disease, and in particular the immunological mechanisms underpinning nasopharyngeal carriage, are poorly understood but are crucial to deciphering why and how carriage may lead to invasive disease as well as the effectiveness of vaccines or therapeutics. I will describe the mechanisms by which *Streptococcus pneumoniae* colonise the nasopharynx without inducing damaging host inflammation and provide insight into the role of bacterial and host constituents that allow and maintain stable carriage, while also describing the factors whose perturbation lead to invasive disease. Similarly, I will discuss our current understanding of the role of immune regulation during invasive disease and how these factors contribute to host susceptibility. Overall, I will highlight the delicate balance between the requirements of the host to prevent inflammatory tissue damage with the need to combat potentially dangerous pathogen colonisation. This equilibrium needs to be considered carefully in the future design of anti-pneumococcal therapeutics and vaccines which may alter the density of pneumococcal carriage and hence affect upper airway immune responses.

PL.04

An ecological perspective on symbiosis between *Streptococcus pneumoniae* and the host

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Respiratory tract infections are a major global health concern, accounting for high morbidity and mortality, especially in young children and elderly individuals. Traditionally, it is thought that bacterial respiratory tract infections, including otitis media and pneumonia, are caused by a limited number of pathogens like *Streptococcus pneumoniae*, and *Haemophilus influenzae*. The ecological niche for these potential pathogens is the upper respiratory tract of humans. They commonly reside here asymptomatically, and at equilibrium with their environment and the host. It is still unclear why in one individual an infection may develop, and in the other one not. However, over the last years the importance of the upper respiratory tract (URT) microbiota in maintaining respiratory health has become more apparent. Analogous to the gut microbiome, the respiratory microbiome is thought to be beneficial to the host by priming the immune system and providing colonisation resistance. In contrast, an imbalanced ecosystem lacking keystone bacteria might predispose to bacterial overgrowth, dissemination and consecutive respiratory infections. Recently, we have characterised the upper respiratory microbiota of a large group of individuals, young and old, during health and disease. We obtained evidence for the existence of different respiratory microbiota profiles, related to environmental drivers, to stability of the ecosystem and susceptibility to respiratory infections. Several of these profiles are positively or negatively related to streptococcal colonisation in general, and *S. pneumoniae* colonisation in particular. In this lecture I will present the current body of evidence regarding the position of *S. pneumoniae* within the nasopharyngeal and oropharyngeal microbial ecosystem, and discuss the current ecological theory regarding pathogenesis of respiratory infections.

Poster abstracts

P1.01

Growth phase regulates the localisation and activity of the pneumococcal autolysin LytA

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In *Streptococcus pneumoniae*, the choline-binding proteins (CBPs) are exposed at the cell surface through association to phosphocholine residues (PCho), which decorate the cell wall teichoic acids. Among them, LytA is an N-acetylmuramoyl-L-alanine amidase responsible for the pneumococcal autolysis in late stationary phase. Many aspects of LytA mechanism remain unknown, such as the molecular mechanism of release, the localisation pattern as well as the regulation of amidase activity. We have developed a version of the superfolder Green Fluorescent Protein (sfGFPop) appropriate to investigate the localisation of secreted proteins at the surface of *S. pneumoniae*. The fusion protein LytA–sfGFPop

has been ectopically expressed in the pneumococcus and live fluorescence microscopy was realised to determine the spatio-temporal localisation of LytA. The results showed that LytA shifts from a homogeneous cytoplasmic localisation during the exponential growth phase to a heterogeneous cytoplasmic localisation when bacterial cell population enters the stationary growth phase. Finally, we observed the extracellular presence of LytA at the septum site along the lytic phase. Respective roles of the choline-binding and amidase domains of LytA were analysed in this relocalisation process. We determined that both domains are required for efficient cell lysis and surface localisation of LytA, which is also linked to the presence of CbpE that regulates the amount of cell wall-associated PCho. In stationary growth phase, cell death occurs before lysis, which suggests that LytA could be released by membrane disruption. Moreover, LytA specifically cleaves the peptidoglycan at the septal sites only when cells enter the stationary growth phase. Studying cell physiology at the early stationary phase will allow deciphering the mechanisms underlying these processes. Preliminary data suggest that the regulation of LytA-mediated cell lysis occurs through the substrate availability. Characterisation of such peptidoglycan modification is currently under investigation.

P1.02

DiiA is a newly identified cell wall protein of *Streptococcus pneumoniae* involved in invasive disease

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Many outer multi-domain proteins play fundamental roles in the virulence of bacterial pathogens in an allele-dependent manner. The hypothetical protein SP1992 of *Streptococcus pneumoniae* TIGR4 contains (from N- to C-terminus): two imperfect repeats (R1 and R2), an unstructured region, and a cell-wall anchor domain. In this study we have investigated 560 clinical isolates, demonstrating that the gene coding for DiiA is part of the core genome and may carry either one (R2) or two (R1R2) repeats. Clonal complexes carrying R1R2 were associated with invasive disease, while those carrying R2 preferentially affected patients with underlying risk factors. Isogenic strains carrying step-wise deletions of *diiA* were constructed to investigate the contribution of the different modules to the infective process. The mutants constructed were: *diiA*-R2 that lacks R1 and mimics the natural short allele; *diiA*-NR that lacks both repeats; and the Δ*diiA* null mutant. Our results show that R1 and R2 are involved in the interaction with lung epithelial cells, and in systemic dissemination using a mice model of pneumonia infection. Moreover, the Δ*diiA* defective strain was severely impaired in its ability to proliferate in blood in a sepsis model. This strain was also less able to avoid complement-mediated immunity and phagocytosis. The latter phenotype may be mediated by the binding of the unstructured region to lactoferrin, for which the DiiA-NR protein had a high affinity for, with a K_D of 2.5 nM. Based on our results, we termed SP1992 DiiA, after Dimorphic invasion-involved A protein.

P1.03

Induction of meningitis by a non-passaged clinical pneumococcal isolate in an infant rat model depends on the presence of capsule

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This study aimed to use an established infant rat model of experimental pneumococcal meningitis to study the potential of a non-passaged clinical pneumococcal isolate to induce disease. A Swiss clinical pneumococcal isolate (106.66) of serotype 6B and its non-encapsulated mutant (106.66 Janus) were administered intracisternally in an established infant rat model without prior passage of the bacteria in animals. Bacterial titres were determined in CSF samples to optimise the model for study of a non-passaged strain and to determine the appropriate time to administer ceftriaxone antibiotic therapy. Clinical parameters were recorded and CSF sampled to determine bacterial titre and cytokine concentrations. Brain damage was quantified by histomorphological analysis. A non-passaged clinical pneumococcal isolate was able

to establish a productive infection and cause meningitis in the model, resulting in significant mortality. Brain damage (cortical damage, hippocampal apoptosis) and leukocyte pleocytosis were evident from histomorphological analysis. The non-encapsulated mutant failed to establish an infection or cause any detectable pathology. The presence of capsule is essential for establishing meningitis in this model. The modified model is appropriate for studying the meningitis-inducing potential of non-passaged clinical isolates.

P1.04

An investigation of the impact of air pollutants on *Streptococcus pneumoniae*

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Despite legislation to reduce air pollution, it is the largest global environmental and health problem we are faced with in current times and was responsible for 1 in 8 deaths worldwide in 2012. One of its most toxic components, particulate matter (PM) is responsible for increases in cardiovascular and respiratory diseases and infections. To date this has been attributed to deleterious and immunomodulatory effects on the human host, including tissue damage, inflammatory responses, and oxidative stress. However, there has been a major oversight in this field as no studies have investigated the direct impact of air pollution on bacteria of the human respiratory tract. This may be a serious omission because bacteria play an essential role in maintaining the health of a host. We have investigated the effect of a specific component of PM and black carbon (BC), which is a by-product of fossil fuel combustion, on the behaviour of *Streptococcus pneumoniae*. *S. pneumoniae* was chosen as it asymptomatically colonises much of the population whilst retaining the ability to cause severe disease. Our data show, for the first time, that there is an interaction between air pollutants and bacteria, with BC inducing species-specific alterations in biofilm formation and structure. Biofilms are vital in both host colonisation and infection, providing protection from the environment and the host immune system. Biofilm alterations will have a significant impact on bacterial survival *in vivo* as well as on the potential for disease progression and transmission. Therefore our data suggests a major effect of air pollution has been overlooked.

P1.05

Studies on pneumococcal esterases

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The genome of pneumococcal strain contains 4 putative esterase genes (SPD_0534 (*estA*), SPD_0932, SPD_1239, and SPD_1506 (*axe*); however, their importance in pneumococcal biology is very sparse. In this study, we determined the contribution of each esterase gene to total esterase activity, evaluated the substrate specificity of pneumococcal esterases, and investigated their role in potentiation of neuraminidase activity and glycoprotein utilisation. The results showed that all mutants displayed significantly less esterase activity than the parental strain EstA, which is responsible for the main esterase activity, and the pneumococcal esterases are specific for short acyl chains. In addition, EstA, but not Axe, was found to catalyse tributyrin, and both EstA and Axe could use acetylated xylan as substrate. It was also found that EstA and Axe use bovine submaxillary mucin (BSM), which is highly acetylated, as substrate to release acetate, and pre-treatment of BSM by either EstA or Axe increases sialic acid release by neuraminidase. Esterases' role in potentiation of neuraminidase activity was further supported by decrease the ability of sialic acid release and growth of esterase-neuraminidase mutants (*ΔestAnanA* and *ΔaxenanA*) in medium containing BSM as sole carbon source compared to each of the respective single mutants. The replacement of S^{121} in EstA and S^{181} in Axe to alanine abolished the catalytic activity of esterases. In addition, the mutation of *estA* alone or in combination with *nanA* reduced the pneumococcal colonisation and virulence significantly after intranasal infection. qRT-PCR results showed that the expression level of *estA* and *axe* were significantly up-regulated when exposed to BSM.

P1.06

Anti-capsular polysaccharide IgG-mediated protection on experimental human pneumococcal carriage

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Recent studies have highlighted the role of mucosal antibodies in blocking acquisition of pneumococcal colonisation through agglutination. We used samples from adults vaccinated with 13-PCV prior to experimental human pneumococcal carriage (EHPC) to study the role of capsule polysaccharide (PS) IgG in mediating protection from carriage after pneumococcal inoculation. Ninety-six healthy adults were intranasally inoculated with live 6B pneumococcus after vaccination (10% carriage in PCV versus 48% in control). We assessed changes in IgG levels to 6B and 23F PS in serum and nasal wash (NW) samples before and after vaccination and before and after inoculation. In the control group, for those colonised, IgG levels against 6B PS but not 23F PS were elevated in both serum and NW at 21 days after challenge when compared to baseline, indicating that carriage boosts IgG levels of existing PS immunity. In PCV-vaccinated subjects, IgG levels to both PS types were increased in serum and NW after vaccination. While IgG levels to both PS types remained increased in sera after challenge, levels of IgG specific to the PS of inoculated type strain dropped at day 2 after challenge, an effect more pronounced in those protected from carriage. No decrease was observed in IgG levels to 23F PS. We hypothesise that PCV protection against carriage is mediated by bacterial agglutination by PS-specific antibodies. Mucosal antibodies are sequestered by the pneumococcal inoculum, blocking bacterial adherence and therefore mediating protection against colonisation. We are evaluating the capacity of NW antibodies from PCV-vaccinated subjects in mediating agglutination of pneumococcus.

P1.07

P4 peptide enhances phagocytic activity of neutrophils acquired from patients admitted to critical care with severe sepsis

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P4 is a 28-aa peptide fragment of pneumococcal surface adhesion A, which shows phagocyte- activating effects *in vitro* and *in vivo*. Passive immunotherapy augmented using P4 is a novel potential therapeutic strategy to enhance phagocytes bacterial killing activity and we have previously shown improved *ex vivo* neutrophil activity in patients with severe community acquired pneumonia. In this study, patients with severe sepsis (respiratory, abdominal, and urogenital) admitted to critical care were recruited and blood samples obtained. We cultured neutrophils *ex vivo* with P4 or control vehicle and assessed phagocyte function, bacterial killing activity and ROS. We took sequential samples at different phases of disease (early, latent, and convalescent) from each subject to perform two biological assays: 1) opsonophagocytosis assay with isolated neutrophils and 2) flow cytometric whole blood phagocytosis assay using fluorophore labelled intraphagosomal reporter (oxidative burst) beads. Our preliminary data demonstrate that P4 peptide significantly increased neutrophil bacterial killing in early and latent phases of disease. In addition, the whole blood phagocytosis assay demonstrated both increased uptake and oxidation of intraphagosomal reporter beads in the early and latent phases. Samples from the convalescent phase are currently being collected. The stimulation of neutrophils with P4 peptide *ex vivo* augments their phagocytic activity in the majority of septic patients. Its administration, in conjunction with intravenous immunoglobulin (IVIG), could be considered as a novel treatment to enhance host immune function and to facilitate the combat against multiple infections.

P1.08

Evaluation of *Streptococcus pneumoniae* respiratory infection in mice with different susceptibility for acute inflammatory responses

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Acute inflammation is an important response against bacterial infections. Here, an invasive respiratory challenge model with a *Streptococcus pneumoniae* serotype 3 strain was studied in two outbred mice lineages, genetically selected for exacerbated (AIRmax) and low (AIRmin) acute inflammatory responses. A dose of 10^4 bacteria per animal induced the death of 100% of AIRmin mice a few days after the challenge. In contrast, only 36.4% of AIRmax mice died after the infection. Bacterial numbers in the airways of AIRmax mice at 48 h post-challenge were significantly lower than the observed in AIRmin mice. Analysis of cytokines in bronchoalveolar lavage fluids, showed peaks of IFN-γ and IL-6 at 12 h post-challenge for AIRmax mice, and significant increases in IL-4, IL-5 and IL-10, at the same time point, for AIRmin mice. No differences were observed for IL-1β, KC or IL-17, when comparing the 2 mice strains. The profile of TNF-α response after the challenge was similar in the 2 mice strains; however, the levels of this cytokine remained high for AIRmin mice at later time points. Preliminary analysis showed no differences in the percentage of alveolar macrophages expressing the CD206 mannose receptor (F4/80+ CD11c+, CD206+), for both mice strains, before or 6 hours post-challenge. However, a significant increase in the expression of CD206 by these cells (measured by the mean fluorescence intensity) was observed in AIRmin mice at 6 hours post-challenge. Further analysis on the role of these cells in the outcome of pneumococcal infection in this model is underway.

P1.09

Stable niche adaptation within clonal lineages of *Streptococcus pneumoniae*

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Individual *Streptococcus pneumoniae* strains differ markedly in their virulence phenotypes, but genetic heterogeneity has complicated attempts to identify any association between a given clonal lineage and propensity to cause a particular disease type. Previously we reported that serotype 3 pneumococci of the same ST-type exhibit distinct *in vitro* and *in vivo* phenotypes in accordance with clinical site of isolation. In this study, serotype 14 blood and ear isolates of the same ST-type (ST15) were investigated for pathogenic phenotype. In a murine intranasal challenge model, the three blood and two ear isolates colonised the nasopharynx to similar extents. However, they exhibited significant differences in bacterial loads in other host niches. At 24 and 2 hours post-challenge, neither of the ear isolates was detectable in the lungs of any of the mice, but the blood isolates were present in the lungs of the majority of mice at both time points. None of the three ST15 blood isolates were detected in the brains of any mice at either time point. However, the ear isolates were detected in the brains and ear compartment of most of the mice at both 24 hours and at 72 hours. Thus, stable niche adaptation within a clonal lineage appears to be a general property of pneumococci. Interestingly, both blood and ear isolates were present in the lungs at similar levels at 6h post-infection, suggesting early immune responses may underpin the distinct virulence phenotypes. Transcriptional analysis of lung tissue from mice infected for 6 hours with blood versus ear isolates using RT-PCR arrays of key immune response genes revealed 8 differentially expressed genes. Two of these were exclusively expressed in response to infection with the ear isolate. These results suggest a link between differential capacity to elicit early innate immune response and the distinct virulence phenotypes of clonally related *S. pneumoniae* strains.

P1.10

The inflammatory response to *Streptococcus pneumoniae* is exaggerated by the polysaccharide capsule

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The inflammatory response to bacteria requires the interaction of pattern recognition receptors (PRR) with bacterial surface constituents. *Streptococcus pneumoniae* expresses a polysaccharide capsule (cps); this essential virulence factor's main function is to inhibit opsonophagocytosis, and would be expected to inhibit interactions with host PRRs, so reducing inflammatory responses. We tested this hypothesis by characterising the effect of *S. pneumoniae* capsule on the inflammatory response using the TIGR4 strain and its unencapsulated derivative Δcps. Despite being more sensitive to phagocytosis by human monocyte derived macrophages (MDM) than TIGR4, RNA transcripts and supernatant levels of TNF, IL1β, and IL6 were reduced in response to Δcps. Furthermore, TIGR4 generated pro-inflammatory cytokine responses and greater neutrophilic infiltrate in a mouse pneumonia model than Δcps, as well as a trend towards greater physiological disturbance in a rat model of septic shock. Whole genome transcriptome analysis demonstrated a generally reduced pro-inflammatory response to the TIGR4cps strain. Notably, preventing phagocytosis preserved these differences. *In vitro* experiments excluded differences in TLR2 signalling, NFκB translocation, antibody recognition, the inflammasome, and scavenger receptor binding as mechanisms driving differences in inflammatory responses between TIGR4 and Δcps. However, a transcription factor array suggested that Δcps activated more transcription factors than TIGR4. These data demonstrate that *S. pneumoniae* capsule causes increased pro-inflammatory responses that are relevant during infection, perhaps by restricting macrophage cell signalling responses. Identifying mechanisms responsible for capsule-dependent inflammation may offer opportunities for adjuvant treatment of *S. pneumoniae* infections.

P1.11

Survival of *Streptococcus pneumoniae* and *Staphylococcus aureus* in sputum from intensive care unit patients

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Pneumonia related to mechanical ventilation in hospitals is referred to as ventilator-associated pneumonia (VAP). VAP results in increased duration of mechanical ventilation and prolonged intensive care unit (ICU) and hospital stay. Both *Streptococcus pneumoniae* and *Staphylococcus aureus* have been associated with VAP. To date, it is not very well known to which stresses these bacteria are exposed in the airways of ventilated patients. The present studies were therefore aimed at establishing an *ex vivo* assay that mimics the *in vivo* situation through the incubation of bacteria in sputum samples collected from different ICU patients. *S. pneumoniae* strains D39 and TIGR4 and *S. aureus* strain HG001 were pre-cultured in different media and, subsequently, incubated for different periods of time in sputum samples from mechanically ventilated ICU patients from the University Medical Center Groningen. Next, bacterial survival in the sputum samples was tested by plating and microscopic inspection. The tested *S. pneumoniae* and *S. aureus* strains showed different rates of survival in sputa from different patients. This did not relate to the way in which sputum samples were pre-treated. Our ongoing research is aimed at assessing both patient parameters that affect bacterial survival in sputum as well as the bacterial gene repertoire that is needed for survival. Results of these analyses will be presented at the meeting.

P1.12

Characterisation of a low molecular weight protein tyrosine phosphatase in *Streptococcus pneumoniae*

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Tyrosine phosphorylation is widely recognised as a critical regulator of bacterial virulence, with the associated protein tyrosine phosphatases (PTPs) and bacterial tyrosine kinases (BY-kinases) major virulence factors in a range of bacterial pathogens including *Streptococcus pneumoniae*. We have previously studied a phospho-regulatory system in the pneumococcus comprising CpsB (a PTP) and CpsC and CpsD, which together form an active BY-kinase. This system plays a crucial role in the regulation of capsule production. While homology searches did not uncover additional BY-kinases, the protein encoded by one open reading frame (designated Sp-PTP) showed homology (46%) to the low molecular weight protein family of tyrosine phosphatase which play a role in capsule regulation in other bacteria, as well as diverse roles that include stress responses and biofilm formation. To determine PTPs role in the pneumococcus, His_6-Sp-PTP purified from *Escherichia coli* enabled us to verify that Sp-PTP is indeed a phosphatase, with specificity against tyrosine. We also found that Sp-PTP's phosphatase activity is inhibited by hydrogen peroxide. We have constructed *ptp* mutations on the pneumococcal chromosome and found that PTP does not affect capsule production. We suggest that the phosphatase may have other roles in the pneumococcus, and predict that tyrosine phosphorylation events in the pneumococcus have wider effects outside the capsule system.

P1.13

Quantification of the agglutinating effect of anti-pneumococcal antibodies analysed by flow cytometry

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Following entry into the airways, pneumococci must first overcome the natural barriers in the nasopharynx before it can establish stable colonisation. The presence of antibodies at mucosal surfaces is thought to inhibit colonisation via multiple mechanisms, including opsonisation-mediated phagocytosis and killing and neutralisation by binding to factors such as bacterial adherence. Recently, a role for antibody-mediated agglutination in the prevention of colonisation was identified in mice. Passive immunisation studies with anti-capsular IgG demonstrated that optimal mucosal protection was independent of Fc-mediated signalling and complement-binding, but occurred through F(ab)2-mediated formation of bacterial aggregates. Agglutination is thought to enhance excretion of bacteria via mucociliary flow or other ways of mechanical clearance. At present, there is no gold standard yet to measure and quantify agglutinating antibodies, although microscopy is frequently used. We therefore developed a novel, fast, standardised, high-throughput flow cytometry-based assay to quantify the agglutinating capacity of antibodies, based on size (FSC) and granularity (SSC) of pneumococcal aggregates. We demonstrate that antibodies against PspA, an antigen that is less abundantly present on the pneumococcal surface than capsular polysaccharides, are able to induce agglutination. Flow cytometry analysis offers the possibility to quantify the agglutinating effect of antibodies on a panel of clinical isolates, thus enabling studies on the correlation with protection.

P1.14

Binding activities and anti-phagocytic properties of PclA, the pneumococcal collagen-like protein A

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The pneumococcal collagen-like protein A (PclA) is covalently linked to the surface of *Streptococcus pneumoniae* via an LPxTG motif and harbours a variable number of repetitive amino acid sequences with high homologies to human collagen. PclA is expressed in up to 45% of tested pneumococcal isolates and mediates pneumococcal adherence to nasopharyngeal cells [1]. Molecular analyses indicated that the collagen-like sequence repeats are clustered in up to 3 collagen-like domains spanning a maximum of 6 repeated amino acid sequences per domain. The total number of collagen-like repeats of PclA ranges from 2 repeats in a serotype 19F isolate, to up to 10 repeats in total in serotype 2 isolates, e.g. R6. Consequently, the molecular weight of PclA varies between 187 kDa and 289 kDa depending on the numbers of collagen-like repeats. The effect of PclA on phagocytic neutralisation was analysed in phagocytic killing assays with macrophage-like U937 cells and with neutrophils prepared from blood of two different human donors. Phagocytosis of a *pclA*-deficient mutant by U937 cells was similar to the corresponding wild type strain. In contrast, depending on the donor, the prepared neutrophils were less efficient in phagocytosis of *pclA*-deficient pneumococcal mutants. Furthermore, we demonstrate for the first time specific binding activity of the collagen-like domain 1 of *S. pneumoniae* strain D39 to human plasma fibronectin and von Willebrand factor. The interaction of the PclA domain with host proteins indicates an important contribution of PclA to pneumococcal colonisation and virulence.

1. Paterson GK, Nieminen L, Jefferies JM, Mitchell TJ. PclA, a pneumococcal collagen-like protein with selected strain distribution, contributes to adherence and invasion of host cells. FEMS Microbiol Lett 2008;285:170–6. PMID:18557785 http://dx.doi.org/10.1111/j.1574-6968.2008.01217.x

P1.15

Delineating the interaction of pneumococcal surface proteins with the human adhesive glycoprotein fibronectin

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The virulence potential of *Streptococcus pneumoniae* is closely associated with its expression and exposure of a diverse repertoire of colonising and virulence factors. Several of these factors belong to microbial surface components recognising adhesive matrix molecules (MSCRAMMs), which exploit eukaryotic host matricellular proteins such as fibronectin, vitronectin, thrombospondin, collagen, or plasminogen as a molecular mediator to interact with host cells. Pneumococcal adherence and virulence factor A (PavA) and B (PavB) are fibronectin-binding proteins (FnBPs), representing a sub-class of MSCRAMMs. PavB is covalently anchored to the pneumococcal cell wall by sortase A, whereas the PavA protein is a member of the so-called non-classical surface proteins (NCSPs) with an ambiguous unknown mechanism of secretion and surface-exposure. In this study direct protein–protein interaction approaches have been used to delineate pneumococcal PavA and PavB interactions with the human glycoprotein fibronectin (Fn). Flow cytometric analysis showed that *S. pneumoniae* recruits fibronectin from human plasma in a dose-dependent manner. Pneumococci interact with the C-terminal part of Fn, while other Gram-positive bacteria bind to the N-terminal Fn type I repeats. In order to assess the underlying molecular mechanism of these PavA/PavB–fibronectin interactions, different Fn fragments and type repeats were used in far western blots and surface plasmon resonance (SPR) studies. Far western blots demonstrated binding of PavA and PavB to C-terminally located type III repeats. The interaction between type III repeats and PavA or PavB was confirmed in our SPR analysis. Among the Fn type III repeats, Fn type III12-14 (heparin binding domain) bound with highest affinity to PavA and PavB. In addition, peptide arrays are used to identify crucial binding motifs in Fn type III repeats. This study shows that FNBPs preferentially bind to type III repeats of Fn molecules

and provides insights into the molecular mechanisms of Fn engagement during pneumococcal-host interactions.

P1.17

Pneumolysin-induced nitric oxide contributes to lysosomal membrane permeabilisation in *Streptococcus pneumoniae*-infected macrophages

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A key phase in the macrophage response to *Streptococcus pneumoniae* is a program of host-mediated apoptosis. This occurs after the macrophage has engulfed bacteria, but becomes overwhelmed. This apoptotic response increases late stage killing of bacteria when conventional phagolysosomal killing has become exhausted. The apical event in this pathway is lysosomal membrane permeabilisation (LMP). This is caused by a currently unknown mechanism that requires the pneumococcal toxin pneumolysin (PLY). PLY is a pore-forming toxin but pore-formation is not necessary to induce LMP, indicating other aspects of PLY activity may be responsible. PLY is known to contribute to nitric oxide (NO) production in macrophages. We hypothesised that PLY-induced NO production by the macrophage contributes to LMP and that this is integral to the host response to pneumococcus. To study this system we employed the use of primary human monocyte derived macrophages (MDMs). MDMs were infected at an MOI of 10 using opsonised D39 *S. pneumoniae*, a PLY negative mutant (Stop), or latex beads. MDMs were assayed for LMP using flow cytometery and western blotting. In some experiments MDMs were treated with inhibitors of ROS (Trolox, DPI) and NO (1400W), NO donors SNAP or NOC-13 and/or TLR agonists lipoteichoic acid (LTA) and lipopolysaccharide (LPS). D39 infected MDM displayed significantly higher levels of LMP than Stop infected cells. Incubation with 1400W and Trolox significantly reduced LMP in D39 infected cells, but DPI had no effect. Conversely, incubation with SNAP and NOC-13 was able to restore LMP in Stop infected cells. Preliminary data suggests that NO is necessary but not sufficient for LMP, with TLR activation and phagocytosis also required for NO to exert its effect; however, further verification is required. These data identify a novel role for NO in LMP and host–pneumococcus interactions.

P1.18

Structure of the pneumococcal L,D-carboxypeptidase DacB and impact of DacB on pathophysiological processes

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The peptidoglycan (PGN) is a complex macromolecule and composed of 2 of the alternating sugars residues, *N*-acetylglucosamine (Glc*N*Ac) and *N*-acetylmuramic acid (Mur*N*Ac), which form oligo-(Glc*N*Ac-Mur*N*Ac) glycan strands. These heteropolymers are cross-linked by short peptides to form a complex three-dimensional scaffold (murein). The PGN forms the essential exoskeleton needed to maintain the shape and osmotic stability of bacteria. Pneumococcal cell wall hydrolases such as L,D- and D,D-carboxypeptidases DacB and DacA respectively, have been shown to be important for cell division and shape. Pneumococcal Δ*dacA* and Δ*dacB* single and double mutants were generated and characterised by immunoblot analysis, growth behaviour, and flow cytometry. The impact of DacB on virulence was tested by phagocytosis assays by applying acute pneumonia mouse infection model in conjunction with real-time bioimaging. To assess the muropeptide species a PGN analysis has been carried out. Importantly, the crystal structure of DacB was solved successfully at high resolution. The L,D-carobxypeptidase DacB is a surface-exposed lipoprotein and its structure is characterised at the atomic level showing radically different structure, regulation and catalytic machinery than the pneumococcal D,D-carboxypeptidase DacA. Importantly, the morphological changes observed in *dac*-mutants are associated with an altered PGN composition and hence lower bacterial fitness under infection-related conditions. The *in vivo* mouse infections and cell cultured-based

adherence and invasion assays indicated loss of function of DacA and/or DacB impaired full-virulence of pneumococci and accelerated uptake by professional phagocytes, while adherence to epithelial cells is decreased. In this study, we further characterised the crucial role of the pneumococcal carboxypeptidases DacA and DacB for PGN architecture, bacterial shape and pathogenesis. By applying *in vivo* and *in vitro* approaches, a close relationship between PGN metabolism and impaired bacterial pathogenesis was discovered.

P1.19

Blockade of *Streptococcus pneumoniae* adhesion receptors of the blood–brain barrier endothelium: a new therapeutic approach for the prevention and cure of pneumococcal meningitis

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Meningitis is thought to occur as the result of pneumococci crossing the blood–brain barrier and invading the central nervous system (CNS). The main route taken by *Streptococcus pneumoniae* to invade the CNS is via the bloodstream by translocating across the vascular endothelium of the blood–brain barrier. Bacteria have the capability to bind to receptors expressed on the plasma membrane of epithelial and endothelial cells and, through this binding, they can invade and cross human cell layers. Platelet endothelial adhesion molecule (PECAM-1) and poly immunoglobulin receptor (pIgR) expressed by brain endothelial cells are cell membrane-receptors which have been recently described to be involved in the adhesion of *S. pneumoniae* to the blood–brain barrier endothelium. It has been shown that pneumococci co-localise with PECAM-1 and pIgR in the brain of mice intravenously infected with *S. pneumoniae*. Furthermore, blockade of pIgR and PECAM-1 with specific antibodies reduced pneumococcal adhesion to human endothelial cells *in vitro*. Firstly, a bacteraemia-derived meningitis model with PECAM-1$^{-/-}$, pIgR$^{-/-}$ and wild-type mice will be used as *in vivo* system to unequivocally demonstrate the role of PECAM-1 and pIgR in pneumococcal meningitis pathogenesis. In the case of a direct role of these 2 receptors, the prediction is that fewer bacteria would translocate across the blood–brain barrier in the knock-out mice and, in addition, the typical symptoms of bacteraemia progressing towards meningitis should be less pronounced. Lastly, antibodies against PECAM-1 and pIgR will be administered systemically in wild-type mice prior to intravenous challenge with *S. pneumoniae* to determine whether the treatment with receptor-specific antibodies counteracts meningitis onset. In the case of a significantly reduced progression towards meningitis, the systemic administration of antibodies to block *S. pneumoniae* adhesion receptors has the realistic potential to become a new therapeutic approach for the prevention and cure of pneumococcal meningitis.

P1.20

Pneumococcal nasopharyngeal carriage in Nepalese children prior to pneumococcal conjugate vaccine introduction

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As part of an impact evaluation of 10-valent pneumococcal conjugate vaccine (PCV10) in Nepal, we undertook a serotype-specific community carriage study among community-based children from Kathmandu in the year prior to PCV10 introduction. Children 6–60 months and 0–8 weeks of age were recruited for the nasopharyngeal (NP) study from immunisation and outpatient clinics, and relatives attending inpatients at Patan Hospital, Kathmandu. Flocked NP swabs were collected according to the WHO guidelines. Swabs were placed into skim-milk-tryptone-glucose-glycerin medium, plated within 8 hours on Columbia blood agar, and incubated overnight at 35–37°C in 5% carbon dioxide. Pneumococcal colonies were identified by morphology and optochin sensitivity. Serotyping was performed by Quellung reaction using sera from Serum Staten Institute, Denmark. Between April and December 2014 we enrolled 1,905 children (1,305 aged 6–60 months, and 600 aged 0–8 weeks). Pneumococcal carriage prevalence was 69.7% (910/1,305) and 18.8% (113/600) in those 6–60 months and 0–8 weeks of age, respectively ($p < 0.0001$). Among those colonised, 32.2% (137/425 [95% CI 27.8–36.9%]) and 22.5% (22/98 [95% CI 14.6–32%]) had PCV10 serotypes in the 6–60 month and 0–8 week age groups,

respectively (p = 0.07). Colonised 0–8-week-old children were more likely to have a nontypeable pneumococcus than colonised 6–60-month-old children, with 33% (32/98 [95% CI 23.5–42.9%]) versus 14.1% (60/425 [95% CI 11–17.8%]; p < 0.0001). Infants in the pre-immunisation age group have a significantly lower prevalence of pneumococcal carriage than vaccine age-eligible children; the youngest colonised children were more likely to have a nontypeable strain than the older children. This may be due to factors such as inter-serotype competition for the nasopharyngeal niche, passively transmitted serotype-specific maternal immunity, or differential exposure.

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P1.21

Phosphoenol-pyruvate phospho-transferase A interactions with putative host target receptors and their derived peptides during *Streptococcus pneumoniae* adhesion

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Streptococcus pneumoniae is a commensal pathogen and the major cause of life threatening diseases, including otitis media, pneumonia, bacteraemia and meningitis. Adhesion of *S. pneumoniae* to the host mucosal cells is prerequisite for disease development. In previous studies several proteins with known enzymatic functions were localised also to the cell wall, and were found to function as adhesins. Current study focuses on one such adhesin, Phosphoenol-pyruvate phospho-transferase A (PtsA), which is the first enzyme of the PTS systems. Putative PtsA target receptors were identified using combinatorial peptide library expressed in filamentous phage followed by a homology based search of the human genome. Immunostaining proved 5 out of 6 PtsA putative target receptors to reside in the lung derived epithelial cells. Microscale thermophoresis (MST) assay confirmed the specificity of rPtsA interaction with each of the 5 putative target receptors derived peptides, yielding affinities in the micro-molar range, in accordance with their inhibitory concentration range in adhesion. To decipher the precise mechanism of PtsA interactions with its putative target receptors and their derived peptides, the 3D structure of PtsA is being resolved using X-ray diffraction. *In vivo* experiments also exhibited lower bacterial load in mice infected with *S. pneumoniae* preincubated with putative receptor derived peptides, indicating the essential role of PtsA in bacterial adhesion and infection. With respect to the increasing antibiotic resistance of *S. pneumoniae*, the putative target receptor derived peptides are currently considered as candidates for alternative effective therapeutics.

P1.22

Contribution of a temperate bacteriophage to the virulence of a *Streptococcus pneumoniae* serotype 1 strain

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Temperate bacteriophages are frequently found in the genomes of clinical isolates of *Streptococcus pneumoniae*. Currently there is a lack of knowledge on how, if at all, these genetic elements affect the virulence of pneumococcal strains. In our study we investigated the effect on virulence of a temperate phage in an invasive clinical isolate of a highly virulent serotype 1 strain of sequence type (ST) 217 by using a mouse model of invasive disease. We found that the presence of the phage was associated with increased virulence and the effect was linked to a gene encoding a phage tail protein, which in *S. mitis* has been described as *pbl*B. In *S. mitis* the *pbl*B platelet binding locus has been shown to mediate binding to platelets and increase virulence in an endocarditis model. We found that in *S. pneumoniae* *pbl*B is required for sustained bacteraemia during invasive disease. Further studies are needed to elucidate the exact mechanisms by which bacteriophages contribute to pathogenesis.

P1.23

Streptococcus pneumoniae cardiac microlesions: pneumolysin-mediated immune evasion and biofilm growth

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Approximately 20% of adults hospitalised for pneumococcal pneumonia experience an adverse cardiac event. We have recently reported on the formation of pneumococci-filled, non-purulent microscopic lesions (i.e. microlesions) within the myocardium of mice with invasive pneumococcal disease (IPD). Microlesion formation was concomitant with adverse cardiac electrophysiology thus offering one possible explanation for the occurrence of adverse cardiac events following IPD. Using a non-anesthetised intraperitoneal mouse challenge model we have characterised the formation of cardiac microlesions in a more physiologically relevant method and in greater detail than our first report. We report the entry of individual pneumococci into the heart as early as 12 hours post-challenge. We show that immune cells are recruited to the site of infection as early as 18 hours, but that these cells are no longer present or limited to the periphery of the microlesion after 24 hours. Fluorescent and electron microscopic analysis of hearts from mice infected with a pneumolysin-deficient mutant showed robust immune cell infiltration of both mononuclear and polymorphonuclear cells, indicating pneumolysin plays a key role in the clearance of immune cells during the early stages of microlesion formation. Pneumococci within microlesions were predominantly found to be the transparent phenotype and consisted of a mixture of live and dead bacteria. Immunohistochemical analysis of the microlesions revealed elevated levels of N-acetylglucosamine and the presence of extracellular DNA from the host. All of which suggest the formation of a biofilm within the cardiac microlesion. Cardiomyocte death was shown to be dependent on pneumolysin and hydrogen peroxide, together inciting activation of caspase and necroptosis signalling cell death pathways.

P1.24

Polyamine transport in the pneumococcus is essential for evading early innate immune responses in pneumococcal pneumonia

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Polyamines are ubiquitous small cationic molecules that are important for growth and virulence of human pathogens, including pneumococcus. An isogenic deletion of polyamine transport operon (Δ*potABCD*) in *Streptococcus pneumoniae* TIGR4 led to attenuation in a mouse model of pneumonia. The aim of this study is to identify specific host innate immune mechanisms that contribute to enhanced resistance in mice challenged with polyamine deficient pneumococci. We inoculated C57BL/6 mice (n = 5) intranasally with 1 x 10^7 CFU of wild-type (WT) TIGR4, Δ*potABCD*. Mice were euthanised 4 hours, 12 hours, and 24 hours post-infection (p.i.) and lungs were harvested. Bacterial CFU enumeration, evaluation of immune cell infiltration, measurement of cytokines/chemokines and mass spectrometry based expression proteomics were carried out with lung homogenates. As early as 4 hours p.i., there was a significant difference in the *in vivo* bacterial burden with WT and *ΔpotABCD*. While the WT strain persisted in the lung 24 hours p.i., *ΔpotABCD* was cleared from the animal. Concentration of G-CSF, LIF, IP-10, KC, GM-CSF, IL-5, MCP-1 and MIP-1a were higher 4 hours p.i. in mice infected with *ΔpotABCD.* Consistent with this observation, we found a significant increase in the infiltration of neutrophils in the lung tissue. Modelling of proteomics data using Ingenuity pathways analysis predicted activation of MyD88 and Interferon gamma 4 hours p.i. in response to *ΔpotABCD* and at 12 hours p.i with WT. These predictions indicate a delay in the activation of early innate immune responses involved in bacterial clearance with the WT. In summary, impaired polyamine transport in *S. pneumoniae ΔpotABCD* resulted in its transition from nasopharynx to lung as early as 4 hours p.i., and its subsequent clearance from the lung and blood by 24 hours p.i. Therefore, polyamine transport in the pneumococcus is essential to evade early innate immune responses.

P1.25

IL-22 contributes to recovery from pneumococcal pneumonia by reducing collagen deposition in lung

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Pneumococcal pneumonia can cause dense pulmonary consolidation, which usually resolves within a few weeks of treatment. IL-22 has been implicated in contributing to the resolution of pulmonary inflammation without fibrosis. We explored the role of IL-22 in resolution of treated pneumococcal pneumonia in a murine model. IL22KO and wild-type mice were infected intra-nasally with 3 x 10^6 cfu of serotype 3 *Streptococcus pneumoniae*. Infection was terminated by administration of ceftriaxone once mice showed clinical signs of infection and again the following day. Lungs were examined histologically and using a soluble collagen assay. Untreated mice developed severe pulmonary consolidation with lung abscess formation and development of empyema. There was no significant difference in outcome between IL-22KO mice and wild-type in outcome of untreated infection with similar histopathological changes and bacterial burden. Treated mice resolved clinical illness and regained lost weight. Histological examination revealed improvement in alveolar infiltration in both mouse types but IL-22KO mice had increased areas of sub-pleural collagen deposition and lungs from IL-22KO mice had increased total collagen content. Treatment of pneumococcal pneumonia leads to resolution of consolidation with relatively little pulmonary fibrosis. IL-22 was required for complete resolution and could represent a novel therapeutic target to augment antibiotic treatment in severe pneumonia.

P1.26

Pneumococcal colonisation and invasive disease studied in the porcine model

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Streptococcus pneumoniae is carried in the nasopharynx and spread by human-to-human transmission causing mild diseases such as otitis media and sinusitis as well as severe diseases including pneumonia, meningitis, and sepsis. Furthermore, *S. pneumoniae* is an important cause of septic arthritis, and endocarditis. Asymptomatic colonisation of the nasopharynx is an essential precursor for pneumococcal disease. Piglets are the natural host for *Streptococcus suis* infections. There are striking similarities between *S. suis* pathogenesis in piglets and *S. pneumoniae* pathogenesis in humans. Piglets develop severe disease like meningitis, sepsis, arthritis, endocarditis or pneumonia upon infection with *S. suis. S. suis* is carried in the oropharynx, the bacterium colonises the tonsil of piglets, similar to *S. pneumoniae* in children. Furthermore, genetically *S. suis* and *S. pneumoniae* are closely related. Because pigs are very similar to humans in terms of anatomy, genetics and physiology, we investigated the use of piglets as a model for human colonisation and for the induction of pneumococcal invasive disease. Piglets inoculated intravenously with *S. pneumoniae* showed persistent bacteraemia, frequently followed by arthritis. Moreover, in piglets inoculated intra-nasally the oropharynx was colonised with *S. pneumoniae* for at least 7 consecutive days. This demonstrates that central aspects of human pneumococcal infections also occur in the porcine model, and advocates the use of piglets in future colonisation, transmission, and intervention studies. There is growing evidence that *S. pneumoniae* infections are associated with an increased risk for cardiovascular events and stroke. Since the porcine and human cardiovascular systems are very similar, the porcine pneumococcal model can be used to gain insight into the pathogenesis of this serious health problem.

P1.27

Variome of the pneumococcal surface-exposed proteins and other virulence factors: a bioinformatics analysis

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The surface of the pneumococcus is decorated by a great amount of proteins, which have been associated with its pathogenic functions such as adhesion, colonisation, transmigration and immune evasion. To date, few studies have reported the genetic variability among the different pneumococcal strains from a global perspective. Therefore, there is still a need to fully understand the conservation and distribution of all the virulence factors this human pathogen employs to cause diseases. Here, the construction of the first variome for *Streptococcus pneumoniae* is reported. Twenty-five pneumococcal strains with fully sequenced and annotated genomes were analysed, estimating the distribution (presence/absence) of the pneumococcal virulence factors and identifying all their allelic and protein variants and mutations. The 25 *S. pneumoniae* strains were distributed in 37 phylogenetic groups with a variable number of represented genomes and 9 strains with a unique representation. A total of 61 different genes and proteins were identified, classified and analysed for the construction of the variome. The genes of the pneumococcal virulence factors are distributed in the genome and located in a co-oriented way in relation with the region were the chromosomal replication begins. The analysis of the gene distribution showed that 24 of them belong to the core genome and 37 to the accessory genome. The estimation of the variability for each of the studied virulence factors allowed us to establish that the virulence factors with the highest conservation in the pneumococcus are Ply, Eno and Usp45; the virulence factors with the highest variability are RrgB, PhtD and NanA. Finally, all these results allow us to identify potential pharmaceutical targets for new antimicrobial therapies, and to confirm highly conserved and well-distributed protein candidates for vaccine development, supporting the idea of a new generation of vaccines based on proteins (adhesins) to fight against *S. pneumoniae* and its associated diseases.

P1.28

Global serotypes and genotypes for *Streptococcus pneumoniae*: a whole genome resource for assessing the impact of global pneumococcal immunisation*

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Pneumococcal serotype and genotype prevalence are known to vary geographically. However, there are substantial gaps in data on global diversity, especially from low and middle-income countries. The Global Pneumococcal Sequencing project (GPS) aims to sequence the genomes of 20,000 pneumococcal isolates with sampling priority for invasive disease isolates from infants in developing countries. A major project goal is to describe geographical differences in global pneumococcal genetic diversity in the context of pneumococcal vaccine implementation. At time of writing, over 8,500 pneumococci, representing >60 serotypes, approximately 90% from disease, have been Illumina sequenced. Isolates originated from >20 different countries with a current focus on the African continent, from between 1991 to 2015, both pre and post PCV, collected predominantly during the last decade. Serotype/genotype designations were derived from whole genome data. A Bayesian clustering program (hierBAPS) was used to define population structure, designating sequence types (STs) in the Multi Locus Sequence Typing database to sequence clusters. These cluster designations were then applied to the GPS dataset using their derived ST. The top ten serotypes were all pneumococcal conjugate vaccine

types, except 15B/C. Over 1000 STs were observed; the most prevalent (*n* = 275) was the highly clonal ST217, expressing serotype 1, exclusively from the African continent. Eight of the 13 major lineages defined by hierBAPS, were present in all nine countries for which >100 isolates had been sequenced, whilst the remaining 5 lineages were present in ≥7 of these countries. All 13 lineages exhibited multiple serotypes but ≤3 serotypes accounted for >50% of isolates in each lineage. Although most lineages were widespread, serotype/genotype profiles varied between countries, potentially overstated by any sampling biases. As PCV use expands globally, characterising lineage and serotype distribution is valuable for assessing changes in pneumococcal diversity, which could potentially impact the effectiveness of pneumococcal prevention and control measures.

*On behalf of all Global Pneumococcal Sequencing project partners: www.pneumogen.net/gps

P1.29

Investigating the pneumococcal phageome using a diverse genome dataset

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Bacteriophages are viruses that infect bacteria: many bacterial species are infected by bacteriophages and the bacteriophages often confer benefits to their host by providing genes that encode toxins or other virulence factors. Pneumococcal bacteriophages were first identified in 1975. Bacteriophages may play an important role in pneumococcal biology and evolution; however, prevalence data has been limited and relatively little is known about their effects on the pneumococcal host. We have a large and diverse global dataset of 336 pneumococcal whole genome sequences (WGS) obtained from 31 different countries between 1916 and 2008. Preliminary analysis of this dataset used bioinformatics tools to interrogate the pneumococcal genomes for sequence-based evidence of bacteriophages. With the view to build a pneumococcal phageome reference database of pneumococcal bacteriophage sequences, the dataset was screened for evidence of bacteriophage DNA using an in-house pipeline and a reference database of previously characterised streptococcal bacteriophage genomes. Initial analyses revealed that 86% of the pneumococcal genomes had bacteriophage DNA integrated within them, with 48 different bacteriophage types identified. In many cases the pneumococcal genomes contained DNA from >1 bacteriophage type: 22 genomes contained 2 full length bacteriophages, and 13 genomes contained ≥5 full length and partial bacteriophages. The results of the screen led to the identification of 10 novel bacteriophages. Four of the novel bacteriophages showed considerable sequence similarity to each other, but were unlike the other 6 novel bacteriophages, and were widespread throughout our collection of South African isolates, suggesting that they are a common feature of the South African pneumococcal population. These results suggest that bacteriophage sequences in the pneumococcus are much more prevalent and diverse than previously recognised, and that they are likely to be contributing to pneumococcal genome evolution.

P1.31

Molecular pneumococcal capsular typing using whole genome sequencing: moving the *Streptococcus pneumoniae* reference service into the genomic era

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We describe a novel bioinformatics method for accurate prediction of 88/92 (95.7%) *Streptococcus pneumoniae* serotypes using WGS data. Our project is part of a large modernisation strategy within Public Health England aiming to replace the current phenotypic pneumococcal species confirmation and serotyping reference service with a unified WGS pipeline. Our bioinformatics pipeline integrating several components provides a comprehensive *S. pneumoniae* workflow delivering species identification, MLST typing and capsular typing for each isolate. The “capsular typing” tool

uses mapping and mutation detection to assign capsular type to bacterial genomic sequence data. The software utilises a two-step approach; firstly, reads are mapped to capsular operon sequences for all known capsular types (*n* = 92). This step can predict 36/92 (39.1%) serotypes to type level and the remaining 56/92 (60.9%) to "genogroup" (group of capsular types with high genetic identity; often serogroups) level. If assigned to a genogroup, reads are fed into step 2 and the software uses genogroup-specific algorithms to determine the serotype. Algorithms have been developed for 18/20 genogroups, and the different approaches include presence/absence of CPS genes, detection of early stop codons, frameshift mutations and other inactivating mutations, detection of differing alleles of genes, and SNP differences. Using this two-step approach we can predict 88/92 serotypes to type level and 4 (32A, 32F, 24B, 24F) to serogroup level. From the relative proportions of isolates of each serotype submitted to RVPBRU during 2014, we predict that the serotyping pipeline will be able to type approximately 96% of circulating strains. The remaining 4% will be identified to genogroup level and will require an additional manual serotyping step. To date, we have sequenced 1344 isolates and have observed 90% concordance with the standard serotyping method. Following side-by-side validation of WGS and standard methods, we anticipate implementation into the reference service at the beginning of 2016.

P1.32

Streptococcus pneumoniae shows no overall systematic genetic adaptation over the course of infections leading to meningitis

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In meningitis caused by *Streptococcus pneumoniae*, the bacteria will typically invade the blood stream of the patient causing bacteraemia, then cross the blood–brain barrier into the cerebrospinal fluid (CSF) causing inflammation leading to meningitis. We sequenced separate pneumococcal isolates from both the blood and the CSF of 675 patients with pneumococcal meningitis. Using the resulting 1,350 whole genome sequences we robustly characterised SNP and indel differences between pairs of strains and found no overall genetic difference between bacteria in the blood and CSF of a single patient. In addition, we obtain equivalent results for 267 patients with meningitis caused by 3 other bacterial species. A Bayesian model was also developed for analysis of sequence data for a phase variable inverting locus affecting virulence. Results for observed average allele frequencies across all samples were consistent with existing mouse infection studies, though no difference was seen between blood and CSF isolates. This work conclusively answers the standing question of whether adaptation of *S. pneumoniae* occurs during an infection: no genetic elements are solely responsible for survival in the blood compared to survival in the CSF. This result is recapitulated in 3 other invasive bacterial species.

P1.33

Genomic and virulence comparisons of non-human isolates of *Streptococcus pneumoniae*

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Streptococcus pneumoniae or the pneumococcus has historically been noted as a human pathogen and commensal. However the pneumococcus has also been isolated from non-human hosts from both carriage and disease [1]. The draft genome sequence of an equine pneumococcal isolate A45 has been compared with other German equine isolates. A novel multi-locus sequence type (MLST) ST6934 and putative clonal complex specific to equine isolates of serotype 3 has been identified. A previously described chromosomal deletion spanning 7Kb from pneumolysin to autolysin, and disrupting both genes [2] appears characteristic and equine strains also appear to harbour a lysogenic prophage which is fully inducible in the A45 strain. The representative pneumolysin-autolysin equine deletion has been transferred into a serotype 4 genetic background. Complemented mutants, with WT Ply on plasmid, have also been generated to compare virulence with parent strains in a murine model of pneumococcal infection. A number of genome sequences of pneumococci from other host species including guinea pig, have also been generated. Comparison of gene content

across these different isolates allows discussion of host restriction within the evolution of *S. pneumoniae* as well as interesting insights into the mechanisms of pathogenesis employed by the pneumococcus.

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P1.34

Analysis into the recombination of the phase variable Type I restriction modification system SpnD39III locus

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We have identified a novel phase variable epigenetic control mechanism in *Streptococcus pneumoniae* with an impact on gene expression and important phenotypes such as virulence in experimental models of infection. This genetic system is based on the rapid inversion of the specificity determinants of the type I restriction modification (RM) system SpnD39III which results in a change in methylation of the bacterial genome. Similarly organised RM loci are present in multiple other species. The objective of this work was to investigate the contribution of the CreX recombinase encoded by this locus to the phase variable rearrangement of epigenetic control. Recombination within the SpnD39III locus reverses the orientation of a *creX* gene encoded in the locus. Deletion of the *creX* gene resulted in an almost complete block of recombination on the sort 15 bp inverted repeat but no significant changes in recombination on the two larger 85 and 333 bp inverted repeats. RNAseq data show that the *creX* gene is expressed two-fold more when co-directional to the hsdRMS genes. Somewhat surprisingly, the strains with increased *creX* expression recombined significantly less than strains with lower *creX* expression. Our data indicate a direct but not exclusive involvement of *creX* in the recombination of the hsdS genes of the SpnD39III locus responsible for the epigenetic control of pneumococcal virulence.

P1.35

Site matters: alternative insertion sites of integrative conjugative element Tn*5253* can influence conjugation frequency in *Streptococcus pneumoniae*

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The integrative and conjugative element (ICE) Tn*5253* of *Streptococcus pneumoniae* confers chloramphenicol and tetracycline resistance, and can be transferred by conjugation. Tn*5253* is found integrated at an 83-bp specific target site (*att*B) located between *spr1042* (*iga*) and *spr1043* (*rbgA*) of the R6 chromosome. A recombinant pneumococcal conjugation recipient was produced using a mutagenic construct containing a Kanamycin resistance cassette joined to an engineered *att*B where the first 63 nucleotides (nts) were deleted and 5 nucleotide changes introduced in the remaining 20 nts. Conjugation frequency of Tn*5253* in the *att*B mutant recipient was considerably lower when compared to a standard recipient (4.8×10^{-7} versus 1.7×10^{-5} transconjugants/donor cells). PCR analysis of transconjugants revealed that: (i) 40% of transconjugants had Tn*5253* integrated into the mutated *att*B; (ii) 45% had Tn*5253* integrated into the original *att*B with loss of the mutagenic construct; and (iii) 15% had Tn*5253* integrated elsewhere in the chromosome. Inverse PCR and sequencing disclosed 5 Tn*5253* alternative insertion sites: *spr1713*, coding for an alpha-galactosidase, *spr0540* coding for a cell wall synthesis enzyme, *spr1534* coding for the substrate binding protein of an ABC sugar transporter, *spr1983* coding for a MFS protein, and *spr0546* (*nrd*) coding for a putative nitroreductase. Transfer of Tn*5253* from transconjugants harbouring Tn*5253* in alternative sites occurred at lower frequencies than the wild-type donor (from 2×10^{-7} to $< 3.6 \times 10^{-8}$). One transconjugant harbouring 3 copies of Tn*5253* was able to transfer the element at a frequency 100-fold higher than the wild-type donor. Our results indicate that Tn*5253* has a strong preference for its primary insertion site, even when it is mutated, but that it can also integrate at different sites. The insertion site of

Tn*5253* affects the transposition rate, which is decreased or abolished when it is integrated in an alternative *att*B.

P1.36

Mutagenesis of one *Streptococcus pneumoniae* serotype 1 strain

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Streptococcus pneumoniae causes pneumonia, bacteraemia, meningitis and otitis media. Despite vaccination, there are still around 1 million deaths each year worldwide. Serotype 1 is amongst the most invasive of serotypes and is extremely important in Africa. It is also a significant cause of meningitis, is unusual in that it is rarely found colonising the nasopharynx, and is frequently associated with outbreaks. Although included in the pneumococcal conjugate vaccine, PCV13, the efficacy against serotype 1 has been questioned. Therefore there is a continuous need to study and understand this particular serotype. In this study, a new method was developed to mutagenise a serotype 1 strain. The method relies on: the known natural competence of *S. pneumoniae*; double recombination; and a delivery plasmid. The delivery plasmid contains a PCR fragment where a portion of the gene of interest has been deleted and substituted by a suitable antibiotic cassette. This is followed by natural transformation. In order to test the method, 3 genes known to be possible to mutate and 1 putative gene were targeted and the mutations achieved. The loss of function mutants will be firstly studied in the insect larvae model *Galleria mellonella,* for decreased virulence, followed by virulence studies in mice. The ability to mutagenise this strain will help pave the way for a better and more extensive understanding of such an important serotype, allowing for improved therapies and better vaccines.

P1.37

Improving molecular detection, identification and serotyping of *Streptococcus pneumoniae* in complex samples

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Molecular methods (*lytA* qPCR and serotyping by microarray) were used to examine pneumococcal carriage in adult caregivers using nasopharyngeal and oropharyngeal (OP) samples collected as part of a PCV10 impact study in Fiji. Results for the OP samples were more difficult to interpret, as microarray analysis indicated many samples contained partial or divergent pneumococcal capsule homologues but also lacked pneumococci, including several *lytA* positive samples. False positives in OP samples due to the presence of capsule and *lytA* gene homologues in non-pneumococcal species have been reported by other groups. Culture of OP samples on gentamicin horse blood agar (gHBA) also revealed a complex mix of bacteria present. Here we dissect the complexity of a subset of these OP samples to better understand the component species present and to improve the sample screening approach by investigating alternative pneumococcal-specific qPCR targets. A representative of each colony morphology on gHBA was subcultured from 11 samples resulting in 92 isolates. Each isolate was tested by standard culture-based pneumococcal identification tests and MALDI-TOF/MS for species identification. A subset were analysed by microarray and serotyped by latex agglutination. Preliminary results have found 3 serotypeable pneumococcal isolates, and 9 non-pneumococcal isolates with latex agglutination serotyping results, most commonly 19B (*n* = 6). MALDI-TOF/MS (*n* = 13) identified *Streptococcus mitis*/*Streptococcus oralis* (*n* = 7), *Streptococcus salivarius* (*n* = 2), *Streptococcus parasanguinis* (*n* = 2) and *Streptococcus anginosus* (*n* = 1), with 1 unidentified isolate. The MALDI-TOF/MS results were concordant with microarray analysis, which also found non-pneumococcal isolates containing pneumococcal capsule gene homologues. Some non-pneumococcal isolates were weakly positive for *lytA* and likely contributing to the weak positivity of some OP samples. Our results thus far highlight the complexity of OP samples and the potential to misidentify and incorrectly serotype pneumococci using existing conventional and molecular methods. Further work examining the performance of alternative pneumococcal identification qPCR screening targets is underway.

P1.38

Genome-wide assessment of pneumococcal antigenic diversity

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Extensive variation in both polysaccharide and protein antigens is evident across pneumococcal populations. The variation in these surface structures can be quantified through whole genome sequencing of systematic samples of pneumococci isolated from carriage. Data from 616 isolates collected in Massachusetts between 2001 and 2007 allowed characterisation of 20 serotype switching events. These revealed a highly significant ($p < 0.0001$) enrichment for within-serogroup switching, based on the observed serotype distribution. However, this pattern could not be fully explained by more frequent, shorter recombinations preferentially driving the smaller changes required to switch between serotypes of the same serogroup. Although a separate dataset indicated vaccine escape sometimes associated with changes in β–lactam resistance, the pattern of switching in this collection was not detectably affected by linkage between *pbp* genes and the capsule polysaccharide synthesis (*cps*) locus. The absence of an explanation based on the properties of transformation suggested the observed pattern was a consequence of selection for preserving serogroup. Hence multiple exchanges of serotypes were experimentally constructed in common genetic backgrounds to test for epistatic interactions between the *cps* locus and other pneumococcal genes. However, these data were not consistent with epistatic interactions between *cps* loci of a particular serogroup and the rest of the genome, meaning they were unlikely to account for the observed distribution of capsule types. These data suggested future work should focus on alternative mechanisms, such as host immunity spanning multiple serotypes within the same serogroups, which might explain this significant trend. Characterisation of proteinaceous antigens found them to be highly diverse across the population. However, individual alleles were stably associated with particular lineages, with only *pspA* and *pspC* undergoing diversification over short timescales. Together, these data show how individual lineages are likely to be restricted in how they diversify under host immune selection pressure.

P1.39

An increase in negative supercoiling in *Streptococcus pneumoniae* induces a global transcriptomic response that regulates the DNA topoisomerase I gene

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The most basic level of transcription regulation in *Streptococcus pneumoniae* results from the organisation of its chromosome into topological domains. We have previously observed a global transcriptional response to DNA-relaxation. In this study, to increase DNA supercoiling, we used seconeolitsine (SCN), an inhibitor of topoisomerase I. Supercoiling density (σ) varied as a function of time as a result of treatment with SCN at 0.5 × MIC: 48%, 74%, and 46% at 5 min, 15 min and 30 min of treatment, respectively. A global transcriptomic response was observed. The number of responsive genes decreased from a high 395 at 5 min to 285 at 15 min, to 150 at 30 min (a drop of more than half). More than one-third of the responsive genes at 15 min were found to be contiguous in the chromosome, forming clusters that showed coordinated regulation. At least 10 clusters were discerned. Clusters were evident at 5 and 15 min. The only DNA topoisomerase gene significantly affected was *topA*, which was 3-fold down-regulated at 15 min. After 30 min, *topA* transcripts were restored to baseline levels, coincident with partial recovery of the supercoiling and reduction of the transcriptomic response. For the first time here, we have identified in bacteria, the existence of a global transcriptomic response triggered by an increase in DNA supercoiling. This response is similar to the one observed during DNA relaxation. This indicates that they are handled globally as a common type of topological stress.

P1.41

Systematic nomenclature for the bacterial two-component regulatory systems, based on genomic, structural and functional analysis of the pan-genome of *Streptococcus pneumoniae*

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Two-component systems (TCSs) represent the single largest paralogous gene family encoding signalling proteins in Archaea and Eukarya. However, despite the importance of these systems for the regulation of different cellular and physiological processes, a clear and uniform nomenclature system allowing for their systematic study does not exist. Here, a thorough bioinformatics search and analysis was performed to identify conserved and distinctive features at the genomic, structural and functional level, with the aim of devising a systematic and expandable model of nomenclature for the operons, genes and proteins comprising the TCSs of the pan-genome of *Streptococcus pneumoniae*. The DNA and protein sequences of 25 pneumococcal strains, whose genomes are completely sequenced and annotated, were analysed employing different bioinformatics tools and databases. Twenty-nine different TCS-proteins (14-HKs, 15-RRs) were identified, indicating a total pan-genomic complement of 15-TCSs, to which this species has access at a population level. However, the modal complement was 13-TCSs and One-Orphan-RR. The TCS-variome estimation (genetic and protein variability) confirmed the high-level of conservation among these pneumococcal regulators. Additionally, through the structural/functional analysis of the TCS-proteins, it was possible to establish the presence of conserved and distinctive features in terms of presence, absence, number, type, organisation and localisation of the different functional domains. Moreover, the common names and diversity of functions under the control of the pneumococcal TCSs was defined by a systematic research of the specialised literature. The identification of both conserved and distinctive features at the genomic, structural and functional level allowed for the establishment of a systematic and uniform model of nomenclature for the operons and proteins comprising the TCSs of the pneumococcal pan-genome. This model has been already extrapolated to other bacterial species and it has the potential to serve as a reference standard to improve research and understanding of these regulatory systems in prokaryotes.

P1.42

Optimisation of the expression of fluorescent proteins in *Streptococcus pneumoniae*

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Streptococcus pneumoniae is a Gram-positive bacterium usually found in association with a range of different types of infections. The understanding of how these bacteria divide or perform specific tasks important for their survival is a requirement for the design of efficient strategies to fight bacterial infections. This implies a detailed knowledge not only of the function of proteins required for the infection process, but also of their localisation and role in complex molecular machineries. In order to determine the correct subcellular localisation of fluorescent proteins in *S. pneumoniae*, we have previously described tools to express derivatives of 4 fluorescent proteins—mCherry, Citrine, CFP and GFP—to levels that allow visualisation by fluorescence microscopy, by fusing the first 10 amino acids of the *S. pneumoniae* protein Wze (the i-tag), upstream of the fluorescent protein. We had proposed that this i-tag extension might facilitate ribosome accessibility to the ribosome-binding site, thus enhancing protein translation. These tools, which we have now confirmed that can also be used in other Gram-positive bacteria, namely *Lactococcus lactis*, *Staphylococcus aureus*, and *Bacillus subtilis*, have been optimised by changing the nucleotide sequence of the i-tag and testing the effect of the first 10 amino acids of other pneumococcal proteins in the increased expression of the fluorescent protein Citrine. We found that manipulating the structure and stability of the 5' end of the mRNA molecule, which may influence the accessibility of the ribosome, is determinant to ensure the expression of a strong fluorescent signal. We therefore propose that the

determination of minimum free energy calculated for the 5' end of the mRNAs molecules originated by these tools may predict whether the expression of a specific fluorescent protein will be successful and may be used to set up a regulated constitutive protein expression.

P1.43

The single transmembrane segment of pneumococcal WalK is required for the perception of an intramembrane or extracellular signal

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The *Streptococcus pneumoniae* genome encodes 13 two-component regulatory systems plus an orphan response regulator. Only one of these systems, WalRK, is essential for viability under laboratory conditions. Despite its importance, the biological role of the WalRK system is not well understood. However, previous studies have shown that it regulates expression of the cross wall splitting enzyme PcsB, and consequently has a crucial role in pneumococcal cell division. Considerable efforts have been made to understand how the system is regulated, but no signal(s) sensed by the WalK histidine kinase has been identified so far. WalK orthologs in most low-GC Gram-positive bacteria are attached to the cytoplasmic membrane via two transmembrane segments separated by a large extracellular loop believed to function as a sensor domain. In contrast, members of the genus *Streptococcus* have WalK histidine kinases that are anchored to the cytoplasmic membrane by a single transmembrane segment. It has been a long-standing question whether this transmembrane segment still serves as a signal-sensing domain, or if it only functions as a membrane anchor. Here, we present data that strongly suggest that the transmembrane segment senses or relays an extracellular or intramembrane signal that regulates the activity of WalK. Moreover, in contrast to what was previously believed, we provide evidence suggesting that the serine/threonine protein kinase StkP upregulates PcsB expression by stimulating the kinase activity or inhibiting the phosphatase activity of WalK rather than by direct phosphorylation of the WalR response regulator.

P2.01

Regulatory responses of *Streptococcus pneumoniae* D39 to ascorbic acid

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We have explored the impact of ascorbic acid on the transcriptome of *Streptococcus pneumoniae* D39. The expression of several genes and operons, including the ula (utilisation of L-ascorbic acid) operon, the AdcR regulon (which has been previously shown to be involved in zinc transport and virulence) and a PTS operon (which we denote here as the ula2 operon) were altered in the presence of ascorbic acid. We have explored the regulatory mechanism of the ula and ula2 operons. Our β-galactosidase assay and microarray data demonstrate that transcriptional regulators UlaR and UlaR2 act as transcriptional activators of the ula and ula2 operons, respectively, in the presence of ascorbic acid. We predict putative regulatory sites for UlaR and UlaR2 binding in PulaA and Pula2, respectively. Furthermore, we have explored the effect of ascorbic acid on the expression of the AdcR regulon in more detail. Our ICP-MS analysis showed that addition of ascorbic acid to the medium causes zinc starvation in the cell that leads to the activation of the AdcR regulon.

P2.02

Altered penicillin-binding protein 2x of *Streptococcus pneumoniae*: a new target of the serine protease HtrA

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The two cefotaxime resistant laboratory *Streptococcus pneumoniae* mutants C405 and C606 derived independently from the laboratory strain R6 [1] contain less essential penicillin-binding protein 2x compared to the parental strain [2]. In transpeptidase domain of PBP2x each of these mutants have 2 or 4 mutations, respectively. In these mutants no effect on the transcription of *pbp2x* gene was detected, and thus the reduced amount of PBP2x was likely to be due to degradation of the protein. Furthermore, both mutants carry mutation in *ciaH*, which encodes the histidine protein kinase of the two-component system CiaRH [3], resulting in hyperactivation of CiaR [4]. One gene product of the CiaRH regulon is the serine protease/chaperon HtrA [5], suggesting that HtrA is responsible for the reduced amount of PBP2x. Analysis of HtrA deletion or active site inactivation of HtrA in C405 and C606 showed that the proteolytic activity of HtrA is responsible for degradation of PBP2x in both mutants. Integration of an ectopic copy of *htrA* in the HtrA deletion mutants restored the phenotype. Introduction of *pbp2x* alleles into the sensitive R6 strain, in which the CiaRH system is not as active as in the mutants, leads to a slight reduction of PBP2x amount, but not as pronounced as in the mutants. This suggests the contribution of other factors to this phenotype. Depletion studies of the $PBP2x_{C405}$ variant in different genetic backgrounds and in presence or absence of HtrA confirmed that HtrA degrades PBP2x in C405. A GFP-$PBP2x_{C405}$ fusion protein still localises at the septum in the absence of HtrA; in the presence of HtrA high cytoplasmatic signals are in agreement with the presence of GFP-$PBP2x_{C405}$ degradation products.

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P2.03

Hydroxyl radicals contribute to the bactericidal effects of the fluoroquinolone moxifloxacin in *Streptococcus pneumoniae*

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We studied the transcriptomic response of *Streptococcus pneumoniae* to moxifloxacin, an inhibitor of DNA gyrase. At 10 × MIC, 30 and 140 responsive genes were detected at 15 and 30 minutes, respectively. This response included the up-regulation of 2 genes converting 6P-sugars to fructose 6P, together with 3 out of 7 genes of the glycolysis pathway, which converts fructose-6P to pyruvate. In addition, an increase in acetyl-coA would be expected from the down-regulation of the acetyl-coA carboxylase. Acetyl-coA would be converted to pyruvate by the action of formate acetyltransferase, which gene was also up-regulated. Since pyruvate is converted to hydrogen peroxide (H_2O_2) by pyruvate oxidase (SpxB),

pyruvate increase would lead to an increase in the intracellular H_2O_2, and in turn, in the amounts of reactive oxygen species (ROS) resulting from the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^\cdot$). ROS damage DNA, lipids and proteins. With moxifloxacin, we observed similar increases in the production of H_2O_2 and ROS, which contributed to the lethality of the drug. In accordance, the lethality of moxifloxacin was attenuated in a strain lacking the *spxB* gene. These results support the model of a universal mechanism of action for bactericidal antibiotics, including fluoroquinolones. These observations are also in agreement with our previous findings that levofloxacin, an inhibitor of topoisomerase IV, triggers the transcriptional activation of iron transport genes. Both drugs stimulate the Fenton reaction in their mechanism of action, by causing an increase in the concentration of either iron or H_2O_2, respectively.

P2.04

Regulation of PavB by TCS08 in *Streptococcus pneumoniae*

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The human pathogen *Streptococcus pneumoniae* (pneumococci) possess 13 two-component regulatory systems (TCSs), which are crucial for bacterial fitness and virulence. Traditionally, these systems consist of a sensor histidine kinase (HK) and an output, the response regulator (RR). For TCS08, its encoding genes are placed downstream of the coding sequence of the adhesin PavB (pneumoccal adherence and virulence factor b), which has been associated with virulence in the pneumococci [1]. Hence, the interaction of the TCS08 proteins and its effect on the regulation of PavB and other pneumococcal surface proteins was evaluated in this work. This study aimed to assess the interaction and regulation of TCS08 proteins and PavB. Maltose binding protein and affinity chromatography were used for TCS08 and PavB proteins purification. Phosphotransfer profiling and EMSA were established to assess the interaction between the recombinant HK08 and RR08, and the RR08 and the promoter region of *pavB*, respectively. The purity of recombinant MBP-HK08 and MBP-RR08 were determined by SDS-PAGE and immunoblot analysis. The HK08 and its cognate RR08 displayed a phosphorylation interaction, suggesting a phosphatase activity and autophosphorylation, respectively. The interaction between the *pavB* promoter fragment and the non-phosphorylated RR08 showed a shift in the electrophoretic mobility of *pavB*. The expression of PavB increased dramatically for the hk08-mutant but no significant differences were found for the rr08- or tcs08-mutants when compared with the wild-type, suggesting a repressor function of the non-phosphorylated RR08 for the expression of PavB. The results revealed no direct relation of the TCS08 with virulence and pathogenicity, but it is involved in the expression of PavB in *S. pneumoniae.*

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P2.05

TpxD serves as a global regulator for pneumococcal response to H_2O_2, and is regulated by CodY

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Streptococcus pneumoniae is a facultative anaerobic pathogen. Although it maintains fermentative metabolism, during aerobic growth pneumococci produce high levels of H_2O_2, which can have adverse effects on cell viability and DNA, and influence pneumococcal interaction with its host. The pneumococcus is unusual in its dealing with toxic reactive oxygen

species, in that it neither has catalase nor the global regulators of peroxide stress resistance such as OxyR and PerR. In this study we investigated TpxD's function as a sensor and global regulator in pneumococcal response to H_2O_2. Microarray analysis revealed that TpxD mediates pneumococcal response to H_2O_2 at the transcriptional level, as mutation of *tpxD* abolished H_2O_2 mediated gene expression. The mechanism underlying TpxD regulatory function was further elucidated by replacing the catalytic cysteine[58] with alanine. This mutation prevented *tpxD* up-regulation upon addition of H_2O_2, and eliminated pneumococcal response to H_2O_2, signifying that cysteine[58] is crucial for TpxD signalling activity. We then aimed to identify the transcription factor governing *tpxD* expression under H_2O_2 stress. Bioinformatic analysis discovered a putative 15-bp consensus CodY binding site in the upstream region of *tpxD*-coding sequence. The functionality of this CodY-box was confirmed by mutations and EMSA analysis. Moreover, no up-regulation of TpxD could be detected in DcodY challenged with H_2O_2. These data identify CodY as the transcription factor modulating *tpxD* expression under H_2O_2 stress. We propose a model in which TpxD functions as a mediator, transferring H_2O_2 signal to down-stream effectors, thereby controlling other targets involved in oxidative stress response.

P2.06

Co2+-dependent transcriptional regulation in *Streptococcus pneumoniae*: opposite effect of Mn2+ and Co2+ on the expression of the virulence genes psaBCA, pcpA and prtA

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Manganese (Mn2+)-, zinc (Zn2+)- and copper (Cu2+)-dependent gene regulation in *Streptococcus pneumoniae* have already been studied extensively. However, the effect of the important transition metal ion cobalt (Co2+) on gene expression of *S. pneumoniae* has not yet been explored. Here, we study the impact of Co2+-stress on the transcriptome of *S. pneumoniae* strain D39. BLAST searches revealed that the genome of *S. pneumoniae* encodes a putative Co2+-transport operon (cbi operon), which we show here to be upregulated under Co2+-stress. By means of transcriptional lacZ-reporter studies, we confirm that the expression of the cbi operon increases with increasing concentrations of Co2+. Furthermore, we show that Co2+, as has been shown previously for Zn2+, can induce derepression of the genes of the PsaR regulon, encoding the Mn2+-uptake system (PsaBCA), the choline-binding protein (PcpA) and the cell-wall associated serine protease (PrtA). The data also corroborate a role for CzcD as a Co2+-efflux pump.

P2.07

Differential complement sensitivity of *Streptococcus pneumoniae* and *Streptococcus mitis*

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Streptococcus pneumoniae and *Streptococcus mitis* are naso-oropharyngeal commensals that are genetically similar. However, *S. pneumoniae* is highly pathogenic and a common cause of pneumonia and septicaemia, whereas *S. mitis* rarely causes disease. We hypothesise that differences in sensitivity to innate immunity may underlie these differences in virulence phenotype. We compared sensitivity of *S. pneumoniae* and *S. mitis* to neutrophil killing. After opsonisation with serum, but not with heat-treated serum or PBS, *S. mitis* was markedly more sensitive to neutrophil killing compared to *S. pneumoniae*. These differences suggested *S. mitis* was relatively complement sensitive, and flow cytometry assays of C3b/iC3b deposition confirmed there was increased complement opsonisation of *S. mitis* compared to *S. pneumoniae*. *S. pneumoniae* resistance to complement is partially dependent on binding of the immune regulator factor H by the surface protein PspC. We investigated factor H binding to *S. mitis* using flow cytometry. The results demonstrated that there was no significant factor H binding to *S. mitis*. By inserting *pspC* of *S. pneumoniae* into *S. mitis,* we demonstrated that expression of PspC enabled *S. mitis* to then bind factor H. Investigation of C3b/iC3b confirmed a decrease in opsonisation. Furthermore, survival in whole human blood of this modified strain showed an increase, when compared to the wild-type strain. These results suggest that an inability to bind factor H might underpin *S. mitis* sensitivity to opsonisation with complement and neutrophil killing compared to *S. pneumoniae*, and therefore contribute to the differences in virulence between these 2 commensal species.

P2.08

Using dual RNA-Seq to characterise gene expression in an animal model of *Streptococcus pneumoniae*

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RNA-Seq is a useful tool for characterising RNA transcription. The ability to generate large numbers of reads allows accurate quantification of the transcriptome of both bacterial pathogens and the host response. We characterised the transcriptome of the serotype 3 strain SRL1 during growth in broth and *in vivo* during a mouse model of infection. RNA was extracted using a bead beating method from broth cultures as well as bronchoalveolar and pleural lavage. Sequencing was conducted using the Illumina Hi-Seq platform. Samples from 3 mice were combined for the final analysis. Ninety-eight million reads from broth culture were successfully mapped to the reference genome while 6.8 million and 4.2 million from BALF and pleura respectively were mapped. Infection samples clustered separately from broth samples on principle component analysis. Three genomic regions were identified as being up-regulated during infection relative to broth: an operon coding for genes involved in purine metabolism and the VanZ glycopeptide resistance gene, the Blp bacteriocin locus, and the PsaA locus. All 3 genomic regions were identified as being upregulated in BALF and pleura. Analysis of the host response showed a significant up-regulation of type I interferon-regulated genes in the pleural space compared to the lung. RNA-Seq is a useful tool to characterise the transcriptome during infection *in vivo*. Consistent gene regulation changes were identified in both infection sites with upregulation of operons associated with metabolism, intra-species competition and virulence. Further work will characterise the role of these genes in infection.

P2.09

Emergence of amoxicillin-resistant variants of Spain^{9V}-ST156 pneumococci expressing serotype 11A correlates with their ability to evade the host immune response

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Capsular switching in *Streptococcus pneumoniae* allows pre-existing clones expressing vaccine serotypes to escape vaccine-induced immunity by acquisition of capsular genes from pneumococci of a non-vaccine serotype. The emergence of penicillin-resistant serotype 11A pneumococci has been identified at the Spanish Pneumococcal Reference Laboratory, resulting in a significant concern as this serotype is not included in the current conjugate vaccines. For this reason, we have analysed the clonal composition of 492 clinical isolates of serotype 11A causing invasive disease in Spain (2000–2012), and their ability to evade the host immune response. Antibiograms, molecular typing and restriction profiles of *pbp2x*, *pbp1a* and *pbp2b* genes were analysed. Interaction with complement components C1q, C3b, C4BP, and factor H was explored. Moreover, opsonophagocytosis assays were performed using HL-60 cells differentiated to neutrophils. Biofilm formation and polymorphisms of the major autolysin LytA were also evaluated. The main genotypes of the 11A pneumococci throughout the period 2000–2012 were ST62 (>65%), followed by ST6521, and ST838. Genotypes ST838 and ST6521 emerged from 2005 displaying penicillin and amoxicillin resistance by harbouring a different *pbp2b* gene. Both genotypes were single (ST838) or double (ST6521) locus variants of the Spain^{9V}-ST156 clone. A different pattern of evasion of complement immunity and phagocytosis was observed between genotypes. Emergence of ST6521^{11A}, a vaccine escape variant of the *S. pneumoniae* Spain^{9V}-ST156 showing a high potential to avoid the host immune response, was observed. Our findings anticipate the possible spread of ST6521^{11A} clone among pneumococcal isolates causing invasive disease.

P2.10

Streptococci of Mitis group, misidentified as *Streptococcus pneumoniae*

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We aimed to perform identification and molecular analysis group of isolates initially identified as *Streptococcus pneumoniae*. Sixty-six *Streptococcus* spp. isolates from upper and lower respiratory tract (mostly from sputum) collected during 1995–2013, were examined for colony morphology on sheep blood agar, optochin sensitivity, bile salts solubility, in mass spectrometry identification systems (MALDI-TOF), by biochemical test API 20 Strep, Pneumotest-Latex, as well as molecular methods such as detection of pneumolysin gene (*ply*), partial sequencing of *lytA* and multi-locus sequence analysis (MLSA). Colony morphology and haemolysis of all isolates resembled one of *S. pneumoniae* strains. Ninety per cent of isolates were optochin sensitive and 70% of isolates were soluble in bile salts. MALDI-TOF identified collected isolates mostly as *S. pneumoniae*. Agglutination was observed with sera from Pneumotest-latex for some isolates. Weak enzymatic activity and fermentation of sugar exhibited by tested isolates prevented unequivocal species identification by API 20 Strep. The *ply* gene was frequent in the analysed group (80%). All studied isolates harboured the 6-bp deletion in the 3' part of the *lytA* gene that excluded their identification as *S. pneumoniae*. MLSA classified isolates as *S. mitis, S. pseudopneumoniae* and single representatives of *S. sanguinis* and *S. oralis*. Species identification of certain strains of alpha-haemolytic streptococci, especially those isolated from respiratory tract, may be problematic and unreliable when limited to the classical microbiological methods.

P2.11

Clonal relationship with the invasive disease potential of pneumococcal serotypes

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Estimation of the invasive disease potential (IDP) of pneumococcal serotypes is an important tool to identify the serotypes with high risk to cause disease. Nowadays, the invasiveness index calculation is labour intensive because it needs to compare the serotype distribution in a wide carrier collection versus the distribution found in patients with invasive pneumococcal disease (IPD). The aim of this study is to determine the IDP of *Streptococcus pneumoniae* serotypes causing IPD in Catalonia without using a carrier collection. We calculate the IDP using the index of clonal diversity of each invasive serotype. All pneumococcal invasive isolates (adults and children) received between 2010 and 2013 from 26 health centres in Catalonia and characterised by the Molecular Microbiology Department at Hospital Sant Joan de Déu, Barcelona, Spain, were included. Capsular typing and clonal analysis were performed by fluorescent Multiplex PCR and MLST, respectively. Clonal diversity was estimated by Simpson's numerical index of discrimination. We received 1,576 pneumococcal invasive isolates. Serotype and clonal analysis were available in 1,551 strains (98.4%). Fifty-two serotypes and 249 clonal types were identified. We classified the invasiveness of the serotypes that were detected in at least 10 isolates. The serotypes with a clonal diversity under the percentile 50 were 10A, 23B, 7F, 12F, 22F, 1, 31,38, 5, 11A, 14, 23A, 8, 3, and 15A. The serotypes with a clonal diversity higher than the percentile 50 were 24F, 18C, 9V, 33F, 23F, 9N, 6C, 6B, 16F, 4, 19A, 15C, 6A, 15B, and 19F. Our results confirmed that serotypes highly associated with invasive disease such as serotype 1 or 7F presented some of the lowest levels of diversity, while serotypes such as 19F and 6C that are associated with carriage showed the highest levels of diversity, suggesting an alternative methodology based only in the invasive strains collection using molecular methods.

P2.12

Pneumococcal carriage in infants and young children in Fiji before and after PCV10 introduction

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Fiji introduced the 10-valent pneumococcal conjugate vaccine (PCV10) in 2012 with no catch up. Annual cross-sectional nasopharyngeal carriage surveys are being conducted to evaluate direct and indirect effects of PCV10. Nasopharyngeal swabs were collected from healthy children aged 12–23 months and infants aged 5–8 weeks before, and for two years after, PCV10 introduction (n ≈ 500 per cohort per year). Swabs were stored in STGG and frozen at -80°C until analysis. Pneumococci were detected by *lytA* qPCR and molecular serotyping performed by microarray. All data are preliminary. For 12- to 23- month-old children, the overall pneumococcal carriage rate was 50% in 2012 (pre-PCV10) compared to 48% in 2013 and 32% in 2014. Of pneumococci identified, 38% were PCV10 serotypes in 2012 compared to 32% in 2013. Post-PCV10, carriage of non-typeable pneumococci has increased, and a higher proportion of pneumococcal-positive samples contain multiple serotypes. For 5- to 8-week-old infants, the overall pneumococcal carriage rate was 34% in 2012 (pre-PCV10) compared to 23% in 2013 and 13% in 2014. Pre-PCV10, 30% of pneumococci identified were PCV10 serotypes. Serotyping is underway for post-PCV10 carriage samples. Overall, pneumococcal carriage rates are decreasing in both age groups following PCV10 introduction. One year post-PCV10, we observed a non-significant decline in PCV10 serotypes, and an increase in multiple serotype and non-typeable pneumococcal carriage in young children. Changes in pneumococcal carriage will be more pronounced in subsequent years, and annual carriage surveys are ongoing.

P2.13

First description of *Streptococcus pneumoniae* serotype 6E in Spain

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The detection of *Streptococcus pneumoniae* serotype 6E isolates is relatively recent and it might be thought it is a result of recent evolution, perhaps obtained by the introduction of the pneumococcal conjugated vaccines (PCV). The presence of serotype 6E isolates before and after PCV was studied in a sample of 169 randomly selected ones (26 from invasive disease) among the 736 serogroup 6 isolates collected at Donostia University Hospital, northern Spain, between 1981 and 2012 and in 5 serogroup 6B reference strains. Serotype 6E investigation was performed by detecting *orf* 1 to 4 specific of serotype 6E. Antimicrobial susceptibility testing was performed by broth microdilution. Overall, 48 serotype 6E (8 invasive) of 165 serogroup 6 isolates were identified: 39/71 (55%) before 2002 and 9/98 (9%) after. Of them, 6 had been previously classified as serotype 6A and 42 as serotype 6B. None of the 29 serotype 6C isolates studied was 6E. Thirty-eight were penicillin non-susceptible (MIC ≥ 0.12 µg/mL), 19 erythromycin-resistant and 4 ciprofloxacin-resistant (MIC ≥ 4 µg/mL). Surprisingly, reference strain of the 6B-2 multidrug-resistant clones *Streptococcus pneumoniae* ATCC 700670 also showed *orf* 1 to 4 described for serotype 6E. Serotype 6E has been circulating since at least 1981, being unlikely that the vaccines have influenced the emergence of its strains. Conversely, PVCs seems to have a protective effect. The *S. pneumoniae* ATCC 700670 should be renamed. Serotype 6E were mainly found among previously identified serotype 6B isolates and frequently showed antimicrobial resistance.

P2.14

Utility of a reverse-hybridisation strip assay for *Streptococcus pneumoniae* serotyping

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Surveillance of pneumococcal serotypes causing disease is essential to evaluate pneumococcal vaccination programs. The Microbiology Department of Donostia University Hospital (San Sebastián, Spain) and the biotechnology company Operon (Zaragoza, Spain) are developing a reverse-hybridisation strip assay to easily determine pneumococcal serotypes. The first prototype developed includes the 13 serotypes of the PCV13 and another 4 related serotypes (6C, 6D and 18A, 18F). This first prototype was initially validated against reference isolates of the 94 pneumococcal serotypes described up to date showing 100% specificity. Besides, it was tested with 66 clinical isolates from the Microbiology Department collection previously serotyped with the Quellung reaction: 59 strains belonging to the 17 serotypes included in the strip and another 7 strains of serotypes not included. All the 66 strains were correctly characterised with the reverse-hybridisation strip assay. However, because of similarity of capsular sequences, serotypes 7A/7F, 9A/9V and 18B/18C could not be differentiated. The reverse-hybridisation strip assay proved to be a good tool for pneumococcal serotyping. An advantage of this technique as compared with the Quellung is the possibility of working in batches of samples. Other advantages are that no experienced staff is required to obtain and interpret results, and that records of the results (hybridised strips) can be stored. With this technique, some related serotypes could not be discriminated because of sequences similarity.

P2.15

Nasopharyngeal colonisation of *Streptococcus pneumoniae* in Colombian children under five years of age, Medellin 2014

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Human colonisation by *Streptococcus pneumoniae* is the main requirement for pneumococcal diseases. In Medellín, Colombia, no studies have documented the behaviour of the pneumococcus and the public health problems it can cause in childhood. Here, the prevalence of nasopharyngeal colonisation by *S. pneumoniae* and its associated factors in children <5 years of age, from the Programa Buen Comienzo (PBC) (Medellín 2014) were investigated. A cross-sectional study was conducted, considering two groups of age (12–24 and 25–60 months). PBC institutions were selected to represent the 16 territorial communes of the city. An informed consent was previously signed for the parents and 629 children <5 years of age constituted the study population. Characteristics concerning children, housing, and institutions were assessed by structured surveys. Anthropometric measurements were taken and vaccination certificates were consulted to assess the vaccination status. Nasopharyngeal swab samples were taken and transported to the laboratory for pneumococcal culture, isolation, identification and testing (bile solubility/Optochin test). Statistical analyses were done with SPSS software. Nasopharyngeal colonisation in children <5 years of age in Medellin was 55.5%. The highest proportion of colonisation occurred in children between 12–24 months of age (60.1%). According to the territorial organisation by communes, the northern and central zones of Medellin had higher rates of colonisation, while the south had lower colonisation levels. Similarly, the institutional modalities based on the daily assistance of children showed higher proportions of colonisation, whereas in those children assisted one day a week, colonisation levels were particularly low

(18.2%). Higher colonisation rates were also associated with the presence of respiratory signs, symptoms and diseases, as well as with the consumption of steroidal medicaments and no affiliation to the health systems (social security). Serotyping, antimicrobial resistance, genotyping and pulsed-field gel electrophoresis and multilocus sequence typing are currently under evaluation. This molecular epidemiology study in Medellín contributes to a better understanding of the scenario of circulating pneumococcal strains and the factors favouring colonisation in children.

P2.16

Serotype distribution and antimicrobial resistance of *Streptococcus pneumoniae* clinical isolates from invasive pneumococcal diseases in Antioquia, Colombia

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Invasive pneumococcal diseases (IPDs) are a leading cause of morbidity and mortality among children <5 years of age. However, the burden of IPDs in Latin American countries remains unknown. In Antioquia, Colombia, the pneumococcal conjugate vaccine PCV7 was introduced in 2006 and replaced in 2010 by PCV10. In 2014, 86.6% of 12-month-old children had received 3 doses of vaccine. Here, the effect of vaccination on IPDs in children and adults after the PCV10 introduction was assessed by estimating the serotype distribution of IPD cases in Antioquia. One hundred and nineteen isolates were collected in 2014 from a hospital network, belonging to the Secretary of Public Health. The age of the patients ranged from 1 to 92 years: 17.6% of the IPD cases were children <5 years, whereas 61.3% were adults >50 years of age. Gender: 41.2% were females and 58.8% males. The geographical distribution was: Medellin (67.2%), and other places of Antioquia (32.1%). The main sources of *Streptococcus pneumoniae* isolation were blood (67.2%), cerebrospinal fluid (16.8%), and other sources (15.9%). The most common diagnoses were sepsis (21.8%), pneumonia (13.4%), meningitis (13.4%), and others (51.4%). The most common pneumococcal serotypes found in Antioquia in 2014 were 14 (9.2%), 19A (9.2%), 3 (7.6%), 6A (6.7%), 19F (5.8%), 12F (5.8%), 4 (5.0%), 23A (4.2%), 12B (3.4%), and 15A (3.4%). These top 10 serotypes accounted for >60% of all IPD cases in the region. According to the Agar diffusion test, the highest rates of antimicrobial resistance were observed with penicillin (NS-45%), tetracycline (R-38%), trimethoprim/sulfamethoxazole (R-35%), erithromycin (R-23%), and clindamycin (R-19%). Traditionally, serotype 14 is the most prevalent serotype in Colombia; however, its incidence has decreased in the last decade. Conversely, the serotype 19A prevalence is increasing in Colombia (emerging serotype) after the PCV introduction. Both serotypes are non-sensible to penicillin and trimethoprim/sulfamethoxazole resistants. The reduced number of IPD cases in children, in comparison with non-PCV-immunised adults, and the shifts in the distribution of IPD serotypes, suggest the potential impact of PCVs on IPDs in Antioquia, Colombia.

P2.17

Vaccination drives changes in metabolic and virulence profiles of *Streptococcus pneumoniae*

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Since the introduction of pneumococcal conjugate vaccines, increases in non-vaccine serotypes have been recorded in several countries: a phenomenon termed vaccine-induced serotype replacement (VISR). Here, using a combination of mathematical modelling and whole genome analysis, we show that targeting particular serotypes through vaccination

also can cause their metabolic and virulence-associated components to transfer through recombination to non-vaccine serotypes: a phenomenon we term vaccine-induced metabolic shift (VIMS). Our results provide a novel explanation for changes observed in the population structure of the pneumococcus following vaccination, and have important implications for strain-targeted vaccination in a range of infectious disease systems.

P2.18

The relevance of a novel rapid quantitative assay to detect up to 40 major *Streptococcus pneumoniae* serotypes directly in clinical nasopharyngeal and blood specimens

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Prior to and following pneumococcal vaccine introduction it is crucial to monitor the distribution and dynamics of *Streptococcus pneumoniae* serotypes. Conventional serotyping methods do not provide rapid or quantitative information on serotype loads. Quantitative serotyping may enable prediction of the invasiveness of a specific serotype compared to other serotypes carried. Here, we describe a novel, rapid multiplex real-time PCR assay for identification and quantification of the 40 most prevalent pneumococcal serotypes and the assay impacts in pneumonia specimen from emerging and developing countries. Two multiplex PCR to specifically detect 29 and 11 serotypes, respectively, were optimised. Quantification was enabled by reference to standard dilutions of known bacterial load. Performance of the assay was evaluated to specifically type and quantify *S. pneumoniae* in nasopharyngeal and blood samples from adult and paediatric patients hospitalised with pneumonia (n = 664) from 5 different countries. Serotype 6AB was widely represented in nasopharyngeal specimens from all 5 cohorts. The most frequent serotypes in the French, South African, and Brazilian cohorts were 1 and 7F, 3 and 19F, and 14, respectively. When both samples were available, the serotype in blood was always present as carriage with other serotypes in the nasopharynx. Moreover, the ability of a serotype to invade the bloodstream may be linked to its nasopharyngeal load. The mean nasopharyngeal concentration of the serotypes that moved to the blood was 2 log-fold higher than the ones only found in the nasopharynx. This novel, rapid, quantitative assay may potentially enable prediction of the *S. pneumoniae* serotypes invasiveness and assessment of pneumococcal serotype distribution pre- and post-introduction of vaccines, and the likely serotype replacement.

P2.19

Streptococcus pneumoniae serotype identification using a rapid, low-cost, automated sequence specific oligonucleotide typing system

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Currently used pneumococcal serotyping techniques are labour intensive, costly and restricted to a limited number of specialist laboratories. A fast, low-cost test that utilises sequence specific oligonucleotides (SSOs) to identify pneumococcal serotypes in an automated procedure has been designed which requires minimal user training. Primers and SSOs were designed from genes located in the capsular polysaccharide synthesis region of the pneumococcal genome. Testing was performed with DNA from pure cultures of pneumococcal, human, and non-pneumococcal bacterial species to ensure no cross-amplification or non-specific SSO signals were detected. We aimed to identify serotypes that are responsible for the majority of invasive pneumococcal disease cases. With our test it is possible to identify a pneumococcal sample to at least the serogroup level, with those protected against by the 23 polyvalent vaccine to the serotype level. However, it is

not possible to differentiate between serotypes that that are caused by protein truncation due the testing method (e.g. 9A and 9V). In addition to pure bacterial culture, identification of serotype direct from extracted DNA of nasopharyngeal and ear swabs as well as serum samples is our next target to ensure rapid diagnosis can be achieved. Within 24 hours of sample acquirement, up to 96 samples can be tested simultaneously before analysis and interpretation with a software program. Furthermore, we aim to be able to identify multiple pneumococcal serotypes from a single sample to reflect multi-serotype pneumococcal carriage. Overall, our system is suitable for high throughput analysis of pneumococcal samples for both the research and medical communities.

P2.20

A novel multiplex real-time PCR assay for identifying and typing of bacterial meningoencephalitis pathogens: development and validation on clinical samples

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Bacterial meningoencephalitis is a major public health concern especially in developing countries. Common identification techniques are time consuming as they require samples culture and often lack sensitivity. Here, we describe a rapid, two-step multiplex real-time PCR method for identifying and typing *Neisseria meningitidis, Streptococcus pneumoniae,* and *Haemophilus influenzae* type b. The first step of the test is a quadruplex, real-time PCR, targeting highly conserved and species specific genes for *S. pneumoniae, N. meningitidis, H. influenzae* type b, and *Streptococcus zooepidemicus* (internal control). The second step consists of 2 multiplex PCR assays for the identification of the 42 most prevalent *S. pneumoniae* serotypes (1, 2, 3, 4, 5, 6A/B, 6C, 7F, 8, 9N/L, 9V, 10A, 10F, 11A, 12F, 13, 14, 15B/C, 16F, 18A/B/C /F, 19A, 19F, 21, 22F, 23A, 23B, 23F, 24F, 31, 33F, 34, 35A, 35B, 35F, 38, 39) and for meningococcal serogroups A, B, C, W135, X and Y. The analytical specificity of all primers/probe pairs has been validated on laboratory strains, representative of each serotype/serogroup targeted by the assay, as well as on closely related members of the genera *Streptococcus, Haemophilus*, and *Neisseria*. The test was then used to analyse 252 samples of cerebrospinal fluid from patients with suspected meningoencephalitis in Antananarivo, Madagascar. Forty-two (17%) were positive for *S. pneumoniae* and we were able to determine the serotype of 35 of them (83%). *N. meningitidis* serogroup W135 was found in 1 sample and another was positive for *N. meningitidis* serogroup C. The major advantage of the proposed molecular assay resides in the possibility to perform a direct analysis of a clinical sample, thus providing results within a couple of hours. It can be also used for monitoring epidemiological trends and serotype replacement upon vaccine introduction.

P2.21

Evolution of *Streptococcus pneumoniae* serotype 14 in Catalonia, Spain, after the introduction of 13-valent pneumococcal conjugate vaccine (PCV13) (2010–2014)

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Serotype 14 (S14) has been considered one of the main serotypes causing invasive pneumococcal disease (IPD). Since 2001, conjugate vaccines are available to prevent IPD caused by different serotypes included S14. Since 2010, PCV13 is the vaccine recommended in children in our region, but it is estimated only 50% are vaccinated. Our aim was to

study S14 evolution in paediatric and adult IPD patients in Catalonia after the introduction of PCV13. All pneumococcal invasive isolates received in the period 2010–2014 from 23 hospitals in Catalonia and characterised by the Microbiology Department at Hospital Sant Joan de Déu, Barcelona, were included. Capsular typing and clonal analysis were performed by fluorescent multiplex PCR and MLST, respectively. One thousand seven hundred and sixty-two IPD episodes were diagnosed during the study period and 1,730 (98.2%) were available for serotyping and clonal study. The IPD rate decreased from 15.4/100,000 habitants in 2010 to 12.9/100,000 in 2014 ($p < 0.001$). A total of 921 (53.2%) isolates were PCV13 serotypes, 126 (7.3%) were S14 (30 from children <5 years). The proportion of PCV13 serotypes decreased from 65.3% in 2010 to 43.8% in 2014 ($p < 0.001$). However, S14 showed a different evolution, without changes during 2010–2013 (6.6% in 2010 vs. 6.4% in 2013) ($p = 0.99$) but with a significant increase in 2014: 6.5% (2010–2013) vs. 11.2% (2014) ($p = 0.007$). Moreover, it achieved the first position among the main serotypes causing IPD in 2014. The proportion of S14 increased mainly in patients between 2–5 years (5% vs. 20.8%) ($p = 0.017$) and >65 years (7.4% vs. 13.1%) ($p = 0.032$). ST156 was the main clonal type identified along the study ($n = 86$; 68.3%). This ST was detected in 63% of S14 isolates during 2010–2013 vs. 82.4% in 2014 ($p = 0.05$). Despite the progressive decrease of IPD rate in Catalonia due to conjugate vaccines, S14 is still a major cause of IPD. Further studies are needed to determine the reasons of this.

P2.22

Conjugate vaccine introduction in Portugal was followed by a decrease of serotypes 6A and 6E but not of serotypes 6B and 6C

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The pneumococcal vaccine PCV13 has been available since 2010 in Portugal. Two serotypes of serogroup 6 (6A and 6B) are included in the vaccine but six others (6C, 6D, 6E, 6F, 6G and 6H) were recently proposed. Their capsule polysaccharides are similar and one recombination or mutation event in the capsular locus may switch serotype. Particularly, a single amino acid change in WciP is sufficient to alter its specificity resulting in a different serotype. Transformation, the main mechanism of horizontal genetic exchange in pneumococci, is regulated by a quorum sensing system controlled by the competence stimulating peptide (CSP). There are 3 variants of CSP and each defines a pherotype with a given strain being able to respond to just 1 variant. The goals of this work were to determine the prevalence of invasive isolates of serogroup 6, to evaluate the potential effect of the conjugate vaccines in this serogroup, and to assess their genetic diversity and determine associations with pherotypes. Out of 239 isolates expressing serogroup 6 recovered between 1999 and 2012, $n = 80$ were 6A, $n = 26$ were 6B, $n = 82$ were 6C, and $n = 51$ were 6E. Serotypes 6E and 6A declined after the introduction of PCV7 in 2001 and PCV13 in 2010, respectively. Serotype 6B remained constant with a low prevalence. After 2010, 6C became the most frequent serotype and there is no evidence of cross-protection conferred by the vaccine. A total of 65 sequence types were identified, reflecting the high genetic diversity of this serogroup. However, the majority belonged to 5 major clonal complexes. Serotypes 6A and 6C were associated with pherotype CSP2 and 6B with CSP1. In conclusion, the majority of previously identified serotype 6B isolates were in fact serotype 6E and these declined after PCV7 introduction. In spite of the presence of serotypes 6A and 6B in PCV13, this did not result in a decline of serotype 6C isolates.

P2.23

PCV13 serotypes were the major constituents of the major clones causing adult IPD in Portugal in the conjugate vaccine era

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We have previously shown that major changes in serotype frequency occurred in the isolates causing adult (>18yrs) invasive pneumococcal disease (IPD) between 2008 and 2011 and in this study we aimed to look at the clonal composition

of this collection. From a total of 1661 isolates, a random sample of at least 50% of the isolates from each serotype was chosen for multilocus sequence typing (MLST) and goeBURST analysis (*n* = 872). We detected a diverse clonal composition with 205 different sequence types (STs) (SID = 0.972 [95% CI 0.967–0.976]) organised in 82 different clonal complexes (CCs) (SID = 0.948 [95% CI 0.942–0.953). Although there was high genetic diversity half of the isolates arranged in only 6 CCs: CC156 (11.6%), CC191 (10.1%), CC180 (8.0%), CC306 (7.8%), CC62 (7.7%), CC230 (5.4%). The major clone was mostly composed of PCV7 serotypes (89%) although these only accounted for 18.1% of the isolates analysed by MLST. The additional serotypes found in PCV13 (42.0% of the genotyped isolates) were the major constituents of 4 of these 6 major clones (CC191, CC180, CC306, and CC230) while PPV23 additional serotypes (20.0%) were the most frequent in CC62. The clonal frequency was greatly influenced by serotype fluctuations, especially of the CC306, represented by serotype 1, which significantly declined from 12.8% (2008) to 2.8% (2011). A few possible capsular switches between vaccine and non-vaccine types were identified. For instance, isolates of ST1201 presented serotypes 19A and 7C, ST230 had serotypes 19A and 24F, ST42 was from serotypes 23A or 6A, and ST241 had both 19A and 18A capsular types. Antimicrobial resistance was more strongly associated with ST than with serotype, with different STs of the resistant serotypes 19A and 14 behaving differently. Since the most important clones causing adult IPD are mainly composed of PCV13 serotypes, major changes in the clonal composition of pneumococci are expected with continued vaccine use.

P2.24

The identification of pneumococci by *lytA*-based identification methods may retrieve false results

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In recent years, molecular strategies targeting *lytA* (coding for the major pneumococcal autolysin), have been proposed to identify pneumococcus. In the clinical setting, the identification of pneumococci based on the amplification of *lytA* by real-time PCR is increasingly used particularly when doing direct detection in clinical samples. In research laboratories, the distinction between atypical pneumococci and closely related species has often been based on the assignment of specific *lytA*-BsaAI-RFLP signatures and/or MLST and multilocus sequence analysis (MLSA) strategies. In our collection, we detected 11 strains displaying conflicting or novel results when identified by *lytA*-BsaAI-RFLP and MLST. Here we report further characterisation of these strains and discuss the utility of using *lytA*-real-time PCR in these cases. MLSA for viridans streptococci divided the 11 strains in 4 pneumococci 2 lineages), 5 *Streptococcus mitis* (5 lineages), and 2 *S. pseudopneumoniae* (1 lineage). Three novel *lytA*-BsaAI-RFLP signatures were found. In addition, 1 pneumococcus displayed the atypical *lytA*-BsaAI-RFLP signature characteristic of non-pneumococci and 2 *S. pseudopneumoniae* displayed the typical *lytA*-BsaAI-RFLP pattern characteristic of pneumococci. *lytA*-real-time PCR misidentified these 3 isolates. DNA sequencing of *lytA* confirmed these observations. Although rare, *lytA*-based identification methods may lead to false results.

P2.25

Characterisation of the *blp* locus of co-colonising pneumococci and its impact on co-colonisation

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Pneumococcal co-colonisation of the nasopharynx is frequent and is important for pneumococcal biology by promoting both evolution and competition. The bacteriocin-like peptides (*blp*) locus has been implicated in intra-species competition but the effect of bacteriocin secretion on co-colonisation remains to be assessed. We aimed to evaluate the impact of the *blp* locus and bacteriocin production on pneumococcal co-existence in the nasopharynx. A collection of 135 co-colonised nasopharyngeal samples from healthy children was used. The inhibitory activity of all strains (*n* = 285)

was assessed through overlay competition assays and their *blp* locus was characterised regarding BlpC type (signalling peptide), integrity of the secretion system, and bacteriocin/immunity content. Approximately one-third of co-colonising strains displayed inhibitory activity (29%) and more than half were cheaters (54%) (expression of immunity without cost of bacteriocin secretion). Cheater strains co-existed with other cheaters (28%) and with inhibitory strains (25%) at a comparable frequency. The 5 known BlpC types were not equally distributed in the population—BlpCT4 was the most prevalent (37%) and BlpCP155 was rare (2%). The proportion of co-colonisation events involving matched (41%) or unmatched (59%) BlpC types was not significantly different ($p = 0.32$). Also, the observed proportions of co-colonisation events predicted to result in inhibition of one strain (41%) or no-inhibition (59%) were not different from the estimated proportions based on the observed frequency in the population ($p = 0.81$). The results show that bacteriocin producers often co-colonise with susceptible strains, suggesting that phenotypes of bacteriocin secretion alone do not explain the co-existence of pneumococci in the nasopharynx.

P2.26

Molecular surveillance on *Streptococcus pneumoniae* carriage in the Netherlands: does the pneumococcus change its niche preference with increasing host age?

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Pneumococcal colonisation of the upper airways is a disease prerequisite, which disproportionally affects infants and elderly. Unlike in children, carriage of *Streptococcus pneumoniae* is rarely detected in elderly by the gold standard method of conventional diagnostic culture. We suspected contemporary carriage rates to be underestimated and that molecular-based analysis of samples from additional niches would enhance pneumococcal carriage detection. Here, we compared the sensitivity of conventional and molecular methods in upper-airway samples from infants, adults and elderly. Nasopharyngeal and saliva samples were collected from asymptomatic 24-month-old children ($n = 289$), parents ($n = 298$) and 135 elderly aged 60–89 (2 time points, $n = 270$). Oropharyngeal samples were also obtained from all adults. Following conventional diagnostic culture on pneumococci-selective media, DNA extracted from all plate growth was tested by qPCR targeting 2 species-specific genes. For all age groups, molecular methods significantly increased the number of carriers detected. There was no significant difference in the number of culture-positive nasopharyngeal samples and qPCR-positive saliva samples from infants, while in both parents and elderly, qPCR-detection of pneumococci in saliva samples was the most sensitive method. Accurate detection of *S. pneumoniae* is essential for monitoring direct and indirect effects of infant pneumococcal vaccination. We demonstrate that no single method of detection is optimal for all age groups, which is an important consideration for future surveillances. Our findings suggest a change in niche preference with age and indicate that carriage rates in adults and elderly are underestimated when based on nasopharyngeal samples alone. We propose that for sensitive pneumococcal carriage detection, nasopharyngeal and saliva samples should be collected from children aged 0–6 years and that oropharyngeal and saliva samples should be collected from all individuals aged 6–80 years.

P2.27

Specificity and sensitivity of molecular methods used for detection of *Streptococcus pneumoniae* and pneumococcal serotypes

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The gold standard method for the detection of upper respiratory tract commensal *Streptococcus pneumoniae* relies on conventional culture. Recent studies investigating culture-independent methods uniformly demonstrate the higher sensitivity of molecular detection. Concerns regarding the specificity of molecular methods have been expressed however, particularly when applied to oropharyngeal samples, representing high microbial diversity including other streptococci that may carry homologues of pneumococcal genes. Here, we investigated the sensitivity and specificity of molecular assays for pneumococcal detection with particular focus on non-pneumococcal streptococci. DNA of streptococcal strains isolated from invasive disease (103 non-pneumococcal, 6 pneumococcal isolates) and asymptomatic carriage (64 infants, 58 elderly; 101 non-pneumococci, 19 pneumococci including 18 non-typeable isolates) was tested for the pneumococcal genes *lytA*, *piaA*, *cps*, *ply,* and spn9802. In addition, pneumococcal serotyping was performed using serotype-specific qPCR assays, as well as capsule sequence typing (CST). Species identification was determined by S2 sequencing. The molecular assay targeting *lytA* was 100% specific and sensitive. Molecular detection of *piaA* was 100% specific but less sensitive for *S. pneumoniae*, while assays targeting *cps*, *ply,* and spn9802 showed a lack of specificity. While CST was highly specific, some streptococcal isolates generated false positive signals in serotype-specific qPCR assays (5, 9A/V, 18B/C, 19F). False positive signals were predominantly generated by *S. pseudopneumoniae* and *S. mitis* strains. Accurate detection and classification of pneumococci is essential for improved understanding of pneumococcal carriage and disease aetiology. We demonstrate that molecular identification of *S. pneumoniae* through *lytA* and *piaA* is highly sensitive and specific. Furthermore, we identified species of bacteria that may confound molecular methods, used for pneumococcal detection and serotype determination when applied to polymicrobial samples from the upper airways.

P2.28

Molecular surveillance on nasopharyngeal carriage of *Streptococcus pneumoniae* in children vaccinated with conjugated polysaccharide pneumococcal vaccines

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As a commensal of the upper respiratory tract, *Streptococcus pneumoniae* is a potential pathogen causing respiratory and invasive diseases. Following implementation of pneumococcal conjugate vaccination (PCV) for infants, surveillance studies have proven essential for monitoring direct (carriage of serotypes targeted by vaccine, VTs) and indirect effects (changes in carriage of non-vaccine serotypes, NVTs). We compared the detection of pneumococcal carriage and serotypes in a unique study setting using both conventional culture and molecular methods, in nasopharyngeal samples from healthy PCV-vaccinated infants in 2 large, cross-sectional surveillance studies on PCV effects in the Netherlands. Nasopharyngeal samples were collected from 1,182 11- and 24-month-old children (n = 591 each) during autumn/winter 2010/11 (n = 584) and 2012/2013 (n = 598). Following conventional culture on plates selective for *S. pneumoniae*, DNA extracted from all bacterial growth was tested by quantitative-PCR (qPCR) for the presence of pneumococci and a panel of serotypes, including serotypes targeted by the 13-valent PCV (PCV13). Molecular diagnostic methods significantly increased both the overall pneumococcal carriage rate and number of serotype carriage events detected as compared

to culture. There was a correlation (Spearman's rho = 0.98; $p < 0.001$) between the frequency of serotypes detected using qPCR and prevalence according to conventional culture. We found no evidence of a hidden circulation of serotypes rarely detected by culture or those targeted by vaccination. Our findings suggest that surveillances based on the culture method alone do not underestimate carriage of VTs in immunised children.

P2.29

PatA/PatB: a multidrug ABC transporter from *Streptococcus pneumoniae* energised by GTP hydrolysis

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The superfamily of ABC transporters (ATP binding cassette) is composed of membrane complexes dedicated to transport a wide variety of compounds through the cell membrane. Transport is carried out by 2 transmembrane domains referred to as permeases and 2 cytoplasmic nucleotide binding domain (NBD) that energise the transporter by hydrolysing ATP. More than 60 ABC transporters have been identified in *Streptococcus pneumoniae*, about 20 of which are carriers putatively involved in the efflux of xenobiotics. PatA/PatB is one of these export systems driving pneumococcal multidrug resistance and a homologue to a multidrug ABC exporter of *Bacillus subtilis*, BmrC/BmrD. It is frequent to face pathogenic strains resistant to antibiotic treatment, which poses problems in patients. To better understand these mechanisms, we performed a functional and structural study of PatA/PatB transporter. PatA/PatB plays a role in the efflux of antibiotic fluoroquinolone and oxazolidinones (linezolid). Single or simultaneous deletion of patA and patB in the pneumococcal genome confers increased susceptibility to ciprofloxacin and norfloxacin; 2 antibiotics belonging to the fluoroquinolone family. Only the heterodimer PatA/PatB has noticeable ATPase and drug transport activities *in vitro* when overexpressed in *Escherichia coli* and using inside-out membrane vesicles; homodimers of PatA or of PatB are unstable and/or inactive. More surprisingly, by incorporating purified PatA/PatB into nanodisc made of phospholipids bilayer, we found that this ABC transporter preferentially hydrolyses GTP as compared to ATP. As a result, the export of drug is increased 6–8-fold in the presence of GTP. Analyses of the intracellular concentrations of ATP and GTP performed by mass spectrometry revealed that these 2 nucleotides are present in similar amounts, indicating that this preference for GTP over ATP has a physiological relevance. Thus, we show for the first time that an ABC transporter has evolved and adapted to hydrolyse GTP. This unexpected adaptation warrants further investigations.

P2.30

Penicillin and erythromycin resistance among pneumococci carried by young children in Portugal: evolution over a 15-year period

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In Portugal, PCV7 was not introduced in the national immunisation plan but was commercially available between 2001 and 2010. We aimed to analyse trends in penicillin and erythromycin resistance among colonising pneumococci over a 15-year period (1996–2010). Nasopharyngeal samples were obtained from children attending day care centres in the urban area of Lisbon and Oeiras. Pneumococci were isolated, serotyped, and antibiotyped by standard procedures. A total of 7,561 children were sampled. Antimicrobial use in the month preceding sampling varied between 13.5%-29.0% and showed a decreasing trend over the years ($p < 0.001$). Although use of PCV7 among children younger than 2 years in 2002 and 2003 was less than 50%, from 2006 onwards PCV7 use was high (range 77.8%–85.2%). Pneumococcal carriage remained high throughout the study (average of 61.2%). High-level resistance to penicillin ranged between 0.7%–8.0%, with a decreasing trend over the years ($p < 0.001$). No sustained changes in the prevalence of low-level resistance to penicillin (range 12.0%-26.5%) or resistance to erythromycin (range 16.4%–27.6%) were observed over time. However,

non-susceptibility to both antimicrobials increased over the years ($p < 0.001$) and was detected in 15–20% of all isolates obtained since 2006. Also, after 2006, most penicillin and/or erythromycin non-susceptible isolates belonged to serotypes not included in PCV7. Our data on antimicrobial resistance among colonising pneumococci suggest that antibiotic pressure remains high in the population. Although a decrease in antibiotic consumption was noted after 2002, it remains high when compared to other countries. These results suggest that additional efforts to contain antibiotic use must be implemented in Portugal.

P2.31

Auranofin-PLGA nanoparticles as an alternative therapeutic tool against pneumococcal infections

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In recent years the effectiveness of the antibiotics for treatment of pneumococcal and other bacterial infections has been compromised by the increasing prevalence of multiresistant strains, which has pushed the development of new drugs with antimicrobial activity. Auranofin, a gold-containing compound approved in 1985 for the treatment of rheumatoid arthritis, has been recently re-profiled by its demonstrated activity against several Gram-positive pathogens. However, the potential pharmacological applications of this hydrophobic compound may be limited by its low solubility in water and biological fluids. One of the most promising strategies for overcoming drug solubility and stability is the use of nanoparticles as drug delivery vehicles. In this work we have explored the use of poly(lactic-co-glycolic acid) (PLGA) nanoparticles loaded with auranofin as antibacterial agent against 2 important streptococcal pathogens i.e. *Streptococcus pneumoniae* and *S. pyogenes*. *In vitro* experiments have shown that cell viability of cultures of several pneumococcal strains, including multiresistant ones, decreased about 3 log units after 6 hours of treatment with 0.5 µM of auranofin, whereas the comparative cultures treated with auranofin-PLGA nanoparticles reached the practical sterility even at lower concentrations (0.25 µM). The different bactericidal effect of auranofin alone or loaded in nanoparticles was more noticeable on the sessile bacterial community (biofilm) of *S. pyogenes*, since auranofin-nanoparticles killed about 4 logs of the bacterial population at 0.25 µM, whereas auranofin alone was virtually ineffective at the same concentration. These promising results have been validated *in vivo* using a zebrafish embryo *S. pneumoniae* infection model. Experiments revealed that treatment with auranofin-nanoparticles resulted in about 15% greater protection compared with the drug alone, in the range of 0.1-0.5 µM.

P2.32

P4 immunotherapy augments neutrophil bacterial killing in patients admitted to critical care with severe community acquired pneumonia

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New treatments are needed to improve outcome in severe pneumonia and sepsis. Immunotherapy using P4 peptide—a 28 amino acid fragment of the pneumococcal protein PsaA—has considerable potential as a novel therapeutic strategy aimed at augmenting phagocytic responses to bacterial infection. To assess the potential clinical efficacy of P4 treatment, we compared neutrophil bacterial killing activity with and without P4 stimulation, using peripheral blood neutrophils obtained from patients admitted to critical care with severe community-acquired pneumonia. Patients admitted to critical care with severe community-acquired pneumonia were recruited to a cross-sectional observational study of outcome and *ex vivo* cellular responses. We assessed neutrophil bacterial killing, cell surface marker, and cytokine expression and

observed clinical course until hospital discharge. Twenty-five patients were recruited and 23 had blood samples tested. Treatment with P4 peptide resulted in significantly increased bacterial killing by neutrophils in 15/23 (65%) of patients (mean killing index 31.4% vs. 20.3%, p = 0.0024). Clinical measures of disease severity (SOFA and APACHE II) did not correlate with P4 peptide activity. Serum levels of IL-10 (GM 12.4 vs. 1.9, p = 0.0061) and IL-8 (GM 59.1 vs. 8.5, p = 0.015) were significantly higher in blood samples that did not respond to P4 stimulation. Treatment with P4 peptide significantly enhanced neutrophil phagocytic activity in the majority of patients with severe community-acquired pneumonia. This study supports the rationale for P4 immunotherapy as a therapeutic strategy in severe community-acquired pneumonia.

P2.33

Recombinant expression of *Streptococcus pneumoniae* capsular polysaccharides in *Escherichia coli*

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Currently, *Streptococcus pneumoniae* is responsible for over 14 million cases of pneumonia worldwide annually, and more than 1 million deaths, the majority of them children. In the UK 2 vaccines are recommended: the pneumococcal polysaccharide vaccine (PPV23), containing purified capsular polysaccharide from 23 serotypes; and the pneumococcal conjugate vaccine (PCV13), containing 13 common serotypes conjugated to CRM197 (mutant diphtheria toxin). Vaccine production costs of pneumococcal conjugate vaccines can be prohibitively high, limiting accessibility of the vaccine in low-income countries. Protein glycan coupling technology (PGCT) can be used to produce recombinant vaccines by expressing the glycan of interest, acceptor protein. and glycosyltransferase enzyme (PglB) within *Escherichia coli*. This approach is flexible and can be exploited to produce an unlimited and purified supply of vaccine at low cost. In order to optimise glycoconjugate yield each of the components must be optimised individually and in concert. Recombinantly expressing a polysaccharide-encoding locus from a Gram-positive bacterium in a Gram-negative bacterium is challenging, not least because transcriptional levels may not be maintained, all the necessary substrates and precursors may not be available, and the resultant components may not be trafficked and assembled in the same way. Our aims were to recombinantly express *S. pneumoniae* capsular polysaccharides, from several different capsule types, within *E. coli*; to find out the minimum set of genes necessary to reliably and efficiently express these polysaccharides heterologously; and finally, to assess the suitability of these polysaccharides to be used with PGCT, coupling them to a variety of immunogenic carrier proteins, to produce an inexpensive and flexible conjugate vaccine. To date, 7 polysaccharides have been recombinantly expressed and detected using a serotype-specific antiserum; of these, 4 have been coupled to a protein using PGCT.

P2.34

A novel cross-protective whole-cell inactivated pneumococcal vaccine

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Generating a pneumococcal vaccine that is serotype-independent and cost effective remains a global challenge. Gamma-irradiation has recently been employed as an inactivation technique for the generation of whole cell bacterial and viral vaccines due to its ability to conserve pathogen structure without disruption of antigenic determinants. In the present study, we utilised gamma irradiation to inactivate an unencapsulated *Streptococcus pneumoniae* strain Rx1 with an unmarked deletion of the autolysin gene *lytA,* and with the pneumolysin gene *ply* replaced with an allele encoding a non-toxic pneumolysoid (PdT) (designated γ-PN vaccine). Intranasal γ-PN vaccination of C57BL/6 mice was shown to be protective in lethal challenge models of pneumococcal bacteraemia, pneumonia and meningitis. Vaccine efficacy was shown to be reliant on B cells, IFN-γ and IL-17A responses. These data are the first to demonstrate the use of gamma-irradiation as a means of generating an effective serotype-independent pneumococcal vaccine that is dependent on both humoral and cellular immunity.

P2.35

Development of a novel, heat shock protein enriched pneumococcal vaccine

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Current vaccination against *Streptococcus pneumoniae*, using vaccines based on capsular polysaccharides from a limited number of serotypes, has led to replacement by non-vaccine serotypes. An alternative approach based on multiple protein antigens enriched with and bound to highly conserved heat-shock proteins (Hsps) will overcome shortcomings of these existing strategies and provide multivalent protection. Hsps are chaperones produced in response to cell stress and bind *S. pneumoniae* proteins to form Hsp-peptide complexes, which improve innate and adaptive immune responses. *S. pneumoniae* cultures were enriched with Hsps by subjecting to heat stress, lysed and purified to form a vaccine preparation. Proteomic analysis was used to confirm enrichment of heat shock proteins and to identify potential protective antigens and their relative expression compared to an untreated homologous lysate. Serum for western blotting, surface IgG-binding, and ELISA analysis of antibody responses were obtained from vaccinated rabbits and mice. Protective efficacy was assessed in mouse models of *S. pneumoniae* infection. The Hsp-enriched vaccine contained several known important antigens and induced robust antibody responses. Vaccinated rabbit sera contained cross-reactive antibodies against multiple serotypes, including non-vaccine serotypes, and sera from vaccinated mice opsonised *S. pneumoniae* with IgG. Active vaccination significantly protected in mouse models of pneumonia infection, whilst passive vaccination of rabbit serum significantly protected against both homologous and heterologous infection mouse models of sepsis. This novel vaccine induced cross-reactive antibodies against multiple serotypes of *S. pneumoniae*, protected against infection and has the potential to provide serotype-independent protection against *S. pneumoniae.*

P2.36

Use of Δ6PLY as an immunomodulator when fused to antigens of unrelated species *Mannheimia haemolytica*

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Pneumolysin (PLY), the cholesterol-dependant cytolysin produced by *Streptococcus pneumoniae,* causes direct tissue damage in the different presentations of pneumococcal infections by pore formation in host cells membranes. The deletion of two amino acids in the PLY sequence produces a non-toxic version known as Δ6PLY, which is non-toxic but retains immunogenicity and is therefore useful for vaccine development. Our research has demonstrated that the fusion of Δ6PLY to other pneumococcal virulence factor such as choline-binding proteins increases their immunogenicity when given mucosally. We hypothesise that the adjuvant properties of PLY may be useful for construction of other vaccines. Therefore we are investigating the use of protein fusions of Δ6PLY with proteins from the ruminant pathogen *Mannheimia haemolytica*, responsible for causing bovine respiratory complex. Current commercial vaccines are produced by the chemical inactivation of the RTX toxin, leukotoxin (Lkt), produced by *M. haemolytica*. Four protein chimeras were designed, 2 of them based on LKT: LktAΔ6PLY, which contains the inactive structural protein LktA, and LktAepΔ6PLY, which is formed by a neutralising epitope of Lkt. The other 2 fusions were designed with a different virulence factor of *M. haemolytica*, the neuraminidase NanH: NanHG63-H585Δ6PLY, which lacks the autotransporter domain, and NanHG63-H435Δ6PLY, which only contains the catalytic domain of NanH. These fusion proteins were expressed in *Escherichia coli* and evaluated for their ability to generate systemic immune responses when administered to mucosal surfaces. We additionally propose the vaccination with crude *E. coli* lysates as a cheaper alternative for veterinary medicine so the proteins will be assessed in a crude and in a purified version.

P2.37

Pneumolysin toxoid as a vaccine adjuvant

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Streptococcus pneumoniae (the pneumococcus) is a major human pathobiont and can cause a range of diseases including pneumonia and meningitis. Currently, available vaccines against the pneumococcus are based on the polysaccharide capsule and as such are serogroup specific. Therefore, there is much interest in developing protein-based vaccines. The pneumococcal pore-forming toxin pneumolysin (PLY) is an important virulence factor, but also a potential protective immunogen. We previously made a non-toxic version of PLY (Δ6PLY) that does not form pores. Δ6PLY is immunogenic in mice and also acts as a mucosal adjuvant for proteins fused to the toxoid. In this study we use a mouse model to demonstrate that mucosal vaccination with protein fusion vaccines can protect against colonisation. The mechanism of protection is IL-17 dependant, as protection against colonisation was abrogated in IL-17 knock-out mice. We will also describe the use of *in vitro* stimulation assays to investigate the possible mechanisms of the adjuvant activity of Δ6PLY. Furthermore, use of IL17A-Cre R26R-tdRFP fate mapping reporter mice will enable us to determine which IL-17A producing cells are involved in the mucosal immune response to Δ6PLY.

P2.38

Clonal expansion of non-susceptible non-vaccine pneumococci in non-invasive isolates following conjugate vaccine introduction results in similar rates of antimicrobial non-susceptibility to the pre-conjugate-vaccine era

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The 7- and 13- valent pneumococcal conjugate vaccines (PCV7/13) were introduced to the Irish childhood immunisation schedules in 2008 and 2010, respectively. We collected 506 paediatric non-invasive pneumococci from 2009 to 2014 at Temple St Children's University Hospital and Our Lady's Children's Hospital Crumlin and AMNCH Tallaght during 2013 to 2014. Serotyping and susceptibility testing to 6 different antimicrobials was performed on isolates. Multilocus sequence typing further characterised 169 isolates non-susceptible to at least 1 antimicrobial. Notable changes occurred in non-susceptible clones and serotypes in non-invasive isolates after conjugate vaccine introduction compared to before conjugate vaccine introduction. In 2007, England^{14}-9 and Spain^{9V}-3 accounted for 12% and 32% of non-susceptible clones, respectively, but in 2009-2014 their prevalence was just 0% and 2.4%. Both England^{14}-9 and Spain^{9V}-3 were associated with PCV7 serotypes 14 and 9V. Furthermore, there was a significant decline in non-susceptible PCV7 serotype 6B belonging to Spain^{6B}-2 and its variants from 2009 to 2014 (p = 0.0024). Fluctuations occurred in clonal complex 320 associated with non-susceptible PCV13 serotype 19A. A significant increase occurred in non-susceptible non-vaccine type (NVT) pneumococci from 12% (3/25) in 2007 to 73% (51/69) in 2014 (p < 0.0001). Serotypes 15A (n = 21) and 35B (n = 22) were the most frequent NVT which were non-susceptible from 2009 onwards. The increase of NVT serotypes 15A and 35B was largely mediated by clonal expansion of Sweden^{15A}-25 and its variants (n = 15) and sequence type 558 (n = 21), respectively. Overall, non-susceptibility rates among non-invasive isolates did not change notably during the study at 23% (2007), 30% (2009), 31% (2010), 21% (2011), 34% (2012), and 41% (2013-2014). This suggests that the increase in clones expressing NVT has resulted in a similar rate of non-susceptibility among non-invasive pneumococci. Continued surveillance will determine if non-susceptible NVT pneumococci increase in circulation with sustained conjugate vaccine use.

P2.39

Salmonella outer membrane vesicles displaying high densities of pneumococcal antigen at the surface offer protection against colonisation

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Bacterial outer membrane vesicles (OMVs) are attractive vaccine formulations because of their intrinsic immunostimulatory properties and non-living nature. In principle, heterologous antigens incorporated into OMVs will elicit specific immune responses, especially if presented at the surface. In this study, we explored the feasibility of the protein expression Hbp platform for vaccine development and present an approach for a broadly protective pneumococcal vaccine. We show that intranasally administered *Salmonella* OMVs displaying high antigen levels at the surface induced strong protection in a murine model of pneumococcal colonisation, without the need for a mucosal adjuvant. Reduction in bacterial recovery from the nasal cavity correlated with local production of antigen-specific IL-17A. Furthermore, the protective efficacy, the production of antigen-specific IL-17A, and local and systemic IgGs, were all improved at a higher concentration of the displayed antigen. As only the α1α2 part of PspA elicited strong protection, we investigated which region(s) in α1α2 could mediate cross-protection between pneumococcal strains using a pneumococcal strain collection of 350 clinical isolates. IgG cross-reactivity between pneumococcal strains appears to be mediated by 2 specific regions of α1α2, which, remarkably, are variants present in almost all clinical isolates. Currently, experiments are ongoing to assess cellular cross-reactivity. As PspA is highly variable among serotypes, the percentage coverage of pneumococcal strains could potentially be increased by combining the cross-reactive regions from different sequences for surface display on *Salmonella* OMVs. Here we demonstrate that intranasally administered OMVs decorated with pneumococcal antigens induce strong protection. This discovery highlights the importance of an efficient antigen expression system for development of recombinant OMV-based vaccines. In conclusion, our findings demonstrate the suitability of the Hbp platform for development of a new generation of OMV vaccines, and illustrate the potential of using this approach to develop a broadly protective mucosal pneumococcal vaccine.

P2.40

Calibration of an individual-based transmission model to pre- and post-PCV pneumococcal carriage

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Predicting the effects of pneumococcal vaccination on carriage of and disease from specific pneumococcal serotypes requires a sufficiently realistic model of the factors underlying serotype-specific incidence and prevalence before and after vaccination. We adapted a previously-published model of pneumococcal population dynamics, which includes serotype-specific variability in duration and within-host competitive ability, as well as serotype-specific and non-specific acquired immunity, to fit the observed prevalence of pneumococcal serotypes, using carriage data from Massachusetts before PCV7, during the PCV7 era, and during the PCV13 era. By varying a single parameter for each serotype that governed both its specific duration and its within-host competitive ability, and varying another parameter governing overall transmission intensity, the model was able to reproduce observed patterns of serotype-specific prevalence before vaccine introduction. Following introduction of vaccine, based on fitting to vaccine-type prevalence, a direct effect of at least a 73% reduction in the acquisition rate of carriage of vaccine types was required to reproduce observed carriage prevalences, post-vaccine introduction. Serotypes that were not in the vaccine but are part of vaccine serogroups expanded post-PCV7 more in observed data than in model predictions. We hypothesise that partial cross-immunity within serogroups may have lowered prevaccine prevalence of these vaccine-related types, and that their disproportionate expansion may be evidence for such partial cross-immunity. We conclude that calibrated, detailed

individual-based modelling of pneumococcal vaccine effects is possible for applications in public health.

P2.41

Evaluation of the efficacy of the immunisation with PspA in a co-colonisation model in mice

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Pneumococcal surface protein A (PspA) is one of the leading candidates for a protein vaccine against pneumococcal infections. PspA shows variability and the great majority of strains express PspA from family 1 (clades 1 and 2) or family 2 (clades 3, 4, and 5). The aim of this study is to evaluate the protection elicited by nasal immunisation with recombinant PspAs from different clades in a mouse model of co-colonisation of the nasopharynx with two strains, one expressing PspA from family 1 and another expressing PspA from family 2. BALB/c mice were immunised intranasally with recombinant PspAs from clades 1 to 4 (rPspA1, rPspA2, rPspA3 and rPspA4) using the whole-cell pertussis vaccine as adjuvant. Immunisation with rPspA1 elicited an increase in anti-rPspA1 and anti-rPspA4 IgG titres measured by ELISA, whereas immunisation with rPspA4 elicited an increase only in anti-rPspA4 IgG titres. Mice were then challenged with a mixture of the strains 491/00 (serotype 6B, PspA1) and 472/96 (serotype 6B, PspA4, trimethoprim resistant) and bacteria were recovered from nasal washes 5 days after challenge. Only mice immunised with rPspA1 showed statistically significant reduction in colonisation with both strains when compared to the control saline group. Animals immunised with rPspA3 and rPspA4 showed statistically significant reduction only in the PspA4 expressing strain when compared to saline. Our preliminary results indicate that rPspA1 and possibly a mixture of rPspA1 and rPspA4 would be suitable for attaining protection against colonisation with strains expressing different PspAs.

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P2.42

New protein vaccines against pneumococcal pneumonia using experimentally induced immunity

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Our experimental human pneumococcal carriage (EHPC) model provides an ethical measurement of vaccine efficacy where colonisation is a surrogate for disease. Factors associated with prevention of colonisation include T-helper 17 cell (Th-17) and humoral immune responses. The conserved yet heterogeneous virulence factor pneumococcal surface protein (PspA) is a promising vaccine candidate, immunogenic and protective in murine disease models. Identification of a region eliciting beneficial Th-17 and humoral responses may inform development of a vaccine conferring protection against pneumonia. Four recombinant PspA fragments (approximately 100 amino acids) were expressed in *Escherichia coli* BL21 and used in *ex vivo* stimulation. Volunteers were inoculated with pneumococcus and mononuclear cells isolated from peripheral blood (PBMCs) and bronchoalveolar lavage (BAL). Th-17 cells were stained for surface phenotyping and intracellular cytokine production. Assay optimisation revealed Th-17 responses were best detected 5 days post-antigen stimulation. Preliminary analysis of PBMC stimulations demonstrates an increase in Th-17 response post-pneumococcal inoculation in all antigens, with elevated responses in one of four fragments. PBMCs of volunteers inoculated with pneumococcus demonstrate an increased Th-17 response on stimulation with all candidate antigens. The PspA fragment inducing the highest Th-17 response in blood and BAL will be used to purify antibodies from plasma of healthy adults susceptible to or protected from colonisation. Resultant antibodies will be employed in functional binding and complement deposition assays to compare ability to bind and potentially clear pneumococci of differing strains.

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P2.43

Novel recombinant glycoconjugate vaccines for prevention of pneumococcal meningitis

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The pneumococcus is a major cause of meningitis around the world. Current vaccination strategies suffer from serious limitations in terms of cost and serotype coverage, and novel approaches are required to overcome these challenges. In this project, we are aiming to produce vaccines comprised of conserved pneumococcal protein antigens conjugated to capsular polysaccharide from serotype 4 pneumococci by making use of an unusual oligosaccharyltransferase from *Campylobacter jejuni* named PglB. This enzyme is able to conjugate glycans containing an acetamido group in the C2 position of their reducing end sugar to proteins containing a specific amino acid sequence (D/E-Y-N-X-S/T). We have introduced this 'glycotag' sequence into 4 pneumococcal protein vaccine candidates: α-glycerophosphate oxidase (GlpO), neuraminidase A (NanA), and the ATP-binding cassette transporters PiuA and Sp0148. All 4 of these proteins have previously shown efficacy as vaccines in animal models of colonisation or meningitis caused by *Streptococcus pneumoniae*. Previous work has allowed the expression of pneumococcal serotype 4 capsule in *Escherichia coli*, and by co-expressing PglB and the target protein in the same strain, we have been able to demonstrate production and small scale purification of protein antigens conjugated to the polysaccharide. Our current efforts are directed towards optimisation of the efficiency of the conjugation process through modifying parameters such as the leader sequence of the protein, the plasmid used to encode its gene, the background strain of *E. coli* and the growth conditions used for expression.

P2.44

Broadly cross-reactive antibodies recognising the proline-rich region of pneumococcal surface protein A variants show cross-reactivity with skeletal muscle

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Pneumococcal surface protein A (PspA) is an important surface-expressed virulence factor of *Streptococcus pneumoniae*. PspA displays high level of sequence variability in clinical isolates but contains a conserved proline-rich region at the C-terminus. PspA was shown to be protective in animal models and has been considered as an attractive vaccine antigen. One of the concerns with PspA is the anecdotal detection of serum antibodies with potential cross-reactivity to human muscle in vaccines. The antigen responsible for inducing tissue cross-reactive antibodies was assumed to be the N-terminal coiled coil region of PspA. We selected 4 human IgG antibodies from a yeast-based antibody library utilising all 5 clade variants of PspA as baits. The resulting mAbs were tested for binding to native PspA by flow cytometry-based surface staining of 61 *S. pneumoniae* clinical isolates. Poly-reactivity of the mAbs was tested against cell membrane extract of Chinese hamster ovary (CHO) cells as well as against porcine myosin. We identified monoclonal antibodies binding to the majority of tested *S. pneumoniae* clinical isolates, expressing clades 1 to 5 of PspA. Confirming the specificity of the mAbs, they do not bind to *S. pneumoniae* D39 ΔpspA/ΔpspC strain. These antibodies were determined to react with a PAPAPKP consensus peptide motif of the proline-rich region of PspA. However, we observed significant binding to CHO cell extract and myosin in ELISA, suggesting that breadth of binding to PspA variants correlated with non-specific reactivity towards mammalian cells and specifically, skeletal muscle. Amino acid homology search identified the presence of the PAPAPKP motif and its variants in several human proteins, including myosin, cell adhesion, and nuclear proteins. In conclusion, broadly cross-reactive PspA monoclonal antibodies targeting the proline-rich region of PspA can bind live bacteria, but they raise concern about off-target tissue cross-reactivity related to this region of PspA.

P2.45

Structural analysis of the choline-binding sites in CbpL from *Streptococcus pneumoniae*

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Four families of surface proteins decorate the cell surface of the human pathogen *Streptococcus pneumoniae*. Besides lipoproteins and LPXTG proteins, also present in other Gram-positive bacteria, the pneumococcus presents the non-classical surface proteins and the choline-binding protein family. Choline binding proteins (CBPs) show a modular organisation including, at least, the choline-binding domain and a domain exerting a biological function. The choline-binding domain interacts with choline molecules from teichoic and lipoteichoic acids, attaching the whole protein to the peptidoglycan layer. Here, we show the three-dimensional structure of the choline-binding domain of CbpL displaying 8 choline-binding sites. Four of them follow the canonical sequence while the other 4 are different. The alternate configuration of canonical and non-canonical sites is a unique property of CbpL among CBP family. Structural analysis and specific features of this module will be provided.

P2.46

Assessment of the specific role of the phosphorylcholine esterase Pce in the modification of pneumococcal teichoic acids

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Pneumococcal diseases are a global burden and *Streptococcus pneumoniae* is one of the most important pathogens in bacterial meningitis, otitis media, and sinusitis. Unlike other Gram-positive bacteria, the lipoteichoic acid (LTA) and wall teichoic acid (WTA) of *S. pneumoniae* possess the same structure within their repeating units. Pneumococci have a unique nutritional requirement for choline for growth to be able to attach phosphorylcholine (*P*-Cho) residues to the *N*-acetylgalactosamine moieties of their teichoic acids (TAs). This structural feature is of physiological importance for the anchoring of surface-localised choline-binding proteins (CBPs) to the pneumococcal cell wall. CBPs like the pneumococcal surface protein C (PspC) and the phosphorylcholine esterase (Pce) have been shown to be involved in pneumococcal adhesion to host cells. Interestingly, Pce hydrolyses about 15–30% of the total *P*-Cho residues attached to pneumococcal TAs. In the study presented here, we investigate which *P*-Cho residues are specifically removed from the TAs by the Pce. LTAs of different pneumococcal strains were isolated by citric buffer/butan-1-ol extraction and purified by hydrophobic interaction chromatography. These LTA preparations were further treated with anhydrous hydrazine to remove fatty acids and D-alanine residues. This procedure has two major advantages: less complex mass spectrometry data can be obtained and ^{1}H as well as ^{31}P NMR spectra show significantly higher resolutions. Importantly, in these spectra of *O*-deacylated and therefore non-aggregated LTA molecules specific moieties can be quantified. We have shown recently that the terminus of the pneumococcal LTA can vary in the *P*-Cho substitution pattern in dependency on the strain (D39 vs. TIGR4) and the culturing conditions. Here, the role of Pce in the modification of pneumococcal TAs will be elucidated by detailed structural investigation of LTA isolated from TIGR4Δ*cps*Δ*pce* as well as by treatment of fully *P*-Cho-decorated pneumococcal LTA with heterologously expressed Pce.