


A mosaic tetracycline resistance gene *tet(S/M)* detected in an MDR pneumococcal CC230 lineage that underwent capsular switching in South Africa

Stephanie W. Lo ^{1*}, Rebecca A. Gladstone¹, Andries J. van Tonder¹, Mignon Du Plessis^{2,3}, Jennifer E. Cornick^{4,5}, Paulina A. Hawkins⁶, Shabir A. Madhi^{7,8}, Susan A. Nzenze^{7,8}, Rama Kandasamy⁹, K. L. Ravikumar¹⁰, Naima Elmdaghri^{11,12}, Brenda Kwambana-Adams^{13,14}, Samanta Cristine Grassi Almeida¹⁵, Anna Skoczynska¹⁶, Ekaterina Egorova¹⁷, Leonid Titov¹⁸, Samir K. Saha¹⁹, Metka Paragi²⁰, Dean B. Everett^{4,21}, Martin Antonio¹⁴, Keith P. Klugman^{2,3,6,7}, Yuan Li²², Benjamin J. Metcalf²², Bernard Beall²², Lesley McGee²², Robert F. Breiman^{6,23}, Stephen D. Bentley¹ and Anne von Gottberg^{2,3} on behalf of the Global Pneumococcal Sequencing Consortium†

¹Parasites and Microbes Programme, The Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge CB10 1SA, UK;

²Centre for Respiratory Disease and Meningitis, National Institute for Communicable Diseases, Johannesburg, South Africa; ³School of Pathology, University of the Witwatersrand, Johannesburg, South Africa; ⁴Malawi Liverpool Wellcome Trust Clinical Research Programme, PO Box 30096, Blantyre, Malawi; ⁵Institute of Infection & Global Health, University of Liverpool, Liverpool L69 7BE, UK;

⁶Hubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA; ⁷Medical Research Council: Respiratory and Meningeal Pathogens Research Unit, University of the Witwatersrand, Johannesburg, South Africa;

⁸Department of Science and Technology/National Research Foundation: Vaccine Preventable Diseases, University of the Witwatersrand, Johannesburg, South Africa; ⁹Oxford Vaccine Group, Department of Paediatrics, University of Oxford, and the NIHR Oxford Biomedical Research Centre, Oxford OX3 9DU, UK; ¹⁰Department of Microbiology, Kempegowda Institute of Medical Sciences Hospital & Research Centre, Bangalore, India; ¹¹Department of Microbiology, Faculty of Medicine and Pharmacy, B.P. 9154, Hassan II University of Casablanca, Casablanca, Morocco; ¹²Bacteriology-Virology and Hospital Hygiene Laboratory, University Hospital Centre Ibn Rochd, Casablanca, Morocco; ¹³NIHR Global Health Research Unit on Mucosal Pathogens, Division of Infection and Immunity, University College London, London, UK; ¹⁴WHO Collaborating Centre for New Vaccines Surveillance, Medical Research Council Unit, The Gambia at The London School of Hygiene and Tropical Medicine, Fajara, The Gambia; ¹⁵National Laboratory for Meningitis and Pneumococcal Infections, Center of Bacteriology, Institute Adolfo Lutz (IAL), São Paulo, Brazil; ¹⁶Department of Epidemiology and Clinical Microbiology, National Medicines Institute, Warsaw, Poland; ¹⁷Laboratory of Clinical Microbiology and Biotechnology, Moscow Research Institute for Epidemiology and Microbiology, Moscow, Russian Federation; ¹⁸Laboratory of Clinical and Experimental Microbiology, The Republican Research and Practical Center for Epidemiology and Microbiology, Minsk, Belarus; ¹⁹Department of Microbiology, Dhaka Shishu (Children's) Hospital, Child Health Research Foundation, Dhaka, Bangladesh; ²⁰Department for Public Health Microbiology, National Laboratory of Health, Environment and Food, Maribor, Slovenia; ²¹University of Edinburgh, The Queens Medical Research Institute, Edinburgh EH16 4TJ, UK; ²²Respiratory Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA; ²³Emory Global Health Institute, Emory University, Atlanta, GA 30322, USA

*Corresponding author. E-mail: sl28@sanger.ac.uk

†Members are listed in the Acknowledgements section.

Received 20 May 2019; returned 15 August 2019; revised 26 September 2019; accepted 16 October 2019

Objectives: We reported *tet(S/M)* in *Streptococcus pneumoniae* and investigated its temporal spread in relation to nationwide clinical interventions.

Methods: We whole-genome sequenced 12 254 pneumococcal isolates from 29 countries on an Illumina HiSeq sequencer. Serotype, multilocus ST and antibiotic resistance were inferred from genomes. An SNP tree was built using Gubbins. Temporal spread was reconstructed using a birth–death model.

Results: We identified *tet(S/M)* in 131 pneumococcal isolates and none carried other known *tet* genes. Tetracycline susceptibility testing results were available for 121 *tet(S/M)*-positive isolates and all were resistant. A majority (74%) of *tet(S/M)*-positive isolates were from South Africa and caused invasive diseases among young children (59% HIV positive, where HIV status was available). All but two *tet(S/M)*-positive isolates belonged to clonal complex (CC) 230. A global phylogeny of CC230 ($n=389$) revealed that *tet(S/M)*-positive isolates formed

a sublineage predicted to exhibit resistance to penicillin, co-trimoxazole, erythromycin and tetracycline. The birth–death model detected an unrecognized outbreak of this sublineage in South Africa between 2000 and 2004 with expected secondary infections (effective reproductive number, R) of ~ 2.5 . R declined to ~ 1.0 in 2005 and < 1.0 in 2012. The declining epidemic could be related to improved access to ART in 2004 and introduction of pneumococcal conjugate vaccine (PCV) in 2009. Capsular switching from vaccine serotype 14 to non-vaccine serotype 23A was observed within the sublineage.

Conclusions: The prevalence of *tet(S/M)* in pneumococci was low and its dissemination was due to an unrecognized outbreak of CC230 in South Africa. Capsular switching in this MDR sublineage highlighted its potential to continue to cause disease in the post-PCV13 era.

Introduction

Streptococcus pneumoniae is a major bacterial cause of disease in young children. Despite the success of pneumococcal conjugate vaccines (PCVs), invasive pneumococcal disease (IPD) remains an important health priority owing to increasing disease incidence caused by pneumococci expressing non-vaccine serotypes, a phenomenon known as serotype replacement.¹ Serotype replacement could be mediated by capsular switching, in which a *cps* locus encoding vaccine-type (VT) capsule is replaced by a *cps* locus encoding non-vaccine-type (NVT) capsule through homologous recombination.² Capsular switching within MDR lineages, especially those recognized by the Pneumococcal Molecular Epidemiology Network (PMEN; <http://spneumoniae.mlst.net/pmen/pmen.asp>), is of increasing concern, as these expansions can reduce overall vaccine effectiveness in preventing IPD and temper the reduction in antimicrobial-resistant pneumococcal infections associated with introduction of PCVs.³ The persistence of the MDR lineage ST156 (Spain9^V-3, PMEN3) in the USA following the introduction of PCV13 provides a clear example of a historically successful lineage that underwent a capsular switch from VT (serotype 9V, 14 and 19A) to NVT (serotype 35B) and continued to cause IPD in the post-vaccine era.^{3–5}

Resistance to tetracycline has been frequently observed in *S. pneumoniae*.⁶ The genetic basis was shown to be *tet(M)*, and less commonly *tet(O)*, which encode for a ribosomal protection protein that prevents tetracycline binding to the bacterial 30S ribosome subunit.^{6,7} Eleven other classes of ribosomal protection proteins such as *tet(S)* and 12 mosaic structures of *tet* genes such as *tet(S/M)* have not been found previously in pneumococci (<http://faculty.washington.edu/marilynr/>). *tet(S)*, originally discovered in *Listeria monocytogenes* strain BM4210,⁸ has occasionally been found in a variety of streptococci, including *Streptococcus suis* (NCBI accession number KX077886),⁹ *Streptococcus infantis* (NCBI accession number JX275965) and *Streptococcus dysgalactiae* (NCBI accession number EF682210)¹⁰ and is associated with a transposase-containing element IS1216, which potentially mediates chromosomal rearrangement. The mosaic *tet(S/M)* has been observed on a Tn916 element in *Streptococcus intermedius*¹¹ and an IS1216 composite in *Streptococcus bovis*.¹² Using a dataset of 12 254 pneumococcal genomes from the Global Pneumococcal Sequencing (GPS) project (<https://www.pneumogen.net/gps/>), we identified a novel genetic basis for tetracycline resistance in *S. pneumoniae*, *tet(S/M)*, and characterized its genetic background in relation to nationwide clinical interventions.

Materials and methods

Isolate collection

In the GPS project, each participating country retrospectively and randomly selected pneumococcal disease isolates collected via laboratory-based surveillance and carriage isolates via cohort studies using the following criteria: $\sim 50\%$ of isolates from children ≤ 2 years old, 25% from children 3–5 years old and 25% from individuals > 5 years old. By May 2017 (last access to the GPS database for this study), 12 254 isolates, representing 29 countries, in Africa (65%), North America (14%), Asia (9%), South America (8%) and Europe (4%), were sequenced, passed quality control and included in this study. The collection spanned 26 years between 1991 and 2016 and included both pneumococcal carriage ($n=4863$) and disease isolates ($n=7391$). We compiled the metadata including age, year of collection, sample source, HIV status and phenotypic antimicrobial susceptibility testing results, where available, from each participating site. In children < 18 months of age, HIV status was confirmed by PCR assay. Tetracycline resistance phenotype of *tet(S/M)*-positive isolates was confirmed by either microbroth dilution or Etest. MIC results were interpreted according to CLSI M100-S24.¹³ When MIC was analysed as ' $>X$ ', MIC was approximated as value 2X for median and IQR calculations.

Genome sequencing and analyses

The pneumococcal isolates were whole-genome sequenced on an Illumina HiSeq platform and raw data were deposited in the European Nucleotide Archive (ENA) (Supplementary metadata, available as [Supplementary data](#) at JAC Online). We inferred serotype, multilocus ST and resistance profile for penicillin, chloramphenicol, co-trimoxazole, erythromycin and tetracycline from the genomic data, as previously described.¹⁴ The *tet(S/M)* gene was identified with a *tet(S/M)* reference sequence (NCBI accession number AY534326) using ARIBA¹⁵ and the promoter region was examined manually by comparing with the reference sequences of *tet(M)* (NCBI accession number M85225) and *tet(S)* (NCBI accession number FN555436). We detected the presence of other *tet* genes in *tet(S/M)*-positive isolates by BLASTing their assemblies against a list of 13 *tet* genes encoding ribosomal protection proteins (Table 1).

To reconstruct a global phylogeny, an additional collection of clonal complex (CC) 230 isolates ($n=130$) from previous studies,^{16–20} together with the CC230 collection ($n=259$) in the GPS dataset were included. The phylogeny was built as previously described.¹⁴ Based on the international genomic definition of pneumococcal lineages, all CC230 isolates in this study belong to GPS Cluster (GPSC)10.¹⁴ The metadata and analysis results of CC230 can be interactively visualized online using the Microreact tool at https://microreact.org/project/GPS_tetSM.

Temporal changes of *tet(S/M)* CC230 sublineage

Coalescent analysis was performed on *tet(S/M)* CC230 sublineage ($n=129$) to date the most recent common ancestor (MRCA) and reconstruct the

Table 1. List of *tet* genes encoding ribosomal protection proteins for BLAST analysis

Gene(s)	NCBI accession number
<i>tet</i> (M)	MH283017
<i>tet</i> (O)	Y07780
<i>tetA</i> (P) and <i>tetB</i> (P)	L20800
<i>tet</i> (Q)	Z21523
<i>tet</i> (S)	FN555436
<i>tet</i> (T)	L42544
<i>tet</i> (W)	AJ222769
<i>tet</i> (32)	AJ295238
<i>tet</i> (36)	AJ514254
<i>tet</i> (44)	FN594949
<i>tet</i> (61)	KY887560
<i>otr</i> (A)	X53401
<i>tet</i>	M74049

The list is adapted from <http://faculty.washington.edu/marilynr/tetweb4.pdf>.

population demographic history. First, we tested the presence of temporal signal by a linear regression of root-to-tip distances against year of collection using TempEst v1.5.²¹ Next, a timed phylogeny was constructed using BEAST v2.4.1.²² The Markov chain Monte Carlo (MCMC) chain was run for 100 million generations, sampled every 1000 states using the general time-reversible (GTR) model of nucleotide substitution and the discrete gamma model of heterogeneity among sites. Finally, the population demographic history was reconstructed using a birth–death model²³ to examine the temporal changes with the *tet*(S/M) CC230 sublineage invasive isolates ($n=105$) but not carriage isolates ($n=24$), because the model assumes that once an individual is diagnosed with IPD, the individual is no longer transmitting due to treatment and recovery, death or being socially removed from susceptible individuals. Thus, it appeared to be logical to apply this model to the disease but not carriage isolates. This model overcomes the limitations of the coalescent-based skyline plot and is able to examine whether introduction of an intervention had an impact on the epidemiological dynamics in a bacterial population.²⁴ The birth–death skyline plot shows the effective reproductive number (R) over time. R is defined as the number of expected secondary infections from an infected individual. $R > 1$ indicates a growing epidemic, whereas $R < 1$ indicates a declining epidemic. Notably, $R \geq 1$ can be reflected in the coalescent-based skyline plot analysis, whereas $R < 1$ cannot. Therefore, we expected the birth–death skyline model would be a better fit for our data. Other Bayesian population-size models (coalescent constant, coalescent exponential and Bayesian skyline), in combination with strict and lognormal-relaxed molecular clocks, were also applied for comparisons using BEAST.

Integrative and conjugative element (ICE)

The ICE was extracted from the *de novo* assemblies of CC230 isolates and compared using EasyFig version 2.2.2. The NCBI accession numbers for the representative ICE sequences in Figure 5 are FM211187 [ICESp23FST81], MH283017 [ICESp14ST230 with *tet*(M)], MH283012 [ICESp14ST230 with *tet*(S/M) and omega cassettes], MH283013 [ICESp14ST230 with *tet*(M) and omega], MH283012 [ICESp14ST230 with *tet*(M) and Tn917], MH283016 [ICESp19AST2013], MH283015 [ICESp17FST8812] and MH283014 [ICESp14ST156].

Results

Prevalence of *tet*(S/M) in a global collection of *S. pneumoniae*

A tetracycline resistance gene, *tet*(S/M), was identified in 131 pneumococcal isolates (1%, 131/12 254) from South Africa ($n=123$), Malawi ($n=5$), Brazil ($n=1$), Mozambique ($n=1$) and the USA ($n=1$). They were isolated from sterile body sites (invasive isolates) [blood ($n=73$), CSF ($n=30$) and pleural fluid ($n=4$)] and from the nasopharynx (carriage isolates) ($n=24$). In South Africa, *tet*(S/M) was found in 3.5% (103/2920) of the invasive isolates that were submitted to the GPS project from 2005 to 2014 and 1.2% (20/1701) of the carriage isolates that were collected in Agincourt and Soweto between 2009 and 2013. Of the 103 invasive isolates, 94% (97/103) were from children with IPD aged ≤ 5 years (Figure S1). HIV status was known in only 44% (54/123) of individuals with *tet*(S/M)-positive pneumococci; 59% (32/54) were HIV positive, of which 94% (30/32) were children aged ≤ 5 years.

MIC of tetracycline was determined for 121 *tet*(S/M)-positive isolates by either Etest ($n=73$) or broth dilution ($n=48$). The remaining 10 isolates were either non-viable ($n=5$) or unable to be tested due to inadequate resources ($n=5$). The median MIC was 8 mg/L (IQR 6–8) by Etest and 16 mg/L by broth dilution. Based on the CLSI guideline, 99% (120/121) and 1% (1/121) were fully (≥ 4 mg/L) and intermediately (2–3 mg/L) resistant to tetracycline, respectively. The *tet*(S/M) in this study showed 100% nucleotide identity, except for one isolate (GPS_ZA_1982) from South Africa, which varied from the others (G1769A) and resulted in the substitution R590Q. This isolate remained resistant to tetracycline with an MIC of >8 mg/L when measured by broth dilution. Unlike the two previously reported *tet*(S/M) alleles from *S. intermedius*¹¹ and *S. bovis*,¹² the amino acid sequence of Tet(S/M) in this study showed 100% identity to Tet(S) (NCBI accession number FN555436) across the first 613 amino acids, with the final 32 amino acids at the C-terminus end being identical to Tet(M) (NCBI accession number M85225) (Figure 1). The promoter region was intact in all *tet*(S/M)-positive isolates and was of *tet*(M) origin, rather than *tet*(S) origin. Between the promoter region and the start codon of *tet*(S/M), a 38 bp stem loop, which is potentially involved in transcriptional regulation,²⁵ was found in all *tet*(S/M) genes (Figure 1), apart from one disease isolate (GPS_ZA_1926) from South Africa. The deletion did not affect the tetracycline resistance level, as the MIC remained at >8 mg/L when measured by the broth dilution method. No other *tet* genes (Table 1) were detected in any *tet*(S/M)-positive isolates, strongly indicating that Tet(S/M) conferred resistance to tetracycline in *S. pneumoniae*.

Phylogeny and characteristics of the *tet*(S/M) CC230 sublineage

Of the 131 *tet*(S/M)-positive isolates, 129 belonged to CC230; one Brazilian and one Malawian isolate belonged to CC156 and ST5359 (a singleton not belonging to any CC), respectively. The global CC230 phylogeny showed that all *tet*(S/M)-positive isolates formed a sublineage predicted to be resistant to penicillin, erythromycin, tetracycline and co-trimoxazole (Figure 2). The *tet*(S/M) sublineage was associated predominantly with VT 14 (98%, 127/129) but was also found in two NVT 23A isolates. The two serotype 23A isolates, which both belonged to ST11106 (a single-locus variant of ST230),

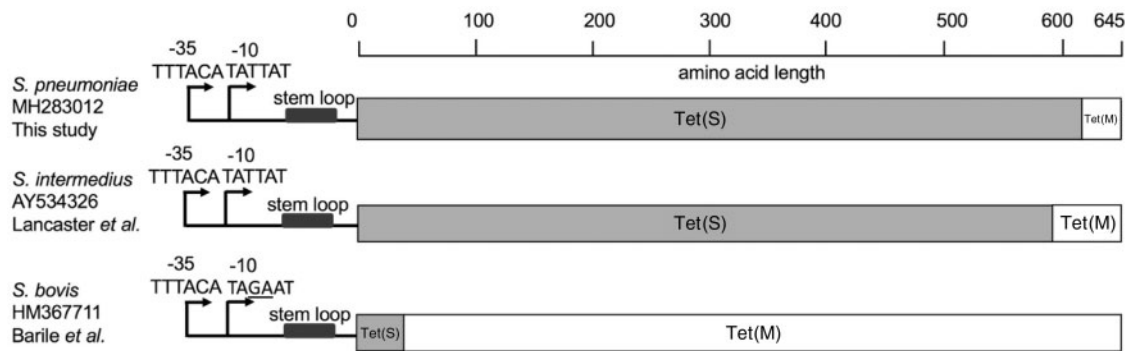


Figure 1. Schematic representation of the mosaic structure of *tet(S/M)* alleles of the current and previous studies. The bars in grey and in white indicate amino acid sequences with high identity to Tet(S) and Tet(M), respectively. The reference sequences for *tet(M)* and *tet(S)* were retrieved from NCBI GenBank using accession numbers M85225 and FN555436, respectively.

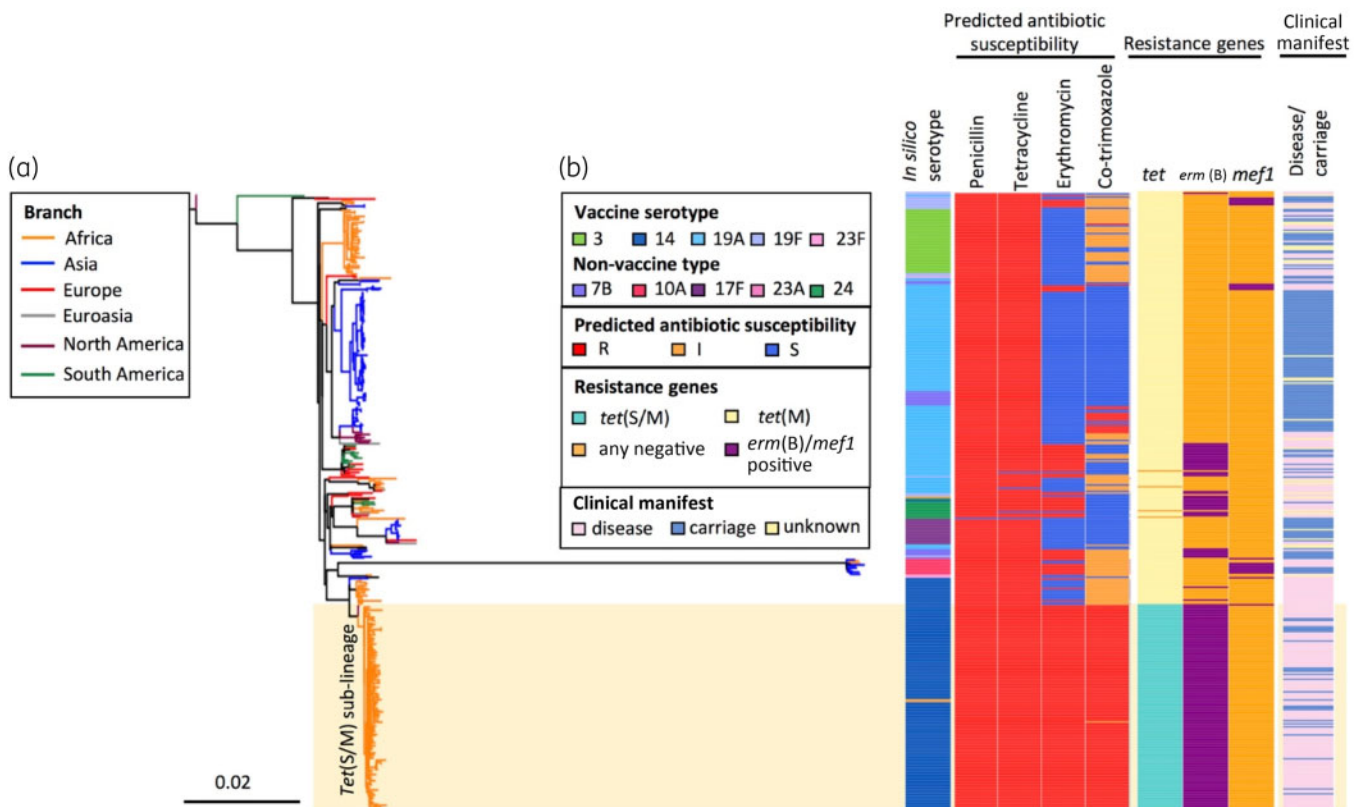


Figure 2. An SNP tree constructed with CC230 *tet(S/M)*-positive isolates ($n=129$) and *tet(S/M)*-negative carriage/disease isolates ($n=260$) collected from 20 countries. The tree was built based on 13 405 SNPs extracted from an alignment outside recombination regions, created by mapping reads of each isolate to the sequence of an ST230 reference strain, PMEN global clone Denmark¹⁴⁻³², PMEN32 (ENA accession number ERS1706837). Penicillin resistance was predicted based on the *pbp1a*, *pbp2x* and *pbp2b* sequences;^{38,39} tetracycline and erythromycin resistance were predicted based on the presence of *tet(M)*, *tet(O)* and *tet(S/M)*, and *erm(B)* and *mef(A)*, respectively. Co-trimoxazole resistance was predicted based on the presence of mutation I100L in *folA* and any indel within amino acid residues 56–67 in *folP*, while the presence of either mutation was predicted to confer a co-trimoxazole-intermediate phenotype.

were recovered from infants after the introduction of PCV13. One was isolated from a nasopharyngeal sample in Soweto in 2012 and the other from blood culture in Johannesburg in 2014. The serotype 23A *cps* locus sequences of these two isolates were

identical and their *cps*-flanking *pbp* loci (*pbp1a* and *pbp2x*) were also identical to the majority of the serotype 14 isolates within the *tet(S/M)* sublineage, exhibiting resistance to penicillin with an MIC of 2 mg/L. To identify the potential donor of the serotype 23A *cps*

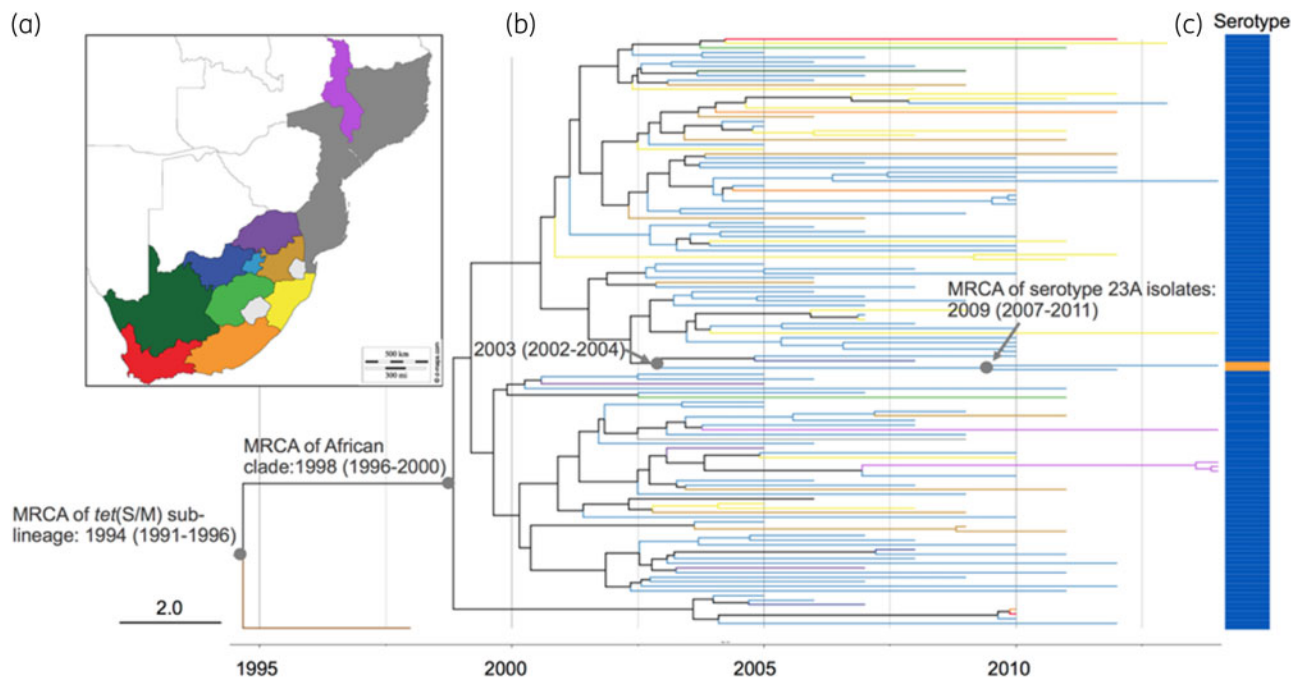


Figure 3. (a) Malawi, Mozambique and administration regions of South Africa. (b) Timed phylogeny for *S. pneumoniae* tet(S/M) CC230 sublineage ($n=129$) reconstructed using BEAST. Tree branches are coloured according to the geographical locations in (a), except for the branch for an isolate collected from the USA, coloured in brown. (c) Vaccine serotype 14 is indicated in blue, whereas non-vaccine serotype 23A is indicated in orange.

locus, a phylogenetic tree was built using the *cps* sequences from all serotype 23A ($n=130$, belong to eight lineages) pneumococci in the GPS database. This analysis showed that the serotype 23A *cps* loci of these two CC230 isolates clustered with those originating from a serogroup 23 lineage GPSC7, which is predominantly (99%, 145/146) represented by CC439 (Figure S2), with pairwise nucleotide similarity of 99.97% (24 818/24 825) and 100% coverage. The seven nucleotide variations were found within the IS630 transposase downstream of *dexB*.

Temporal spread of the tet(S/M) CC230 sublineage

The sublineage showed a temporal signal in terms of SNP accumulation against time ($R^2 = 0.4094$, $P = 0.001$; Figure S3). Using a birth–death model in BEAST, the tet(S/M) sublineage was estimated to emerge around 1994 [95% highest posterior density (HPD): 1991–96]; the MRCA for the African clade was 1998 (95% HPD: 1996–2000) and for the two serotype 23A isolates was 2009 (95% HPD: 2007–11) (Figure 3). The temporal changes of spread were reconstructed based on a birth–death skyline plot and a coalescent-based skyline plot (Figure 4). Both skyline plots showed that the tet(S/M) sublineage expanded at the beginning of the year 2000 and growth continued until around 2004. The decline of the tet(S/M) sublineage was only captured by the birth–death skyline plot in or around 2005, from expected secondary infections (R) of ~ 2.5 to ~ 1 , and steadily declined until 2012 when the median and HPD of R were < 1 , indicating a declining epidemic. The coalescent-based skyline plot failed to detect the impact of the epidemic decline, as described in a previous study.²³

ICE carrying tet(S/M)

The acquisition of tetracycline and erythromycin resistance determinants by CC230 was the result of the insertion of a Tn5253-type ICE, which shared a similar structure to ICESp23FST81 identified in PMEN1 (Figure 5). Both the tet(M) ($n=255$) and tet(S/M) ($n=131$) genes detected in this study were carried on a conserved conjugative Tn916 transposon (Figure S4). Of the 172 macrolide-resistant isolates, insertions of either the ‘omega’ element ($n=165$) or Tn917 ($n=7$) harbouring *erm(B)* were found upstream or downstream of the tet gene, respectively (Figure 5). The insertion of the ‘omega’ element truncated the gene encoding the replication initiation factor, creating an 8 bp DR, CAAAAAAA. The insertion of Tn917 disrupted the gene *orf9*, which encodes a putative conjugative transposon regulator; no DRs were found.

Discussion

We used WGS to identify a novel mosaic structure of tet(S/M) in *S. pneumoniae*. This approach overcame the limitation of PCR that requires specific primers to detect known antibiotic resistance genes. Compared with tet(M), the prevalence of tet(S/M) was low. They were mainly found in a CC230 sublineage that predominantly expressed VT 14 and exhibited MDR in South Africa. Together with its conserved nucleotide sequence and genomic location, our finding strongly suggested a clonal expansion of tet(S/M)-positive CC230 isolates within South Africa prior to the introduction of PCVs.

Unlike what was observed in other countries, CC230 in South Africa predominantly expressed a highly invasive serotype 14 capsule¹⁴ and was the clone that represented most of the serotype 14 isolates (43%) in the pre-vaccine era, when serotype 14 was the most prevalent serotype causing IPD in South Africa.²⁶ Any

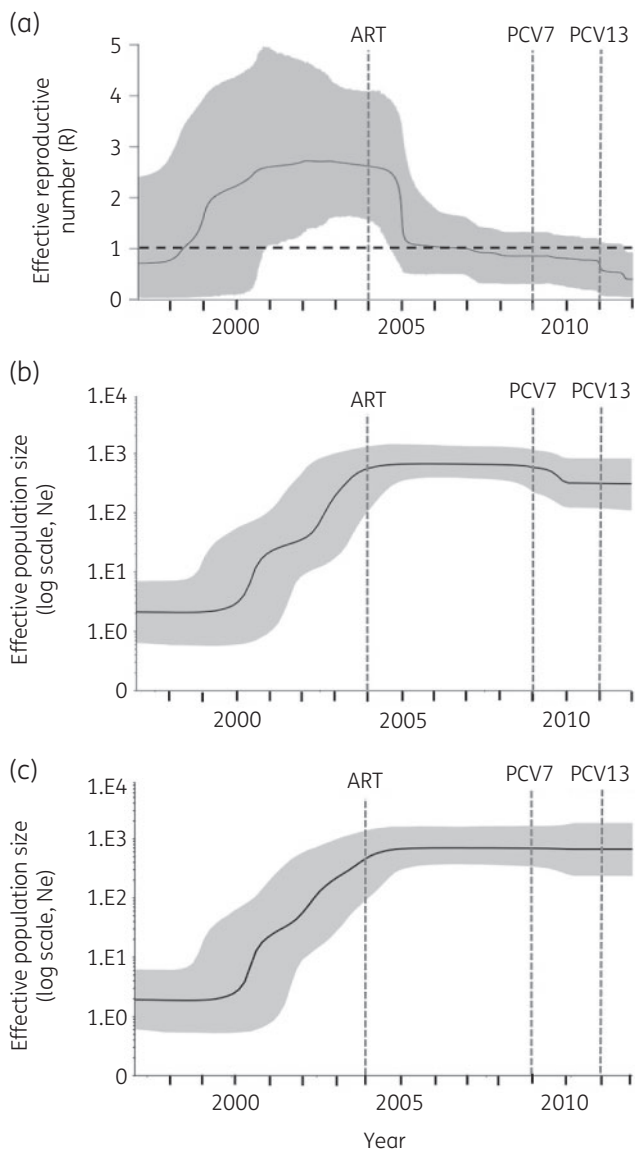


Figure 4. (a) Birth–death skyline plot of inferred changes in R of *S. pneumoniae tet(S/M)* CC230 sublineage using IPD isolates ($n=105$). (b) Coalescent-based skyline plot of inferred changes in the effective population size (N_e) of the *S. pneumoniae tet(S/M)* CC230 sublineage using both IPD and carriage isolates ($n=129$) and (c) using only IPD isolates ($n=105$). The black continuous line shows the median of R in (a) and N_e in (b) and (c). The background area represents the 95% HPD intervals. $R>1$ indicates a growing epidemic, whereas $R<1$ indicates a declining epidemic.

controlling measure to decrease this lineage would not only result in a reduction in the IPD burden but also MDR IPD incidence.

The birth–death model estimated that the decline of the *tet(S/M)* CC230 sublineage started around 2005, one year after the national ART programme was launched to treat HIV-infected individuals in South Africa.²⁷ The prediction was consistent with a 41% reduction of IPD incidence among HIV-infected children after the introduction of ART.²⁷ Among the IPD caused by *tet(S/M)* CC230 isolates, almost 60% occurred in HIV-positive children.

This observational evidence strengthened the suggestion that ART was likely to contribute to the decline before PCV introduction. In contrast, the large-scale use of co-trimoxazole as prophylaxis to prevent bacterial infections among HIV-positive individuals was unlikely to be responsible for the decline, as the sublineage was resistant to co-trimoxazole. The further decline in 2012 predicted by the model echoed the epidemiological finding that IPD caused by VT pneumococci significantly decreased among children in 2012.²⁸ Our finding demonstrated that we could effectively reconstruct the temporal spread of an epidemic using genomic data and highlighted the possible use of routine genomic surveillance to model outbreaks as they occur.

The MRCA of two CC230 isolates expressing NVT 23A was dated to emerge around 2009, the year when PCV7 was introduced. However, the long branch leading to the MRCA from the internal node that was shared with the closely related serotype 14 isolates indicated that the window of time for capsular switching could be between 2002 and 2011. Although the invasive disease potential for serotype 23A is low,¹⁴ a significant increase of this serotype in IPD cases was reported from England,²⁹ Stockholm³⁰ and Taiwan³¹ after the implementation of PCV13. Serotype 23A is primarily associated with CC338 (GPSC5, PMEN26) and CC439 (GPSC7) and is thus rarely found in a CC230 genetic background. Such serotype and genotype combinations were only identified in two ST9396 isolates (single-locus variant of ST230) from China in 2013 and one ST10921 isolate (double-locus variant of ST230) from Poland in 2013 in the MLST database. In South Africa, CC439 accounted for 62% of serotype 23A (both carriage and disease) isolates¹⁴ and is the potential donor of the serotype 23A *cps* to the *tet(S/M)* CC230 sublineage, highlighting that capsular switching with the prevalent NVT lineage could enable a VT lineage to evade the vaccine. Capsular switching is usually a result of homologous recombination. When compared with 620 other GPSCs, GPSC10, including 98% (258/262) of CC230 isolates, is a very recombinogenic lineage, which had a significantly high recombination rate [ratio of base substitutions predicted to have been imported through recombination to those occurring through point mutation for GPSC10: 10.9 versus median of 35 dominant GPSCs: 8.3 (IQR, 5.7–10.7), $P<0.0001$, Wilcoxon signed-rank test].¹⁴ Given this recombinogenic nature, together with the established MDR genotypes, it is of concern that any further capsular switching may increase the chance of this MDR lineage surviving and continuing to cause invasive disease.

Like *tet(M)*, *tet(S/M)* was also carried by Tn916, which was reported as a mobile conjugative transposon with a broad host range.¹¹ The conserved genetic environment of *tet(M)* and *tet(S/M)* indicates that the recombination resulting in the mosaic structure of *tet(S/M)* probably occurred after the acquisition of the gene by Tn916. Comparison of *tet(M)* sequences in the current collection also revealed a high degree of allelic variations that were probably due to homologous recombination.³² This finding is consistent with previous studies that suggested that *tet* evolved separately from Tn916.^{6,32} However, the driving force behind the evolution of *tet* genes remains unclear, given that tetracycline was not used as a first-line antibiotic to treat pneumococcal disease and was seldom used in young children.³³ The allelic diversity of *tet* may be maintained by: (i) frequent recombination among *S. pneumoniae* and with closely related species such as normal nasopharyngeal resident *Streptococcus mitis*³⁴ and the zoonotic pathogen *S. suis*;³⁵

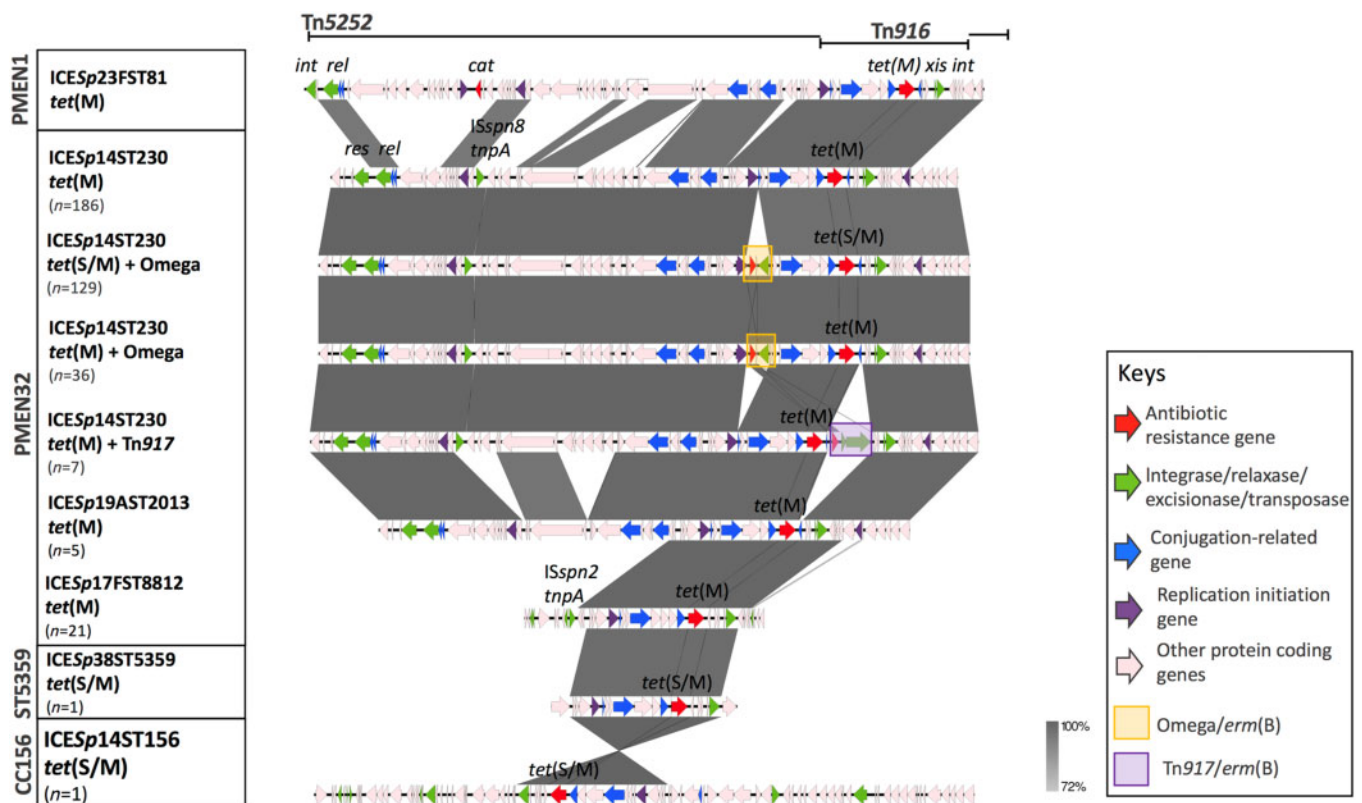


Figure 5. Comparison of ICE identified in CC230 with Spain23^F-1 (PMEN1). Grey bands between the sequences indicate BLASTN matches.

and (ii) antibiotic-selective pressure via the food chain, as tetracycline is widely used in agriculture³⁶ and its residue is detected in milk.³⁷ Future studies that investigate the driving force behind the evolution of *tet* will improve our understanding to develop preventive measures to reduce tetracycline resistance in *S. pneumoniae*.

In conclusion, we identified the tetracycline-resistance determinant *tet(S/M)* in *S. pneumoniae* and showed that its dissemination is due to a clonal expansion of the MDR lineage CC230 in South Africa, where the HIV burden is high. With genomic data, we successfully detected the decline in transmission of this MDR lineage using a birth–death model and the fall of this lineage may correlate with the improved treatment of HIV-infected individuals and the implementation of PCVs. Capsular switching within this lineage is potentially of public health importance and may erode the beneficial effect brought about by the implementation of PCVs. The capacity for continuous genomic surveillance in the post-vaccine era provides critical opportunities for monitoring and forecasting the rise of MDR pneumococcal lineages that may also undergo vaccine evasion through capsular switching events.

Acknowledgements

We would like to acknowledge the Bill & Melinda Gates Foundation (grant code OPP1034556) and the Wellcome Sanger Institute (core Wellcome grants 098051 and 206194) for funding this study, as part of the GPS project. We deeply appreciate all members of the Global Pneumococcal Sequencing Consortium for their collaborative spirit and

determination in the monumental task of sampling, extracting data and for their intellectual input to this manuscript. We extend our special thanks to Linda De Gouveia from the National Institute for Communicable Diseases of the National Health Laboratory Service, South Africa who helped us with the antimicrobial susceptibility testing. We appreciated critiques and suggestions from all team members in the Genomics of Pneumonia and Meningitis (and neonatal sepsis) group and technical support from the Pathogen Informatic Team in the Parasites and Microbe Programme at the Wellcome Sanger Institute.

Members of the Global Pneumococcal Sequencing Consortium

Abdullah W. Brooks, Alejandra Corso, Alexander Davydov, Alison Maguire, Andrew J. Pollard, Anmol Kiran, Anna Skoczynska, Benild Moiane, Betuel Sigauque, David Aanensen, Deborah Lehmann, Diego Faccone, Ebenezer Foster-Nyarko, Ebrima Bojang, Elena Voropaeva, Eric Sampene-Donkor, Ewa Sadowy, Geetha Nagaraj, Godfrey Bigogo, Helio Mucavele, Houria Belabbès, Idrissa Diawara, Jennifer Moisi, Jennifer Verani, Jeremy Keenan, Jyothish N. Nair Thulasee Bhai, Kedibone M. Ndlangisa, Khalid Zerouali, Leon J. Bentley, Linda De Gouveia, Maaiké Alaerts, Maria-Cristina de Cunto Brandileone, Margaret Ip, Md Hasanuzzaman, Metka Paragi, Mushal Ali, Nicholas Croucher, Nicole Wolter, Noga Givon-Lavi, Özgen Köseoglu Eser, Pak Leung Ho, Patrick E. Akpaka, Paul Turner, Paula Galletti, Peggy-Estelle Tientcheu, Philip E. Carter, Pierra Law, Rachel Benisty, Rafal Mostowy, Rebecca Ford, Rebecca Henderson, Roly Malaker, Ron Dagan, Sadia Shakoar, Sanjay Doiphode, Shamala Devi Sekaran, Somporn Srifuengfung, Shamala Devi Sekaran, Somporn Srifuengfung, Stephen Obaro, Stuart C. Clarke, Tamara Kastrian, Theresa J. Ochoa, Waleria Hryniewicz, Veeraraghavan Balaji and Yulia Urban.

Funding

This work was co-funded by the Bill & Melinda Gates Foundation (grant code OPP1034556), the Wellcome Sanger Institute (core Wellcome grants 098051 and 206194) and the US Centers for Disease Control and Prevention.

Transparency declarations

R. A. Gladstone reports a PhD studentship from Pfizer, outside the submitted work. A. Skoczynska reports grants from Pfizer, assistance to attend scientific meetings and honoraria for lecturing from GlaxoSmithKline and Pfizer, and participation in Advisory Boards of GlaxoSmithKline and Pfizer, outside the submitted work. S. D. Bentley reports personal fees from Pfizer and personal fees from Merck, outside the submitted work. A. von Gottberg reports grants and other from Pfizer, during the conduct of the study, and grants and other from Sanofi, outside the submitted work. All other authors: none to declare.

Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Supplementary data

Supplementary metadata and Figures S1 to S4 are available as [Supplementary data](#) at JAC Online.

References

- Lo SW, Gladstone RA, van Tonder AJ *et al.* Pneumococcal lineages associated with serotype replacement and antibiotic resistance in childhood invasive pneumococcal disease in the post-PCV13 era: an international whole-genome sequencing study. *Lancet Infect Dis* 2019; **19**: 759–69.
- Wyres KL, Lambertsen LM, Croucher NJ *et al.* Pneumococcal capsular switching: a historical perspective. *J Infect Dis* 2013; **207**: 439–49.
- Metcalfe BJ, Gertz RE Jr, Gladstone RA *et al.* Strain features and distributions in pneumococci from children with invasive disease before and after 13-valent conjugate vaccine implementation in the USA. *Clin Microbiol Infect* 2016; **22**: 60.e9–29.
- Chochua S, Metcalfe BJ, Li Z *et al.* Invasive serotype 35B pneumococci including an expanding serotype switch lineage, United States, 2015–2016. *Emerg Infect Dis* 2017; **23**: 922–30.
- Olarte L, Kaplan SL, Barson WJ *et al.* Emergence of multidrug-resistant pneumococcal serotype 35B among children in the United States. *J Clin Microbiol* 2017; **55**: 724–34.
- Wyres KL, van Tonder A, Lambertsen LM *et al.* Evidence of antimicrobial resistance-conferring genetic elements among pneumococci isolated prior to 1974. *BMC Genomics* 2013; **14**: 500.
- Widdowson CA, Klugman KP, Hanslo D. Identification of the tetracycline resistance gene, *tet(O)*, in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1996; **40**: 2891–3.
- Charpentier E, Gerbaud G, Courvalin P. Characterization of a new class of tetracycline-resistance gene *tet(S)* in *Listeria monocytogenes* BM4210. *Gene* 1993; **131**: 27–34.
- Huang J, Ma J, Shang K *et al.* Evolution and diversity of the antimicrobial resistance associated mobilome in *Streptococcus suis*: a probable mobile genetic elements reservoir for other streptococci. *Front Cell Infect Microbiol* 2016; **6**: 118.
- Liu LC, Tsai JC, Hsueh PR *et al.* Identification of *tet(S)* gene area in tetracycline-resistant *Streptococcus dysgalactiae* subsp. *equisimilis* clinical isolates. *J Antimicrob Chemother* 2008; **61**: 453–5.
- Lancaster H, Roberts AP, Bedi R *et al.* Characterization of Tn916S, a Tn916-like element containing the tetracycline resistance determinant *tet(S)*. *J Bacteriol* 2004; **186**: 4395–8.
- Barile S, Devirgiliis C, Perozzi G. Molecular characterization of a novel mosaic *tet(S/M)* gene encoding tetracycline resistance in foodborne strains of *Streptococcus bovis*. *Microbiology* 2012; **158**: 2353–62.
- CLSI. *Performance Standards for Antimicrobial Susceptibility Testing—Twenty-Fourth Edition: M100*. 2014.
- Gladstone RA, Lo SW, Lees JA *et al.* International genomic definition of pneumococcal lineages, to contextualise disease, antibiotic resistance and vaccine impact. *EBioMedicine* 2019; **43**: 338–46.
- Hunt M, Mather AE, Sanchez-Buso L *et al.* ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. *Microb Genom* 2017; **3**: e000131.
- Chewapreecha C, Harris SR, Croucher NJ *et al.* Dense genomic sampling identifies highways of pneumococcal recombination. *Nat Genet* 2014; **46**: 305–9.
- Quirk SJ, Haraldsson G, Erlendsdottir H *et al.* Effect of vaccination on pneumococci isolated from the nasopharynx of healthy children and the middle ear of children with otitis media in Iceland. *J Clin Microbiol* 2018; **56**: e01046–18.
- Chaguzo C, Cornick JE, Andam CP *et al.* Population genetic structure, antibiotic resistance, capsule switching and evolution of invasive pneumococci before conjugate vaccination in Malawi. *Vaccine* 2017; **35**: 4594–602.
- Cremers AJ, Mobegi FM, de Jonge MI *et al.* The post-vaccine microevolution of invasive *Streptococcus pneumoniae*. *Sci Rep* 2015; **5**: 14952.
- Gladstone RA, Devine V, Jones J *et al.* Pre-vaccine serotype composition within a lineage signposts its serotype replacement – a carriage study over 7 years following pneumococcal conjugate vaccine use in the UK. *Microbial Genomics* 2017; **3**: e000119.
- Rambaut A, Lam TT, Max Carvalho L *et al.* Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol* 2016; **2**: vew007.
- Bouckaert R, Heled J, Kuhnert D *et al.* BEAST 2: a software platform for Bayesian evolutionary analysis. *PLoS Comput Biol* 2014; **10**: e1003537.
- Stadler T, Kuhnert D, Bonhoeffer S *et al.* Birth–death skyline plot reveals temporal changes of epidemic spread in HIV and hepatitis C virus (HCV). *Proc Natl Acad Sci USA* 2013; **110**: 228–33.
- Kuhnert D, Coscolla M, Brites D *et al.* Tuberculosis outbreak investigation using phylodynamic analysis. *Epidemics* 2018; **25**: 47–53.
- Chopra I, Roberts M. Tetracycline antibiotics: mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiol Mol Biol Rev* 2001; **65**: 232–60.
- Ndlangisa KM, du Plessis M, Wolter N *et al.* Population snapshot of *Streptococcus pneumoniae* causing invasive disease in South Africa prior to introduction of pneumococcal conjugate vaccines. *PLoS One* 2014; **9**: e107666.
- Nunes MC, von Gottberg A, de Gouveia L *et al.* The impact of antiretroviral treatment on the burden of invasive pneumococcal disease in South African children: a time series analysis. *AIDS* 2011; **25**: 453–62.

- 28** von Gottberg A, de Gouveia L, Tempia S et al. Effects of vaccination on invasive pneumococcal disease in South Africa. *N Engl J Med* 2014; **371**: 1889–99.
- 29** Moore CE, Paul J, Foster D et al. Reduction of invasive pneumococcal disease 3 years after the introduction of the 13-valent conjugate vaccine in the Oxfordshire region of England. *J Infect Dis* 2014; **210**: 1001–11.
- 30** Galanis I, Lindstrand A, Darenberg J et al. Effects of PCV7 and PCV13 on invasive pneumococcal disease and carriage in Stockholm, Sweden. *Eur Respir J* 2016; **47**: 1208–18.
- 31** Su LH, Kuo AJ, Chia JH et al. Evolving pneumococcal serotypes and sequence types in relation to high antibiotic stress and conditional pneumococcal immunization. *Sci Rep* 2015; **5**: 15843.
- 32** Oggioni MR, Dowson CG, Smith JM et al. The tetracycline resistance gene *tet(M)* exhibits mosaic structure. *Plasmid* 1996; **35**: 156–63.
- 33** WHO. Scientific basis of WHO recommendations for treatment of pneumonia. In: *Revised WHO Classification and Treatment of Pneumonia in Children at Health Facilities: Evidence Summaries*. 2014. <https://www.ncbi.nlm.nih.gov/books/NBK264159/>.
- 34** Kilian M, Riley DR, Jensen A et al. Parallel evolution of *Streptococcus pneumoniae* and *Streptococcus mitis* to pathogenic and mutualistic lifestyles. *MBio* 2014; **5**: e01490–14.
- 35** Ye C, Bai X, Zhang J et al. Spread of *Streptococcus suis* sequence type 7, China. *Emerg Infect Dis* 2008; **14**: 787–91.
- 36** Granados-Chinchilla F, Rodriguez C. Tetracyclines in food and feeding-stuffs: from regulation to analytical methods, bacterial resistance, and environmental and health implications. *J Anal Methods Chem* 2017; **2017**: 1315497.
- 37** de Albuquerque Fernandes SA, Magnavita AP, Ferrao SP et al. Daily ingestion of tetracycline residue present in pasteurized milk: a public health problem. *Environ Sci Pollut Res Int* 2014; **21**: 3427–34.
- 38** Li Y, Metcalf BJ, Chochua S et al. Penicillin-binding protein transpeptidase signatures for tracking and predicting β -lactam resistance levels in *Streptococcus pneumoniae*. *MBio* 2016; **7**: e00756–16.
- 39** Li Y, Metcalf BJ, Chochua S et al. Validation of β -lactam minimum inhibitory concentration predictions for pneumococcal isolates with newly encountered penicillin binding protein (PBP) sequences. *BMC Genomics* 2017; **18**: 621.