selection by higher throughput methods. Higher throughput methods suitable for assessing smaller differences in capsule quantity between multiple strains include ELISA or flow cytometry using anti-capsular antibody (12,16). Alternatively, flow cytometry assays can rapidly compare complement resistance or antibody binding to subcapsular protein antigens for dozens of strains and therefore identify important phenotypes that vary with capsule thickness or chemical composition (4,5,10). These assays give an indirect indication of changes in capsule width. When preparing *S. pneumoniae* for assays measuring capsule content, culture under

stress conditions specifically affecting carbohydrate availability or metabolism could be important as this might accentuate subtle effects of *cps* loci mutations on capsule synthesis. Those strains showing significant differences with the higher throughput assays could then be investigated using electron and/or atomic force microscopy.

Whether any differences in the capsule phenotype translate into effects on virulence can be assessed using competitive infection experiment in mouse models of infection. These are highly sensitive at identifying relatively small effects on virulence, and can assess interactions between bacterial genetic factors and host immune effectors (17). For all phenotype analyses any differences identified between strains will be confounded by genetic variation outside of the *cps* loci. Hence, linking any specific phenotypes directly to genetic change in the *cps* locus will require reconstructing the mutations in another strain and repeating the phenotype analyses. Although SNPs that cause changes in *S. pneumoniae* resistance to immune effectors and in virulence in mice could be biologically important, ultimately this needs to be demonstrated by epidemiology studies showing they associate with differences in colonization or invasive disease in humans. The authors found no association between the 19A SNPs with whether a strain was isolated from the nasopharynx or from invasive infection. However, it is likely only a small number of the SNPs (or combinations of certain SNPs) could have serious functional consequences. To identify these will require longitudinal studies that can identify whether specific serotype subtypes rapidly become dominant in humans compared to other subtypes of the same capsular serotype.

The capsule is vital for virulence and disease caused by multiple pathogens, so do the findings by Arends *et al*. have any broader relevance for non-pneumococcal pathogens? In fact, SNPs have already been shown to affect the quantity of capsule expressed by *Streptococcus agalactiae* (16). Hence, minor genetic changes affecting capsule genes could indeed have important functional consequences for other pathogens. When compared to *S. pneumoniae* most pathogens have very limited variation in the chemical structure of their capsule between

strains and therefore limited numbers of capsular serotypes. This perhaps might make minor genetic variation in capsule genes more important for other pathogens compared to *S. pneumoniae*, as any effects on disease potential will be less obscured by variations in virulence between serotypes.

The description of genetic variation within the *cps* loci of *S. pneumoniae* strains expressing the same capsular serotype by Arends *et al*. potentially adds another layer of complexity to the investigation of the consequences of genetic and epigenetic variation between strains on disease pathogenesis. Further detailed phenotyping of multiple strains will be needed to ascertain whether genetic variation within the *cps* locus does result in significant biological effects independent of capsular serotype.

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