Purpose of review
Streptococcus pneumoniae (the pneumococcus) remains an important cause of invasive disease including bacteraemia. This review highlights recent findings related to pneumococcal bacteraemia, virulence factors, and multiple colonization, including strain competition, biofilm formation, and competence.

Recent findings
Countries with no vaccination programmes see vaccine serotypes still prevalent in disease, whereas the emergence of nonvaccine serotypes in nasopharyngeal carriage and invasive disease is seen in countries with conjugate vaccination in place. Co-colonizing strains are being uncovered with more sensitive methods, and may act synergistically or compete with each other for survival. Several factors such as iron uptake, quorum signalling and the luxS gene, involved in colonization and virulence, are discussed. The role of quorum sensing signalling molecules and formation of biofilms are being explored.

Summary
Epidemiological data suggest that the latest serotype-based conjugate vaccines should provide heightened protection, although serotype replacement is now being seen. Much remains to be elucidated about its biology during multiple colonization, when evolution and adaptation to its host take place. The modes of colonization (biofilm, intracellular or surface adherence to the mucosal epithelium), and whether organisms that cause invasive disease have attenuated ability to colonize the nasopharynx remain to be elucidated.

Keywords
invasive pneumococcal disease, nasopharyngeal colonization, pneumococcal vaccines, pneumococcus, serotypes, Streptococcus pneumoniae

INTRODUCTION
Streptococcus pneumoniae (the pneumococcus) is a common colonizer of the human nasopharynx. It also causes infections including otitis media and pneumonia, and invasive pneumococcal diseases (IPDs) such as meningitis and bacteraemia. The organism is estimated to be responsible for more than 1.6 million deaths per year, most of which occur in children and the elderly [1].

The organism is associated with more than 90 capsular serotypes differentiated by the composition and linkage of capsule polysaccharides [2–4]. These capsules are targets of pneumococcal vaccines, and the first pneumococcal conjugate vaccine (PCV7) is specific for seven of the most common serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F). In 2010, a 13-valent vaccine was introduced, containing six additional serotypes (PCV7 serotypes and 1, 3, 5, 6A, 7F, and 19A). It is likely that the use of PCV13 will increase the protective coverage of the vaccine [5].

This review summarizes recent epidemiological findings pertaining to pneumococcal bacteraemia. In addition, studies investigating roles of virulence factors are described. Finally, we highlight recent studies on pneumococcal carriage with special attention to multiple colonization, and roles of quorum sensing systems and the formation of biofilms during asymptomatic colonization.

RECENT EPIDEMIOLOGICAL FINDINGS
Pneumococcal bacteraemia remains a concern in sub-Saharan Africa, where conjugate vaccination has only been implemented in a few countries. In Nigeria, where S. pneumoniae is not the leading causative agent in childhood bacteraemia, it is, however, the leading cause of death among bacteraemia cases [6]. In the absence of vaccines, PCV7 serotypes made up 75% of bacteraemia cases.
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KEY POINTS

- Given the high prevalence of bacteraemia cases caused by vaccine serotypes, the recent introduction of conjugate vaccines in Africa should provide a heightened level of protection, whereas other forms of treatment, such as HAART therapy, may also prevent pneumococcal diseases in immunocompromised groups.
- Sensitive methods in detecting multiple colonization in asymptomatic children indicate that a high proportion of hosts are colonized by heterogeneous strains together allowing the organisms to adapt rapidly by horizontal gene transfer.
- Interaction and competition between cells during colonization involve complex processes including bacteriocin production, biofilm formation, competence activation, and requirement for other surface proteins.

Similarly, serotypes 1, 6A, and 6B constitute over 40% of IPDs in children under 16 years old in Malawi [7]. In contrast, in areas where PCV7 has been introduced, a reduction in PCV7 serotype disease incidence was met with rises in IPD caused by nonvaccine serotypes (NVTs) including serotype 6C. A US study on paediatric isolates found high prevalence of 6C, with 44% of these isolated from blood [8*]. Multilocus sequence typing (MLST) revealed that some of these serotype 6C sequence types were previously associated with vaccine serotypes 6B, 19A, and 23F, suggestive of capsular switching. A post-PCV7 study in Spain also identified NVTs 19A and 7F as the leading serotypes in bacteraemia [9].

Pneumococcal bacteraemia remains one of the most common complications in HIV-positive individuals. A recent study showed that pneumococcal bacteraemia prevalence decreased by over 90% within 4 years following highly active antiretroviral therapy (HAART) [10]. Remarkably, HAART was superior to conjugate vaccination in reducing bacteraemic HIV-positive patients after 2 years of treatment. It may be that for such high-risk groups, reduction of IPD incidence can be achieved by means other than pneumococcal vaccination.

Despite no clear sex differences in IPD incidence, animal studies revealed that disease susceptibility and mortality were greater in male mice compared with female mice in models of pneumonia and sepsis [11*]. More male mice succumbed to infection by 48 h compared to female mice. In addition to significant weight loss and a reduction in body temperature, infected male mice were also associated with increased expression of cytokines such as interleukin (IL)-5, IL-7, regulated on activation normal T cell expressed and secreted, and KC (keratinocyte chemattractant; keratinocyte-derived chemokine; formerly CXCL1). To our knowledge this is the first to compare sex with disease susceptibility.

It is known that some serotypes are more invasive than others. To assess and compare the disease potential of serotypes, a study was conducted in Israel to compare serotypes prevalent in the nasopharynx of healthy children with those admitted to hospital with community-acquired alveolar pneumonia (CAAP) [12]. The most common serotypes identified in the nasopharynx of CAAP children included 1, 5, 22F, 7F, 14, and 9V. Conversely, odds ratios (ORs) for colonization prevalence in CAAP compared to healthy individuals were less than 1 for serotypes 6A, 6B, 23A, and 35B, suggesting that these serotypes have lower disease potentials. Age-related differences in colonization were also examined, and serotypes 1 and 5 showed increased colonization with age, whereas serotypes 14 and 19F decreased, regardless of health status.

VIRULENCE/COLONIZATION FACTORS

An understanding of the biology of the organism may provide opportunities for new therapeutic platforms. Current epidemiological studies have been complemented by research into virulence factors that may also play roles in colonization.

The polysaccharide capsule enables the pneumococcus to evade opsonophagocytosis [13]. Given the important role of the spleen in host immunity, Lammers et al. [14] investigated the role of capsule in disease susceptibility in asplenic mice. All aspelenic mice intranasally inoculated with high concentrations (4 × 10^7 CFU/ml) of capsulate pneumococci survived after 48 h, compared to the death of over 85% of mice inoculated with encapsulated D39 even at a two-logs lower concentration (4 × 10^5 CFU/ml).

Pneumolysin (encoded by the gene ply) is an important virulence factor that exerts its haemolytic activity by binding to the host cholesterol receptor and forming an oligomeric pore [15]. Recently, a variant ply allele (ply4496) in a hypervirulent lineage of serotype 1 showed reduced haemolytic activity, and while strain D39 expressing this allele was attenuated in murine sepsis, higher bacterial count in blood was detected compared to the wild type, suggesting that reduced haemolytic activity confers a growth advantage in blood [16].

Immune evasion is a principal mechanism for pneumococcal survival. The pneumococcal surface protein C (PspC) binds to the host complement factor H (fH), escaping recognition of the alternative complement pathway. The potential of the
fH-binding region of PspC as a vaccine target was investigated recently [17]. Immunization with a booster in mice increased IgG levels of this epitope, and intravenous injection of the homologous strain into vaccinated mice showed increased survival compared to nonimmunized mice, accompanied with increased C3 binding, lowered fH binding, and increased susceptibility to phagocytosis. However, challenge with heterogeneous strains showed lowered levels of protection, possibly dependent on the variation of PspC amino acid sequences between the vaccine antigen and that of the challenge strain. Given the high sequence diversity of pspC [18], it is unlikely that PspC alone as a vaccine antigen can provide protection from across different pneumococcal strains.

Metal ions are known to be required for pneumococcal cellular function and pathogenicity [19,20]. Bayle et al. [21] reported the requirement of zinc in normal pneumococcal cellular function, as mutations of two components involved in zinc transport, AdcA and AdcAII, were associated with abnormalities in cell division, extended lag phase, and attenuation in murine colonization, pneumonia, and sepsis.

**NASOPHARYNGEAL COLONIZATION**

Infections caused by the pneumococcus are rare compared to colonization. During colonization, microevolution and adaptation arise by horizontal gene transfer (HGT) of capsular genes and genes encoding antibiotic resistance determinants and surface antigens under selection [22,23]. This results in a supragenome (or pan-genome) that is larger than the genome of any single organism [24–26]. Therefore, the co-colonization (or multiple colonization) of pneumococcal strains increases the repertoire of genes available for adaptation and virulence, enhancing its survival as a species [27].

Multiple colonization was documented as early as the 1930s [28], but few studies since have determined multiple colonization by serotyping multiple colonies on blood agar, and even fewer studies accompanied serotype results with genetic analysis of strains, or performed serotyping with more sensitive DNA-based methods. This results in an underestimation of strain diversity. Recently, two studies [29**,30**] addressed this issue using DNA-based serotyping methods in association with MLST to investigate strain diversity in the nasopharynx of Gambian and Tanzanian children (Table 1). Cohort differences and detection methods used may account for some of the difference seen in co-colonization rates. Leung et al. [30**] observed the presence of unrelated strains of the same serotype, highlighting the underestimation of co-colonizations by characterizing serotype alone. The same study also documented the simultaneous colonization of strains with different susceptibility to penicillin and trimethoprim–sulfamethoxazole. Both Gambian and Tanzanian studies found a large proportion of sequence types exclusive to Africa, as well as novel sequence types. They hypothesize that both the community prevalence of multiple colonization and the extent of strain diversity within the nasopharynx encourage HGT between strains, leading to frequent assortments of MLST gene fragments giving rise to these novel sequence types. Donkor et al. [29**] also reported evidence of capsular switching.

Detection of co-colonizations requires sensitive methods. Three methods were compared in Thai children (Table 1) [31**]. In addition to high rates of co-colonization, microarray detected a high prevalence of nontypable pneumococci. Nontypable pneumococci may comprise acapsulate strains, which had traditionally thought to be rare colonizers due to the loss of protective capsule. However, the use of DNA-based methods may increase the likelihood of detecting nontypables by as much as three-fold [32**]. Genetic analysis indicated that most nontypables were associated with ST304, a sequence type associated with conjunctivitis, blood infections, and multidrug resistance [32**,33]. These data should be treated cautiously as DNA-based methods do not reveal only current co-colonizations.

**Table 1. Recent studies of multiple colonization using sensitive detection methods**

<table>
<thead>
<tr>
<th>Country</th>
<th>Age group (months)</th>
<th>Methoda</th>
<th>Prevalence (%)</th>
<th>No. max serotype</th>
<th>Referenceb</th>
</tr>
</thead>
<tbody>
<tr>
<td>The Gambia</td>
<td>≤24</td>
<td>Microarray and CS</td>
<td>19</td>
<td>3</td>
<td>[29**]</td>
</tr>
<tr>
<td>Tanzania</td>
<td>≤72</td>
<td>CS of 20 colonies and sequence-based sequeotyping</td>
<td>12</td>
<td>5</td>
<td>[30**]</td>
</tr>
<tr>
<td>Thailand</td>
<td>≤24</td>
<td>CS on three colonies</td>
<td>11</td>
<td>2</td>
<td>[31**]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sweep colony with latex agglutination</td>
<td>43</td>
<td>4</td>
<td>[31**]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Microarray</td>
<td>48</td>
<td>9</td>
<td>[31**]</td>
</tr>
</tbody>
</table>

*aCS, conventional serotyping by Quellung reaction.

bIn the study by Turner et al. [31**], three methods were performed on the same cohort.
The method of Simoes et al. [32**] also distinguished pneumococci from *S. pseudopneumoniae*. Identification of *S. pseudopneumoniae* is difficult due to its strong genotypic and phenotypic resemblance to nontypable pneumococci. However, *S. pseudopneumoniae* appears to be differentiable from nontypable pneumococci by the absence of *lytA* targeted by primers described. It must be noted only two pseudopneumococcal strains were included in this study, and *lytA*-positive strains of *S. pseudopneumoniae* have been reported [34].

The pneumococcus shares the same ecological niche with *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus*. The prevalence of multiple colonization of these organisms in Gambian infants was assessed using PCR targeting species-specific regions [35]. More than 90% of children were detected to be colonized by at least one of these four opportunistic pathogens by one year of age, and may acquire these species as early as 3 weeks old. There also appears to be a strong and more modest correlation between pneumococcal colonization and that of *H. influenzae* and *M. catarrhalis*, respectively, in which colonization point-prevalence for all three organisms from newborn to 27 weeks increased. Remarkably, the opposite was observed for *S. aureus*, in which case colonization prevalence declined by approximately 80% in the same period. Such information is crucial in understanding the effects of pneumococcal vaccination on colonization of other potential pathogens.

**BACTERIOCINS, BIOFILM FORMATION, AND COMPETENCE**

Competition between pneumococcal strains is mediated by small antimicrobial peptides (bacteriocins). Bacteriocin secretion is modulated by the two-component system encoded by the *blp* locus consisting of an ABC transporter (*blpAB*), a peptide pheromone (*blpC*), a histidine kinase receptor (*blpH*), and response regulator (*blpR*). Pneumococci are protected by their own bacteriocins by the synthesis of immunity proteins. A lineage of high number of clinical isolates was detected to contain a 4-bp repeat insertion sequence in *blpA* that renders these strains unable to secrete BlpC, but they are still responsive to BlpC secreted by other strains, and could produce bacteriocin and immunity proteins [36]. These ‘cheater’ strains are thought to have acquired the insertion via HGT, and are likely to confer a survival advantage by benefiting from bacteriocin production from nearby strains. Son et al. [36] hypothesizes that such an advantage drives the diversification of BlpC so that cheater strains do not respond to heterologous pheromones.

Pneumococci are thought to colonize as a biofilm, a complex polymicrobial community initiated by adherence to the mucosal epithelium. Biofilm formation is related to competence, a transient physiological state in which pneumococci can acquire DNA. Similar to *blp* activation, competence in pneumococci is modulated by the two-component system encoded by the *com* locus, and the signalling molecule involved in competence activation (*ComC*) exists in allelic variants (or pherotypes) [37]. Competence is also responsible for the activation of fratricide, when a pherotype lyzes non-competent strains. The co-colonization of different pherotypes was documented for the first time in asymptomatic children [38**]. The authors rationalized that the co-existence of different pherotypes provides evidence that fratricide appears to have little effect on competing pherotypes.

Competence leads to the expression of stress response genes [39], and was shown to be activated in response to erroneous protein translation and antibiotics [40]. Pneumococcal HtrA (heat-shock-induced, surface-associated serine protease) appears to modulate this regulation. Whereas the mechanism of how HtrA influences activation of competence is unclear, CSP (competence stimulating peptide) may be degraded by HtrA. It is unknown whether host responses that affect pneumococcal protein misfolding also affect competence [40].

The role of competence in biofilm formation depends on the biofilm model [41*]. In microtitre plates, pherotype-specific CSP seemed to affect the late phase of biofilm formation for both exponential and stationary-phase cells, as *comC* and *comD* deletion mutants had significantly reduced biofilm cell count by 24h. Interestingly, the addition of a different exogenous pherotype did not support the maintenance of a biofilm, despite previous indications of possible pherotype cross-talk [42]. CSP does not seem to have a role in continuous biofilm formation, whereas the lack of capsule was associated with increased biofilm thickness, cell count, and surface area [41*,43].

Although adhesion and biofilm formation are thought to occur in colonization, it is uncertain whether this occurs with invasive strains. Comparison of different serotypes captured from IPD revealed that biofilm development is a general pneumococcal property [43]. Some serotypes (serotypes 6A, 6B, 7F) formed better biofilms than others (serotype 3). However, the genetic background and antibiotic susceptibility may also contribute to biofilm formation.

Competence, quorum sensing, and biofilm formation have been linked to the *luxS* gene, in which early biofilm formation was associated with maximal
expression (up to 300-fold) of luxS during mid-log phase, with increases in ply and lytA mRNA levels [44]. Strains with inactivated luxS had reduced biofilm formation, with a lower count of attached cells and a lack of biofilm architecture. The involvement of LuxS in biofilm formation was further supported by Trappetti et al. [45], when LuxS activity and biofilm growth appeared to be dependent on acquisition of extracellular iron. Mutants deficient in luxS may have weakened ability to acquire iron, as a reduced expression of the iron acquisition lipoprotein PiuA was seen. The authors further associated luxS expression and availability of iron to up-regulation of genes involved in competence and drastic. These current studies on biofilm development and its regulation indicate a complex process involving multiple cellular signalling pathways and the expression of a wide array of proteins in response to different environmental factors.

CONCLUSION
Pneumococcal bacteraemia is a common cause of death in African children. With the recent introduction of PCV13, a reduction in IPD caused by vaccine serotypes is anticipated. However, replacement serotypes may follow, as seen in countries with PCV7 implemented. Increasing our understanding of virulence colonization factors may be beneficial in unravelling pneumococcal pathogenicity. Multiple pneumococcal colonization is an emerging concept, as shown by efforts to increase the sensitivity of detection. Finally, understanding the interaction of these different strains during colonization, such as the biofilm mode of growth, competition and communication between species, may provide new therapeutic platforms to prevent pneumococcal disease in the face of the global increase in antibiotic resistance and serotype replacement post vaccination.

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Conflicts of interest
There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 000—000).

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This publication revealed a high number of novel sequence types, suggested to arise from gene exchange which is enhanced in areas of high rates of colonization.


This study uncovered a high extent of genetic diversity within a colonization, with co-colonization of antibiotic-susceptible and nonsusceptible strains, and unrelated strains of the same serotype, indicating that serotype alone underestimates true heterogeneity of carriage.


Using microarray, the authors have uncovered up to nine serotypes colonizing the same host at one time, with fold-changes of increase compared to traditional culture methods.


This publication emphasized the finding that colonization by nontypable pneumococci is higher than previously thought, and may play an important role in pneumococcal adaptation.


This is the first publication highlighting the colonization of multiple competence phenotypes.


Findings in this publication allow a more categorical assessment of previous works on roles of competence on biofilm formation based on the different biofilm models employed.


The finding that LuxS plays roles in affecting biofilm formation, competence, and fratricide may soon allow a greater understanding of the interconnection of these complex processes.