A novel mosaic tetracycline resistance gene *tet*(S/M) detected in a multidrug-resistant pneumococcal CC230 lineage that underwent capsular switching in South Africa

Stephanie W. Lo,^{a#} Rebecca A. Gladstone,^a Andries J. van Tonder,^a Mignon du Plessis,^{b,c} Jennifer E. Cornick,^{d,e} Paulina A. Hawkins,^f Shabir A. Madhi,^{h,i} Susan A. Nzenze,^{h,i} Rama Kandasamy,^j KL Ravikumar,^k Naima Elmdaghri,^{l,m} Brenda Kwambana-Adams,^{n,o} Samanta Cristine Grassi Almeida,^p Anna Skoczynska,^q Ekaterina Egorova,^r Leonid Titov,^s Samir K. Saha,^t Metka Paragi,^u Dean B. Everett,^{d,v} Martin Antonio,^o Keith P. Klugman,^{b,c,f,h} Yuan Li,^g Benjamin J Metcalf,^g Bernard Beall,^g Lesley McGee,^g Robert F. Breiman,^{f,w} Stephen D. Bentley,^{a#} Anne von Gottberg,^{b,c#}, on behalf of The Global Pneumococcal Sequencing Consortium †

^aParasites and Microbes programme, The Wellcome Sanger Institute, Wellcome Genome Campus, Hinxton, Cambridge, CB10 1SA, UK

^bCentre for Respiratory Disease and Meningitis, National Institute for Communicable Diseases, Johannesburg, South Africa

^cSchool of Pathology, University of the Witwatersrand, Johannesburg, South Africa

^dMalawi Liverpool Wellcome Trust Clinical Research Programme, P.O. Box 30096, Blantyre, Malawi

^eInstitute of Infection & Global Health, University of Liverpool, Liverpool, L69 7BE, UK

^fHubert Department of Global Health, Rollins School of Public Health, Emory University, Atlanta, GA 30322, USA

^gRespiratory Diseases Branch, Centers for Disease Control and Prevention, Atlanta, GA 30333, USA

^hMedical Research Council: Respiratory and Meningeal Pathogens Research Unit, University of the Witwatersrand, Johannesburg, South Africa

- 26 ⁱDepartment of Science and Technology/National Research Foundation: Vaccine Preventable
- 27 Diseases, University of the Witwatersrand, Johannesburg, South Africa
- 28 ^jOxford Vaccine Group, Department of Paediatrics, University of Oxford, and the NIHR
- 29 Oxford Biomedical Research Centre, Oxford, OX3 9DU, UK
- 30 ^kDepartment of Microbiology, Kempegowda Institute of Medical Sciences Hospital &
- 31 Research Centre, Bangalore, India
- 32 ^lDepartment of Microbiology, Faculty of Medicine and Pharmacy, B.P. 9154, Hassan II
- 33 University of Casablanca, Morocco
- 34 ^mBacteriology-Virology and Hospital Hygiene Laboratory, University Hospital Centre Ibn
- 35 Rochd, 1, Rue des, Casablanca, Morocco
- 36 ⁿNIHR Global Health Research Unit on Mucosal Pathogens, Division of Infection and
- 37 Immunity, University College London, London, United Kingdom.
- 38 ^oWHO Collaborating Centre for New Vaccines Surveillance, Medical Research Council Unit
- 39 The Gambia at The London School of Hygiene and Tropical Medicine, Fajara, The Gambia.
- 40 ^pNational Laboratory for Meningitis and Pneumococcal Infections, Center of Bacteriology,
- 41 Institute Adolfo Lutz (IAL), São Paulo, Brazil
- 42 ^qDepartment of Epidemiology and Clinical Microbiology, National Medicines Institute,
- 43 Warsaw, Poland.
- 44 ^rLaboratory of Clinical Microbiology and Biotechnology, Moscow Research Institute for
- 45 Epidemiology and Microbiology, Moscow, Russia Federation
- 46 ^sLaboratory of Clinical and Experimental Microbiology, the Republican research and
- 47 practical center of Epidemiology and Microbiology, Minsk, Belarus
- 48 ^tDepartment of Microbiology, Dhaka Shishu (Children) Hospital, Child Health Research
- 49 Foundation, Dhaka, Bangladesh

^uDepartment for Public Health Microbiology, National Laboratory of Health, Environment and Food, Maribor, Slovenia

^vUniversity of Edinburgh, The Queens Medical Research Institute, Edinburgh, EH16 4TJ, UK

^wEmory Global Health Institute, Emory University, Atlanta, GA 30322, USA

[†]Members are listed in the Acknowledgement section

#Anne von Gottberg annev@nicd.ac.za

Respiratory and Meningeal Pathogens Research Unit,

National Institute for Communicable Diseases,

Private Bag X4, Sandringham, 2131,

Gauteng, South Africa

Tel: +27 0117171000

Stephen D. Bentley sdb@sanger.ac.uk

D247, Sulston Building,

Wellcome Sanger Institute, Hinxton,

Cambridgeshire, CB10 1SA, United Kingdom

Tel: +44 01223494942

Stephanie W. Lo, sl28@sanger.ac.uk

D203-204, Sulston Building,

Wellcome Sanger Institute, Hinxton,

Cambridgeshire, CB10 1SA, United Kingdom

Tel: +44 01223497253

Synopsis

Objective We reported a novel tetracycline-resistant gene in *Streptococcus pneumoniae* and investigated its temporal spread in relation to nationwide clinical interventions.

Methods We whole genome sequenced 12,254 pneumococcal isolates from twenty-nine countries on an Illumina HiSeq Sequencer. Serotypes, sequence types and antibiotic resistance were inferred from genomes. Phylogeny was built based on single-nucleotide variants. Temporal changes of spread were reconstructed using a birth-death model.

Results We identified *tet*(S/M) in 131 pneumococcal isolates, 97 (74%) caused invasive pneumococcal diseases among young children (59% HIV-positive, where HIV status was available) in South Africa. A majority of *tet*(S/M)-positive isolates (129/131) belong to clonal complex (CC)230. A global phylogeny of CC230 (n=389) revealed that *tet*(S/M)-positive isolates formed a sub-lineage that exhibited multidrug-resistance. Using the genomic data and a birth-death model, we detected an unrecognised outbreak of this sub-lineage in South Africa between 2000 and 2004 with an expected secondary infections (R) of ~2.5. R declined to ~1.0 in 2005 and <1.0 in 2012. The declining epidemic coincided and could be related to the nationwide implementation of anti-retroviral treatment (ART) for HIV-infected individuals in 2004 and PCVs in late 2000s. Capsular switching from vaccine serotype 14 to non-vaccine serotype 23A was observed within the sub-lineage.

Conclusions The prevalence of *tet*(S/M) in pneumococci was low and its dissemination was due to an unrecognised outbreak of CC230 in South Africa prior to ART and PCVs. However, capsular switching in this multidrug-resistant sub-lineage highlighted its potential to continue to cause disease in the post-PCV13 era.

Running title: Novel tetracycline resistant gene detected in pneumococcal CC230 in South Africa

Introduction

Streptococcus pneumoniae is a major bacterial cause of disease in young children. Since 2000, pneumococcal conjugate vaccines (PCVs) targeting up to 13 serotypes were gradually introduced into childhood immunisation programmes in many countries and have significantly reduced pneumococcal deaths globally by 51% and 75% in HIV-uninfected and HIV-infected children aged <5 years, respectively, resulting in saving an estimated 375,000 lives annually when compared with the estimated mortality rate in the pre-vaccine era ¹. However, increasing invasive pneumococcal disease (IPD) caused by non-vaccine serotype pneumococci has been observed in numerous locations including England and Wales ², France ³, Germany ⁴ and Israel ⁵, a phenomenon known as serotype replacement. 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 ⁶. Capsular switching within multidrug-resistant lineages, especially those recognised 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 ⁷. The persistence of the multidrug resistant lineage ST156 (Spain ^{9V}-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 ⁷⁻⁹.

Resistance to tetracycline has been frequently observed in *S. pneumoniae* ¹⁰. The genetic basis was shown to be the *tet*(M), less commonly *tet*(O), which encode for a ribosomal protection protein that prevents tetracycline binding to the bacterial 30S ribosome subunit ^{10, 11}. Eleven other classes of ribosomal protection proteins such as *tet*(S) and twelve mosaic structure of *tet*

genes such as *tet*(S/M) have not been found previously in pneumococci (<http://faculty.washington.edu/marilynr/>). The *tet*(S), originally discovered in *Listeria monocytogenes* strain BM4210¹², was occasionally found in a variety of streptococci, including *S. suis* (NCBI accession number KX077886)¹³, *S. infantis* (NCBI accession number JX275965), and *S. dysgalactiae* (NCBI accession number EF682210)¹⁴ and is associated with a transposase-containing element *IS1216*, which potentially mediates chromosomal rearrangement. The mosaic *tet*(S/M) was observed on a *Tn916* element in *S. intermedius*¹⁵ and an *IS1216* composite in *S. bovis*¹⁶. 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 *tet*(S/M) in *S. pneumoniae* and characterised its genetic background in relation to nationwide clinical interventions.

Material and Methods

Isolate collection

In the GPS project, each participating country randomly selected disease isolates collected via laboratory-based surveillance and carriage isolates via cohort-studies using the following criteria: ~50% isolates were from children ≤ 2 years, 25% from children 3-5 years, and 25% from individuals >5 years. By May 2017 (last accessed 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 carriage (n=4,863) and disease isolates (n=7,391). 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. MIC results were interpreted according to Clinical

Laboratory Standards Institute M100-S24¹⁷. When MIC was analysed as “>X”, MIC was approximated as value 2X for median and interquartile range 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). We inferred serotype, multilocus sequence types (MLSTs) and resistance profile for penicillin, chloramphenicol, cotrimoxazole, erythromycin and tetracycline from the genomic data as previously described¹⁸. The *tet*(S/M) gene was identified with a *tet*(S/M) reference sequence (NCBI accession number AY534326) using ARIBA¹⁹.

To reconstruct a global phylogeny, an additional collection of CC230 isolates (n=130) from previous studies²⁰⁻²⁴, together with the CC230 collection (n=259) in the GPS dataset were included. The phylogeny was built as previously described¹⁸. Based on the international genomic definition of pneumococcal lineages, all CC230 isolates in this study belong to Global Pneumococcal Sequence Cluster (GPSC)10¹⁸. 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 sub-lineage

Coalescent analysis was performed on *tet*(S/M) CC230 sub-lineage (n=129) to date the most recent common ancestor (MRCA) and reconstruct the 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 v1.5²⁵. Next, a timed phylogeny was constructed using BEAST v2.4.1²⁶. 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 model²⁷ to examine

the temporal changes with the *tet*(S/M) CC230 sub-lineage 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 population²⁸. The birth-death skyline plot shows the effective reproductive number (R) over time. R is defined as the number of expected secondary infections of 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 were 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 novel tetracycline-resistant gene *tet*(S/M) was identified in 131 pneumococcal isolates (1%, 131/12,254) from South Africa (n=123), Malawi (n=5), and one each from Brazil, Mozambique, and the USA. They were isolated from sterile body sites (invasive isolates): blood (n=73), cerebrospinal fluid (n=30), 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 2005-2014 and 1.2% (20/1701) 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 (Fig. S1). HIV status was known in only 44% (54/123) of individuals with *tet*(S/M)-positive pneumococci; 59% (32/54) were HIV-positive, in which 94% (30/32) were children aged ≤ 5 years.

Among the *tet*(S/M)-positive isolates, the minimum inhibitory concentration (MIC) to tetracycline was determined by either E-test (n=56) or broth dilution (n=48). E-test showed a median MIC of 8 mg/L with an interquartile range of 6-9 mg/L and 16 mg/L by broth dilution. Based on the CLSI guideline, 99% (103/104) and 1% (1/104) were fully and intermediately 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 at G1769A and resulted in a substitution R590Q. This isolate remained resistant to tetracycline with a MIC of >8 mg/L when measured by broth dilution. Unlike the two previously reported *tet*(S/M) alleles from *S. intermedius*¹⁵ and *S. bovis*¹⁶, 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 EFU09422) (Figure 1). Examining the promoter regions revealed that the -10 (TATTAT) and -35 (TTTACA) promoter sequence was of *tet*(M) origin, rather than *tet*(S) origin. Between the promoter region and start codon of

tet(S/M), a 38-bp stem loop which is potentially involved in transcriptional regulation²⁹ 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.

Phylogeny and characteristics of *tet*(S/M) CC230 sub-lineage

All 131 *tet*(S/M)-positive isolates belonged to CC230, except for one Brazilian and one Malawian isolate belonging 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 sub-lineage which predicted to be resistance to penicillin, erythromycin, tetracycline and cotrimoxazole (Figure 2). The *tet*(S/M) sub-lineage 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), 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) sub-lineage, exhibiting resistance to penicillin with MIC of 2 mg/L. To identify the potential donor of the serotype 23A *cps* 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 sub-lineage

The sub-lineage showed a temporal signal in terms of SNP accumulation against time ($R^2 = 0.4094$, p value = 0.001; Figure S3). Using a birth-death model in BEAST, the *tet*(S/M) sub-lineage was estimated to emerge around 1994 (95% highest posterior density [HPD]: 1991-1996); the MRCA for the African clade was 1998 (95% HPD: 1996-2000) and for the two serotype 23A isolates was 2009 (95% HPD: 2007-2011) (Figure 3). The temporal changes of spread were reconstructed based on a birth-death skyline plot and coalescent-based skyline plot (Figure 4). Both skyline plots showed that the *tet*(S/M) sub-lineage expanded at the beginning of the year 2000 and growth continued until around 2004. The decline of the *tet*(S/M) sub-lineage 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 below one, indicating a declining epidemic. The coalescent-based skyline plot failed to detect the impact of the epidemic decline as described in a previous study ²⁷.

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) or *tet*(S/M) 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 up- 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 direct repeat, CAAAAAAA. The insertion of Tn917 disrupted the gene *orf9* which encodes a putative conjugative transposon regulator. No direct repeats 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 sub-lineage that predominantly expressed VT 14 and exhibited multidrug resistance 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.

The convergence of antimicrobial resistance and virulence in the CC230 sub-lineage probably contributed to its expansion in disease-causing populations prior to the introduction of PCVs. Unlike what was observed in other countries, CC230 in South Africa predominantly expressed a highly invasive serotype 14 capsule¹⁸ 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 Africa³⁰. Any controlling measure to decrease this lineage would not only result in a reduction in the IPD burden but also multidrug-resistant IPD incidence.

The birth-death model estimated that the decline of the *tet*(S/M) CC230 sub-lineage started around 2005, one year after the national ART programme was launched to treat HIV-infected individuals in South Africa³¹. The prediction was consistent with a 41% reduction of IPD incidence among HIV-infected children after the introduction of ART³¹. Among the IPD caused by *tet*(S/M) CC230 isolates, almost 60% occurred in HIV-positive children. This observational evidence strengthened that ART was likely to contribute to the decline before PCV introduction. In contrast, the large-scale use of cotrimoxazole as prophylaxis to prevent bacterial infections among HIV-positive individuals was unlikely to be responsible for the decline, as the sub-lineage was resistant to cotrimoxazole. The further decline in 2012 predicted by the model echoed the epidemiological finding that IPD caused by VT pneumococci significantly decreased among children in 2012³². 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 identify outbreaks as they occur in the future.

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 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 ¹⁸, a significant increase of this serotype in IPD cases was reported from England ^{2, 33}, Stockholm ³⁴ and Taiwan ³⁵ 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 combination 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) isolates ¹⁸ is the potential donor of the serotype 23A *cps* to the *tet*(S/M) CC230 sub-lineage, 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 other 620 GPSCs, GPSC10 which included 98% (258/262) of CC230 isolates is a very recombinogenic lineage which had a significantly high recombination rate [GPSC10 r/m: 10.9 vs median of 35 dominant GPSCs: 8.3 (1st – 3rd quartile, 5.7-10.7) p value < 0.0001, Wilcoxon signed-rank test] ¹⁸. Given this recombinogenic nature, together with the established multidrug resistant genotypes, it is of concern that any further capsular switching may increase the chance of this multidrug-resistant lineage surviving and continuing to cause invasive disease.

Like *tet(M)*, *tet(S/M)* was also carried by a highly mobile conjugative transposon, Tn916, with a broad host range. 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 recombination³⁶. This finding is consistent with previous studies which suggest that the *tet* evolved separately from Tn916^{10,36}. However, the driving force behind the evolution of *tet* genes remains unclear, given that tetracycline is not used as a first-line antibiotic to treat pneumococcal disease and was seldom used in young children³⁷. The allelic diversity of *tet* gene may be maintained by 1) frequent recombination among *S. pneumoniae* and with closely related species such as normal nasopharyngeal resident *S. mitis*³⁸ and zoonotic pathogen *S. suis*³⁹; 2) antibiotic-selective pressure via food chain, as tetracycline is widely used in agriculture⁴⁰ and its residue is detected in milk⁴¹. Future studies that investigate the driving force behind will improve our understanding to develop preventive measure to reduce tetracycline resistance in *S. pneumoniae*.

In conclusion, we identified a novel tetracycline-resistant determinant *tet(S/M)* in *S. pneumoniae* and showed that its dissemination is due to a clonal expansion of the multidrug-resistant lineage CC230 in South Africa where the HIV burden is high. With genomic data, we successfully detected the declines in transmission of this multidrug-resistant lineage using a birth-death model, and the fall of this lineage may correlate to 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 multidrug-

resistant pneumococcal lineages that may also undergo vaccine evasion through capsular switching events.

Acknowledgements

We deeply appreciate all members of the GPS consortium for their collaborative spirit and determination for 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 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, Andrew J Pollard, Anna Skoczynska, Betuel Sigauque, Deborah Lehmann, Diego Faccone, Elena Voropaeva, Eric Sampane-Donkor, Ewa Sadowy, Godfrey Bigogo, Helio Mucavele, Houria Belabbès, Idrissa Diawara, Jennifer Moïsi, Jennifer Verani, Jeremy Keenan, Khalid Zerouali, Maria-Cristina de Cunto Brandileone, Margaret Ip, Md Hasanuzzaman, Nicole Wolter, Noga Givon-Lavi, Özgen Köseoglu Eser, Pak Leung Ho, Patrick E Akpaka, Paul Turner, Paula Gagetti, Peggy-Estelle Tientcheu, Philip E. Carter, Pierra Law, Rachel Benisty, Rebecca Ford, Ron Dagan, Sadia Shakoor, Sanjay Doiphode, Shamala Devi Sekaran, Somporn Srifuengfung, Stephen Obaro, Stuart C Clarke, Tamara Kastrin, Theresa J. Ochoa, Waleria Hryniewicz, Veeraraghavan Balaji and Yulia Urban.

Funding

This study was co-funded by the Bill and 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 Declaration

Dr. Gladstone reports PhD studentship from Pfizer, outside the submitted work; Dr. Lees reports grants from Pfizer, outside the submitted work. Dr. von Gottberg reports grants and other from Pfizer, during the conduct of the study and grants and other from Sanofi, outside the submitted work; Dr. Bentley reports personal fees from Pfizer, personal fees from Merck, outside the submitted work. Professor A. Skoczynska reports grants from Pfizer, assistance to attend scientific meetings and honoraria for lecturing funded from GlaxoSmithKline and Pfizer, and participation in Advisory Board of GlaxoSmithKline and Pfizer, outside the submitted work.

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.

References

1. Wahl B, O'Brien KL, Greenbaum A et al. Burden of Streptococcus pneumoniae and Haemophilus influenzae type b disease in children in the era of conjugate vaccines: global, regional, and national estimates for 2000-15. *Lancet Glob Health* 2018; **6**: e744-e57.
2. Ladhani SN, Collins S, Djennad A et al. Rapid increase in non-vaccine serotypes causing invasive pneumococcal disease in England and Wales, 2000-17: a prospective national observational cohort study. *Lancet Infect Dis* 2018; **18**: 441-51.

- 396 3. Ouldali N, Levy C, Varon E et al. Incidence of paediatric pneumococcal meningitis and
397 emergence of new serotypes: a time-series analysis of a 16-year French national survey.
398 *Lancet Infect Dis* 2018; **18**: 983-91.
- 399 4. Weinberger R, von Kries R, van der Linden M et al. Invasive pneumococcal disease in
400 children under 16years of age: Incomplete rebound in incidence after the maximum effect
401 of PCV13 in 2012/13 in Germany. *Vaccine* 2018; **36**: 572-7.
- 402 5. Rokney A, Ben-Shimol S, Korenman Z et al. Emergence of *Streptococcus pneumoniae*
403 Serotype 12F after Sequential Introduction of 7- and 13-Valent Vaccines, Israel. *Emerg Infect*
404 *Dis* 2018; **24**: 453-61.
- 405 6. Wyres KL, Lambertsen LM, Croucher NJ et al. Pneumococcal capsular switching: a
406 historical perspective. *J Infect Dis* 2013; **207**: 439-49.
- 407 7. Metcalf BJ, Gertz RE, Jr., Gladstone RA et al. Strain features and distributions in
408 pneumococci from children with invasive disease before and after 13-valent conjugate
409 vaccine implementation in the USA. *Clin Microbiol Infect* 2016; **22**: 60 e9- e29.
- 410 8. Chochua S, Metcalf BJ, Li Z et al. Invasive Serotype 35B Pneumococci Including an
411 Expanding Serotype Switch Lineage, United States, 2015-2016. *Emerg Infect Dis* 2017; **23**:
412 922-30.
- 413 9. Olarte L, Kaplan SL, Barson WJ et al. Emergence of Multidrug-Resistant
414 Pneumococcal Serotype 35B among Children in the United States. *J Clin Microbiol* 2017; **55**:
415 724-34.
- 416 10. Wyres KL, van Tonder A, Lambertsen LM et al. Evidence of antimicrobial resistance-
417 conferring genetic elements among pneumococci isolated prior to 1974. *BMC Genomics*
418 2013; **14**: 500.

- 419 11. Widdowson CA, Klugman KP, Hanslo D. Identification of the tetracycline resistance
420 gene, tet(O), in *Streptococcus pneumoniae*. *Antimicrob Agents Chemother* 1996; **40**: 2891-3.
- 421 12. Charpentier E, Gerbaud G, Courvalin P. Characterization of a new class of
422 tetracycline-resistance gene tet(S) in *Listeria monocytogenes* BM4210. *Gene* 1993; **131**: 27-
423 34.
- 424 13. Huang J, Ma J, Shang K et al. Evolution and Diversity of the Antimicrobial Resistance
425 Associated Mobilome in *Streptococcus suis*: A Probable Mobile Genetic Elements Reservoir
426 for Other Streptococci. *Front Cell Infect Microbiol* 2016; **6**: 118.
- 427 14. Liu LC, Tsai JC, Hsueh PR et al. Identification of tet(S) gene area in tetracycline-
428 resistant *Streptococcus dysgalactiae* subsp. *equisimilis* clinical isolates. *J Antimicrob*
429 *Chemother* 2008; **61**: 453-5.
- 430 15. Lancaster H, Roberts AP, Bedi R et al. Characterization of Tn916S, a Tn916-like
431 element containing the tetracycline resistance determinant tet(S). *J Bacteriol* 2004; **186**:
432 4395-8.
- 433 16. Barile S, Devirgiliis C, Perozzi G. Molecular characterization of a novel mosaic
434 tet(S/M) gene encoding tetracycline resistance in foodborne strains of *Streptococcus bovis*.
435 *Microbiology* 2012; **158**: 2353-62.
- 436 17. Wayne P. *CLSI. Performance Standards for Antimicrobial Susceptibility Testing:*
437 *Twenty-Fourth Information Supplement*: Clinical and Laboratory Standards Institute, 2014.
- 438 18. Gladstone RA, Lo SW, Lees JA et al. International genomic definition of
439 pneumococcal lineages, to contextualise disease, antibiotic resistance and vaccine impact.
440 *EBioMedicine* 2019.
- 441 19. Hunt M, Mather AE, Sanchez-Buso L et al. ARIBA: rapid antimicrobial resistance
442 genotyping directly from sequencing reads. *Microb Genom* 2017; **3**: e000131.

- 443 20. Chewapreecha C, Harris SR, Croucher NJ et al. Dense genomic sampling identifies
444 highways of pneumococcal recombination. *Nat Genet* 2014; **46**: 305-9.
- 445 21. Quirk SJ, Haraldsson G, Erlendsdottir H et al. Effect of Vaccination on Pneumococci
446 Isolated from the Nasopharynx of Healthy Children and the Middle Ear of Children with
447 Otitis Media in Iceland. *J Clin Microbiol* 2018; **56**.
- 448 22. Chaguza C, Cornick JE, Andam CP et al. Population genetic structure, antibiotic
449 resistance, capsule switching and evolution of invasive pneumococci before conjugate
450 vaccination in Malawi. *Vaccine* 2017.
- 451 23. Cremers AJ, Mobegi FM, de Jonge MI et al. The post-vaccine microevolution of
452 invasive *Streptococcus pneumoniae*. *Sci Rep* 2015; **5**: 14952.
- 453 24. Gladstone RA, Devine V, Jones J et al. Pre-vaccine serotype composition within a
454 lineage signposts its serotype replacement – a carriage study over 7 years following
455 pneumococcal conjugate vaccine use in the UK. *Microbial Genomics* 2017; **3**.
- 456 25. Rambaut A, Lam TT, Max Carvalho L et al. Exploring the temporal structure of
457 heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol* 2016; **2**:
458 vew007.
- 459 26. Bouckaert R, Heled J, Kuhnert D et al. BEAST 2: a software platform for Bayesian
460 evolutionary analysis. *PLoS Comput Biol* 2014; **10**: e1003537.
- 461 27. Stadler T, Kuhnert D, Bonhoeffer S et al. Birth-death skyline plot reveals temporal
462 changes of epidemic spread in HIV and hepatitis C virus (HCV). *Proc Natl Acad Sci U S A*
463 2013; **110**: 228-33.
- 464 28. Kuhnert D, Coscolla M, Brites D et al. Tuberculosis outbreak investigation using
465 phylodynamic analysis. *Epidemics* 2018; **25**: 47-53.

- 466 29. Chopra I, Roberts M. Tetracycline Antibiotics: Mode of Action, Applications,
467 Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular*
468 *Biology Reviews* 2001; **65**: 232-60.
- 469 30. Ndlangisa KM, du Plessis M, Wolter N et al. Population snapshot of Streptococcus
470 pneumoniae causing invasive disease in South Africa prior to introduction of pneumococcal
471 conjugate vaccines. *PLoS One* 2014; **9**: e107666.
- 472 31. Nunes MC, von Gottberg A, de Gouveia L et al. The impact of antiretroviral
473 treatment on the burden of invasive pneumococcal disease in South African children: a time
474 series analysis. *AIDS* 2011; **25**: 453-62.
- 475 32. von Gottberg A, de Gouveia L, Tempia S et al. Effects of Vaccination on Invasive
476 Pneumococcal Disease in South Africa. *New England Journal of Medicine* 2014; **371**: 1889-
477 99.
- 478 33. Moore CE, Paul J, Foster D et al. Reduction of invasive pneumococcal disease 3 years
479 after the introduction of the 13-valent conjugate vaccine in the Oxfordshire region of
480 England. *J Infect Dis* 2014; **210**: 1001-11.
- 481 34. Galanis I, Lindstrand A, Darenberg J et al. Effects of PCV7 and PCV13 on invasive
482 pneumococcal disease and carriage in Stockholm, Sweden. *Eur Respir J* 2016; **47**: 1208-18.
- 483 35. Su LH, Kuo AJ, Chia JH et al. Evolving pneumococcal serotypes and sequence types in
484 relation to high antibiotic stress and conditional pneumococcal immunization. *Sci Rep* 2015;
485 **5**: 15843.
- 486 36. Oggioni MR, Dowson CG, Smith JM et al. The tetracycline resistance gene tet(M)
487 exhibits mosaic structure. *Plasmid* 1996; **35**: 156-63.

37. Organization WH. Revised WHO Classification and Treatment of Pneumonia in Children at Health Facilities: Evidence Summaries. Geneva: World Health Organization. *Scientific basis of WHO recommendations for treatment of pneumonia*, 2014.
38. 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.
39. Ye C, Bai X, Zhang J et al. Spread of *Streptococcus suis* sequence type 7, China. *Emerg Infect Dis* 2008; **14**: 787-91.
40. Granados-Chinchilla F, Rodriguez C. Tetracyclines in Food and Feedingstuffs: From Regulation to Analytical Methods, Bacterial Resistance, and Environmental and Health Implications. *J Anal Methods Chem* 2017; **2017**: 1315497.
41. 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.

Figure 1. Schematic representation of the mosaic structure of *tet(S/M)* alleles of the current and previous studies. The reference sequences for *tet(M)* and *tet(S)* were retrieved from NCBI Genbank using accession number EFU09422 and FN555436, respectively.

Figure 2. A SNP tree constructed with CC230 *tet(S/M)*-positive isolates (n=129) and *tet(S/M)*-negative carriage/disease isolates (n=260) collected from twenty 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 a ST230 reference strain, PMEN global clone Denmark¹⁴⁻³², PMEN32 (ENA accession number ERS1706837). Penicillin resistance was predicted based on the *pbp1a*, *pbp2x*, *pbp2b* sequences (1, 2); tetracycline and erythromycin resistance were predicted based on the presence of *tet(M)* and *tet(S/M)*, and *erm(B)* and *mef(A)*, respectively. Cotrimoxazole resistance was predicted based on the presence of mutation I100L in *folA* and any indel within amino acid residue 56-67 in *folP* while presence of either mutation predicted to confer cotrimoxazole-intermediate phenotype.

Figure 3. (A) Malawi, Mozambique and administration regions of South Africa (B) Timed phylogeny for *S. pneumoniae* tet(S/M) CC230 sub-lineage (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 United States coloured in brown. (C) Vaccine serotype 14 is indicated in blue, whereas non-vaccine serotype 23A in orange.

Figure 4. (A) Birth-death skyline plot of inferred changes in effective reproductive number (R) of *S. pneumoniae* tet(S/M) CC230 sub-lineage using IPD isolates ($n=105$). (B) Coalescent-based skyline plot of inferred changes in the effective population size (N_e) of *S. pneumoniae* tet(S/M) CC230 sub-lineage using both IPD and carriage isolates ($n=129$) and (C) using only IPD isolates ($n=105$). The black solid line shows the median of R in (A) and N_e in (B) and (C), respectively. The background area represents the 95% highest posterior density intervals. $R>1$ indicates a growing epidemic, whereas $R<1$ indicates a declining epidemic. ART, antiretroviral treatment; PCV7, seven-valent pneumococcal conjugate vaccine; PCV13, thirteen-valent pneumococcal conjugate vaccine.

Figure 5. Comparison of integrative and conjugative element (ICE) identified in clonal complex (CC)230 with Spain^{23F}-1 (PMEN1). Grey bands between the sequences indicate BLASTN matches.

Figure S1. Number of *tet*(S/M)-positive isolates, by age and clinical manifest

Figure S2. Maximum likelihood phylogenetic tree was constructed using 2,178 SNPs extracted from a 21,296-bp alignment of serotype 23A *cps* locus sequences from the serotype 23A *S. pneumoniae* isolates (n=130) in the GPS curated dataset. This analysis used the serotype 23F *cps* locus reference sequence (accession number CR931685) as the outgroup on which to root the tree. The serotype 23A *cps* reference sequence (accession number CR931683) was included. The primary clonal complex (CC) or sequence type (ST) associated with Global Pneumococcal Sequence Cluster (GPSC) was indicated in parentheses.

Figure S3. Linear regression of root-to-tip distance against time on *tet*(S/M)-CC230 lineage (n=129) using TempEST v1.5. TempEst detected a significant positive correlation of year of collection with its genetic distance from the root, indicating a signal of a ‘molecular clock’, with which isolates measurably diversifying from their last common ancestor over time.

Figure S4. Comparison of Integrative and conjugative element (ICE) carrying *tet(S/M)* and *tet(M)* in clonal complex (CC)230. The yellow arrows indicated protein coding region. The grey band between the sequence indicates BLASTN match and black vertical lines shows the unmatched nucleotide bases.