

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

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75 **Synopsis**

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

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

82 **Results** We identified *tet(S/M)* in 131 pneumococcal isolates, 97 (74%) caused invasive  
83 pneumococcal diseases among young children (59% HIV-positive, where HIV status was  
84 available) in South Africa. A majority of *tet(S/M)*-positive isolates (129/131) belong to clonal  
85 complex (CC)230. A global phylogeny of CC230 (n=389) revealed that *tet(S/M)*-positive  
86 isolates formed a sub-lineage that exhibited multidrug-resistance. Using the genomic data and  
87 a birth-death model, we detected an unrecognised outbreak of this sub-lineage in South Africa  
88 between 2000 and 2004 with an expected secondary infections (R) of  $\sim 2.5$ . R declined to  $\sim 1.0$   
89 in 2005 and  $< 1.0$  in 2012. The declining epidemic coincided and could be related to the  
90 nationwide implementation of anti-retroviral treatment (ART) for HIV-infected individuals in  
91 2004 and PCVs in late 2000s. Capsular switching from vaccine serotype 14 to non-vaccine  
92 serotype 23A was observed within the sub-lineage.

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

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

99

## 100 **Introduction**

101 *Streptococcus pneumoniae* is a major bacterial cause of disease in young children. Since 2000,  
102 pneumococcal conjugate vaccines (PCVs) targeting up to 13 serotypes were gradually  
103 introduced into childhood immunisation programmes in many countries and have significantly  
104 reduced pneumococcal deaths globally by 51% and 75% in HIV-uninfected and HIV-infected  
105 children aged <5 years, respectively, resulting in saving an estimated 375,000 lives annually  
106 when compared with the estimated mortality rate in the pre-vaccine era <sup>1</sup>. However, increasing  
107 invasive pneumococcal disease (IPD) caused by non-vaccine serotype pneumococci has been  
108 observed in numerous locations including England and Wales <sup>2</sup>, France <sup>3</sup>, Germany <sup>4</sup> and Israel  
109 <sup>5</sup>, a phenomenon known as serotype replacement. Serotype replacement could be mediated by  
110 capsular switching, in which a *cps* locus encoding vaccine-type (VT) capsule is replaced by a  
111 *cps* locus encoding non-vaccine-type (NVT) capsule through homologous recombination <sup>6</sup>.  
112 Capsular switching within multidrug-resistant lineages, especially those recognised by the  
113 Pneumococcal Molecular Epidemiology Network (PMEN,  
114 <http://spneumoniae.mlst.net/pmen/pmen.asp>), is of increasing concern, as these expansions can  
115 reduce overall vaccine effectiveness in preventing IPD and temper the reduction in  
116 antimicrobial-resistant pneumococcal infections associated with introduction of PCVs <sup>7</sup>. The  
117 persistence of the multidrug resistant lineage ST156 (Spain <sup>9V</sup>-3, PMEN3) in the USA  
118 following the introduction of PCV13 provides a clear example of a historically successful  
119 lineage that underwent a capsular switch from VT (serotype 9V, 14 and 19A) to NVT (serotype  
120 35B) and continued to cause IPD in the post-vaccine era <sup>7-9</sup>.

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

125 genes such as *tet(S/M)* have not been found previously in pneumococci  
126 (<http://faculty.washington.edu/marilynr/>). The *tet(S)*, originally discovered in *Listeria*  
127 *monocytogenes* strain BM4210<sup>12</sup>, was occasionally found in a variety of streptococci,  
128 including *S. suis* (NCBI accession number KX077886)<sup>13</sup>, *S. infantis* (NCBI accession number  
129 JX275965), and *S. dysgalactiae* (NCBI accession number EF682210)<sup>14</sup> and is associated with  
130 a transposase-containing element *IS1216*, which potentially mediates chromosomal  
131 rearrangement. The mosaic *tet(S/M)* was observed on a *Tn916* element in *S. intermedius*<sup>15</sup> and  
132 an *IS1216* composite in *S. bovis*<sup>16</sup>. Using a dataset of 12,254 pneumococcal genomes from  
133 The Global Pneumococcal Sequencing (GPS) project (<https://www.pneumogen.net/gps/>), we  
134 identified a novel genetic basis for tetracycline resistance *tet(S/M)* in *S. pneumoniae* and  
135 characterised its genetic background in relation to nationwide clinical interventions.

136

## 137 **Material and Methods**

### 138 **Isolate collection**

139 In the GPS project, each participating country randomly selected disease isolates collected via  
140 laboratory-based surveillance and carriage isolates via cohort-studies using the following  
141 criteria: ~50% isolates were from children  $\leq 2$  years, 25% from children 3-5 years, and 25%  
142 from individuals  $>5$  years. By May 2017 (last accessed to the GPS database for this study),  
143 12,254 isolates, representing 29 countries, in Africa (65%), North America (14%), Asia (9%),  
144 South America (8%), and Europe (4%), were sequenced, passed quality control and included  
145 in this study. The collection spanned 26 years between 1991 and 2016 and included both  
146 carriage (n=4,863) and disease isolates (n=7,391). We compiled the metadata including age,  
147 year of collection, sample source, HIV status and phenotypic antimicrobial susceptibility  
148 testing results, where available, from each participating site. In children  $< 18$  months of age,  
149 HIV status was confirmed by PCR assay. MIC results were interpreted according to Clinical

150 Laboratory Standards Institute M100-S24 <sup>17</sup>. When MIC was analysed as “>X”, MIC was  
151 approximated as value 2X for median and interquartile range calculations.

## 152 **Genome sequencing and analyses**

153 The pneumococcal isolates were whole genome sequenced on an Illumina HiSeq platform and  
154 raw data were deposited in the European Nucleotide Archive (ENA) (Supplementary  
155 metadata). We inferred serotype, multilocus sequence types (MLSTs) and resistance profile for  
156 penicillin, chloramphenicol, cotrimoxazole, erythromycin and tetracycline from the genomic  
157 data as previously described <sup>18</sup>. The *tet(S/M)* gene was identified with a *tet(S/M)* reference  
158 sequence (NCBI accession number AY534326) using ARIBA <sup>19</sup>.

159 To reconstruct a global phylogeny, an additional collection of CC230 isolates (n=130) from  
160 previous studies <sup>20-24</sup>, together with the CC230 collection (n=259) in the GPS dataset were  
161 included. The phylogeny was built as previously described <sup>18</sup>. Based on the international  
162 genomic definition of pneumococcal lineages, all CC230 isolates in this study belong to Global  
163 Pneumococcal Sequence Cluster (GPSC)10 <sup>18</sup>. The metadata and analysis results of CC230 can  
164 be interactively visualized online using the Microreact tool at  
165 [https://microreact.org/project/GPS\\_tetSM](https://microreact.org/project/GPS_tetSM).

## 166 **Temporal changes of *tet(S/M)* CC230 sub-lineage**

167 Coalescent analysis was performed on *tet(S/M)* CC230 sub-lineage (n=129) to date the most  
168 recent common ancestor (MRCA) and reconstruct the population demographic history. First,  
169 we tested the presence of temporal signal by a linear regression of root-to-tip distances against  
170 year of collection using TempEst v1.5 <sup>25</sup>. Next, a timed phylogeny was constructed using  
171 BEAST v2.4.1 <sup>26</sup>. The Markov chain Monte Carlo (MCMC) chain was run for 100 million  
172 generations, sampled every 1000 states using the general time-reversible (GTR) model of  
173 nucleotide substitution and the discrete gamma model of heterogeneity among sites. Finally,  
174 the population demographic history was reconstructed using a birth-death model <sup>27</sup> to examine

175 the temporal changes with the *tet*(S/M) CC230 sub-lineage invasive isolates (n=105) but not  
176 carriage isolates (n=24), because the model assumes that once an individual is diagnosed with  
177 IPD, the individual is no longer transmitting due to treatment and recovery, death, or being  
178 socially removed from susceptible individuals. Thus, it appeared to be logical to apply this  
179 model to the disease but not carriage isolates. This model overcomes the limitations of the  
180 coalescent-based skyline plot and is able to examine whether introduction of an intervention  
181 had an impact on the epidemiological dynamics in a bacterial population<sup>28</sup>. The birth-death  
182 skyline plot shows the effective reproductive number (R) over time. R is defined as the number  
183 of expected secondary infections of an infected individual.  $R > 1$  indicates a growing epidemic,  
184 whereas  $R < 1$  indicates a declining epidemic. Notably,  $R \geq 1$  can be reflected in the coalescent-  
185 based skyline plot analysis, whereas  $R < 1$  cannot. Therefore, we expected the birth-death  
186 skyline model would be a better fit for our data. Other Bayesian population size models  
187 (coalescent constant, coalescent exponential and Bayesian skyline) in combination with strict  
188 and lognormal-relaxed molecular clocks were also applied for comparisons using BEAST.

### 189 **Integrative and conjugative element (ICE)**

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

196

## 197 **Results**

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



199 A novel tetracycline-resistant gene *tet(S/M)* was identified in 131 pneumococcal isolates (1%,  
200 131/12,254) from South Africa (n=123), Malawi (n=5), and one each from Brazil,  
201 Mozambique, and the USA. They were isolated from sterile body sites (invasive isolates):  
202 blood (n=73), cerebrospinal fluid (n=30), pleural fluid (n=4), and from the nasopharynx  
203 (carriage isolates) (n=24). In South Africa, *tet(S/M)* was found in 3.5% (103/2920) of the  
204 invasive isolates that were submitted to the GPS project from 2005-2014 and 1.2% (20/1701)  
205 carriage isolates that were collected in Agincourt and Soweto between 2009 and 2013. Of the  
206 103 invasive isolates, 94% (97/103) were from children with IPD aged  $\leq 5$  years (Fig. S1).  
207 HIV status was known in only 44% (54/123) of individuals with *tet(S/M)*-positive  
208 pneumococci; 59% (32/54) were HIV-positive, in which 94% (30/32) were children aged  $\leq 5$   
209 years.

210 Among the *tet(S/M)*-positive isolates, the minimum inhibitory concentration (MIC) to  
211 tetracycline was determined by either E-test (n=56) or broth dilution (n=48). E-test showed a  
212 median MIC of 8 mg/L with an interquartile range of 6-9 mg/L and 16 mg/L by broth dilution.  
213 Based on the CLSI guideline, 99% (103/104) and 1% (1/104) were fully and intermediately  
214 resistant to tetracycline, respectively. The *tet(S/M)* in this study showed 100% nucleotide  
215 identity, except for one isolate (GPS\_ZA\_1982) from South Africa which varied from the  
216 others at G1769A and resulted in a substitution R590Q. This isolate remained resistant to  
217 tetracycline with a MIC of  $>8$  mg/L when measured by broth dilution. Unlike the two  
218 previously reported *tet(S/M)* alleles from *S. intermedius*<sup>15</sup> and *S. bovis*<sup>16</sup>, the amino acid  
219 sequence of *tet(S/M)* in this study showed 100% identity to Tet(S) (NCBI accession number  
220 FN555436) across the first 613 amino acids, with the final 32 amino acids at the C-terminus  
221 end being identical to Tet(M) (NCBI accession number EFU09422) (Figure 1). Examining the  
222 promoter regions revealed that the -10 (TATTAT) and -35 (TTTACA) promoter sequence was  
223 of *tet(M)* origin, rather than *tet(S)* origin. Between the promoter region and start codon of

224 *tet(S/M)*, a 38-bp stem loop which is potentially involved in transcriptional regulation<sup>29</sup> was  
225 found in all *tet(S/M)* genes (Figure 1), apart from one disease isolate (GPS\_ZA\_1926) from  
226 South Africa. The deletion did not affect the tetracycline resistance level, as the MIC remained  
227 at >8 mg/L when measured by the broth dilution method.

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

229 All 131 *tet(S/M)*-positive isolates belonged to CC230, except for one Brazilian and one  
230 Malawian isolate belonging to CC156 and ST5359 (a singleton not belonging to any CC),  
231 respectively. The global CC230 phylogeny showed that all *tet(S/M)*-positive isolates formed a  
232 sub-lineage which predicted to be resistance to penicillin, erythromycin, tetracycline and  
233 cotrimoxazole (Figure 2). The *tet(S/M)* sub-lineage was associated predominantly with VT 14  
234 (98%,127/129) but was also found in two NVT 23A isolates. The two serotype 23A isolates,  
235 which both belonged to ST11106 (a single-locus variant of ST230), were recovered from  
236 infants after the introduction of PCV13. One was isolated from a nasopharyngeal sample in  
237 Soweto in 2012 and the other from blood culture in Johannesburg in 2014. The serotype 23A  
238 *cps* locus sequences of these two isolates were identical, and their *cps*-flanking *pbp* loci (*pbp1A*  
239 and *pbp2X*) were also identical to the majority of the serotype 14 isolates within the *tet(S/M)*  
240 sub-lineage, exhibiting resistance to penicillin with MIC of 2 mg/L. To identify the potential  
241 donor of the serotype 23A *cps* locus, a phylogenetic tree was built using the *cps* sequences  
242 from all serotype 23A (n=130, belong to eight lineages) pneumococci in the GPS database.  
243 This analysis showed that the serotype 23A *cps* loci of these two CC230 isolates clustered with  
244 those originating from a serogroup 23 lineage GPSC7, which is predominantly (99%, 145/146)  
245 represented by CC439 (Figure S2), with pairwise nucleotide similarity of 99.97%  
246 (24,818/24,825) and 100% coverage. The seven nucleotide variations were found within the  
247 IS630 transposase downstream of *dexB*.

### 248 **Temporal spread of the *tet(S/M)* CC230 sub-lineage**

249 The sub-lineage showed a temporal signal in terms of SNP accumulation against time ( $R^2 =$   
250 0.4094,  $p$  value = 0.001; Figure S3). Using a birth-death model in BEAST, the *tet*(S/M) sub-  
251 lineage was estimated to emerge around 1994 (95% highest posterior density [HPD]: 1991-  
252 1996); the MRCA for the African clade was 1998 (95% HPD: 1996-2000) and for the two  
253 serotype 23A isolates was 2009 (95% HPD: 2007-2011) (Figure 3). The temporal changes of  
254 spread were reconstructed based on a birth-death skyline plot and coalescent-based skyline plot  
255 (Figure 4). Both skyline plots showed that the *tet*(S/M) sub-lineage expanded at the beginning  
256 of the year 2000 and growth continued until around 2004. The decline of the *tet*(S/M) sub-  
257 lineage was only captured by the birth-death skyline plot in or around 2005, from expected  
258 secondary infections ( $R$ ) of  $\sim 2.5$  to  $\sim 1$ , and steadily declined until 2012 when the median and  
259 HPD of  $R$  were below one, indicating a declining epidemic. The coalescent-based skyline plot  
260 failed to detect the impact of the epidemic decline as described in a previous study<sup>27</sup>.

### 261 *ICE carrying tet(S/M)*

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

271

## 272 **Discussion**

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

280 The convergence of antimicrobial resistance and virulence in the CC230 sub-lineage probably  
281 contributed to its expansion in disease-causing populations prior to the introduction of PCVs.  
282 Unlike what was observed in other countries, CC230 in South Africa predominantly expressed  
283 a highly invasive serotype 14 capsule<sup>18</sup> and was the clone that represented most of the serotype  
284 14 isolates (43%) in the pre-vaccine era, when serotype 14 was the most prevalent serotype  
285 causing IPD in South Africa<sup>30</sup>. Any controlling measure to decrease this lineage would not  
286 only result in a reduction in the IPD burden but also multidrug-resistant IPD incidence.

287 The birth-death model estimated that the decline of the *tet*(S/M) CC230 sub-lineage started  
288 around 2005, one year after the national ART programme was launched to treat HIV-infected  
289 individuals in South Africa<sup>31</sup>. The prediction was consistent with a 41% reduction of IPD  
290 incidence among HIV-infected children after the introduction of ART<sup>31</sup>. Among the IPD  
291 caused by *tet*(S/M) CC230 isolates, almost 60% occurred in HIV-positive children. This  
292 observational evidence strengthened that ART was likely to contribute to the decline before  
293 PCV introduction. In contrast, the large-scale use of cotrimoxazole as prophylaxis to prevent  
294 bacterial infections among HIV-positive individuals was unlikely to be responsible for the  
295 decline, as the sub-lineage was resistant to cotrimoxazole. The further decline in 2012 predicted  
296 by the model echoed the epidemiological finding that IPD caused by VT pneumococci  
297 significantly decreased among children in 2012<sup>32</sup>. Our finding demonstrated that we could

298 effectively reconstruct the temporal spread of an epidemic using genomic data and highlighted  
299 the possible use of routine genomic surveillance to identify outbreaks as they occur in the  
300 future.

301 The MRCA of two CC230 isolates expressing NVT 23A was dated to emerge around 2009,  
302 the year when PCV7 was introduced. However, the long branch leading to the MRCA from the  
303 internal node that shared with the closely related serotype 14 isolates indicated that the window  
304 of time for capsular switching could be between 2002 and 2011. Although the invasive disease  
305 potential for serotype 23A is low <sup>18</sup>, a significant increase of this serotype in IPD cases was  
306 reported from England <sup>2, 33</sup>, Stockholm <sup>34</sup> and Taiwan <sup>35</sup> after the implementation of PCV13.  
307 Serotype 23A is primarily associated with CC338 (GPSC5, PMEN26) and CC439 (GPSC7),  
308 and is thus rarely found in a CC230 genetic background. Such serotype and genotype  
309 combination were only identified in two ST9396 isolates (single locus variant of ST230) from  
310 China in 2013 and one ST10921 isolate (double locus variant of ST230) from Poland in 2013  
311 in the MLST database. In South Africa, CC439 accounted for 62% of serotype 23A (both  
312 carriage and disease) isolates <sup>18</sup> is the potential donor of the serotype 23A *cps* to the *tet(S/M)*  
313 CC230 sub-lineage, highlighting that capsular switching with the prevalent NVT lineage could  
314 enable a VT lineage to evade the vaccine. Capsular switching is usually a result of homologous  
315 recombination. When compared with other 620 GPSCs, GPSC10 which included 98%  
316 (258/262) of CC230 isolates is a very recombinogenic lineage which had a significantly high  
317 recombination rate [GPSC10 r/m: 10.9 vs median of 35 dominant GPSCs: 8.3 (1<sup>st</sup> – 3<sup>rd</sup> quartile,  
318 5.7-10.7) p value < 0.0001, Wilcoxon signed-rank test] <sup>18</sup>. Given this recombinogenic nature,  
319 together with the established multidrug resistant genotypes, it is of concern that any further  
320 capsular switching may increase the chance of this multidrug-resistant lineage surviving and  
321 continuing to cause invasive disease.

322 Like *tet(M)*, *tet(S/M)* was also carried by a highly mobile conjugative transposon, Tn916, with  
323 a broad host range. Conserved genetic environment of *tet(M)* and *tet(S/M)* indicates that the  
324 recombination resulting in the mosaic structure of *tet(S/M)* probably occurred after the  
325 acquisition of the gene by Tn916. Comparison of *tet(M)* sequences in the current collection  
326 also revealed a high degree of allelic variations that were probably due to homologous  
327 recombination<sup>36</sup>. This finding is consistent with previous studies which suggest that the *tet*  
328 evolved separately from Tn916<sup>10,36</sup>. However, the driving force behind the evolution of *tet*  
329 genes remains unclear, given that tetracycline is not used as a first-line antibiotic to treat  
330 pneumococcal disease and was seldom used in young children<sup>37</sup>. The allelic diversity of *tet*  
331 gene may be maintained by 1) frequent recombination among *S. pneumoniae* and with closely  
332 related species such as normal nasopharyngeal resident *S. mitis*<sup>38</sup> and zoonotic pathogen *S.*  
333 *suis*<sup>39</sup>; 2) antibiotic-selective pressure via food chain, as tetracycline is widely used in  
334 agriculture<sup>40</sup> and its residue is detected in milk<sup>41</sup>. Future studies that investigate the driving  
335 force behind will improve our understanding to develop preventive measure to reduce  
336 tetracycline resistance in *S. pneumoniae*.

337 In conclusion, we identified a novel tetracycline-resistant determinant *tet(S/M)* in  
338 *S. pneumoniae* and showed that its dissemination is due to a clonal expansion of the multidrug-  
339 resistant lineage CC230 in South Africa where the HIV burden is high. With genomic data, we  
340 successfully detected the declines in transmission of this multidrug-resistant lineage using a  
341 birth-death model, and the fall of this lineage may correlate to the improved treatment of HIV-  
342 infected individuals and the implementation of PCVs. Capsular switching within this lineage  
343 is potentially of public health importance and may erode the beneficial effect brought about by  
344 the implementation of PCVs. The capacity for continuous genomic surveillance in the post-  
345 vaccine era provides critical opportunities for monitoring and forecasting the rise of multidrug-

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

348

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384

### 385 **Disclaimer**

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

388

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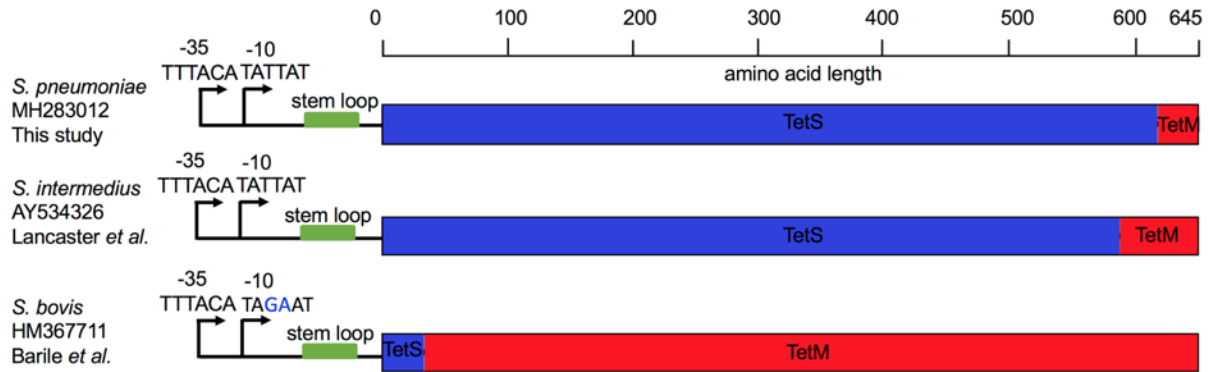
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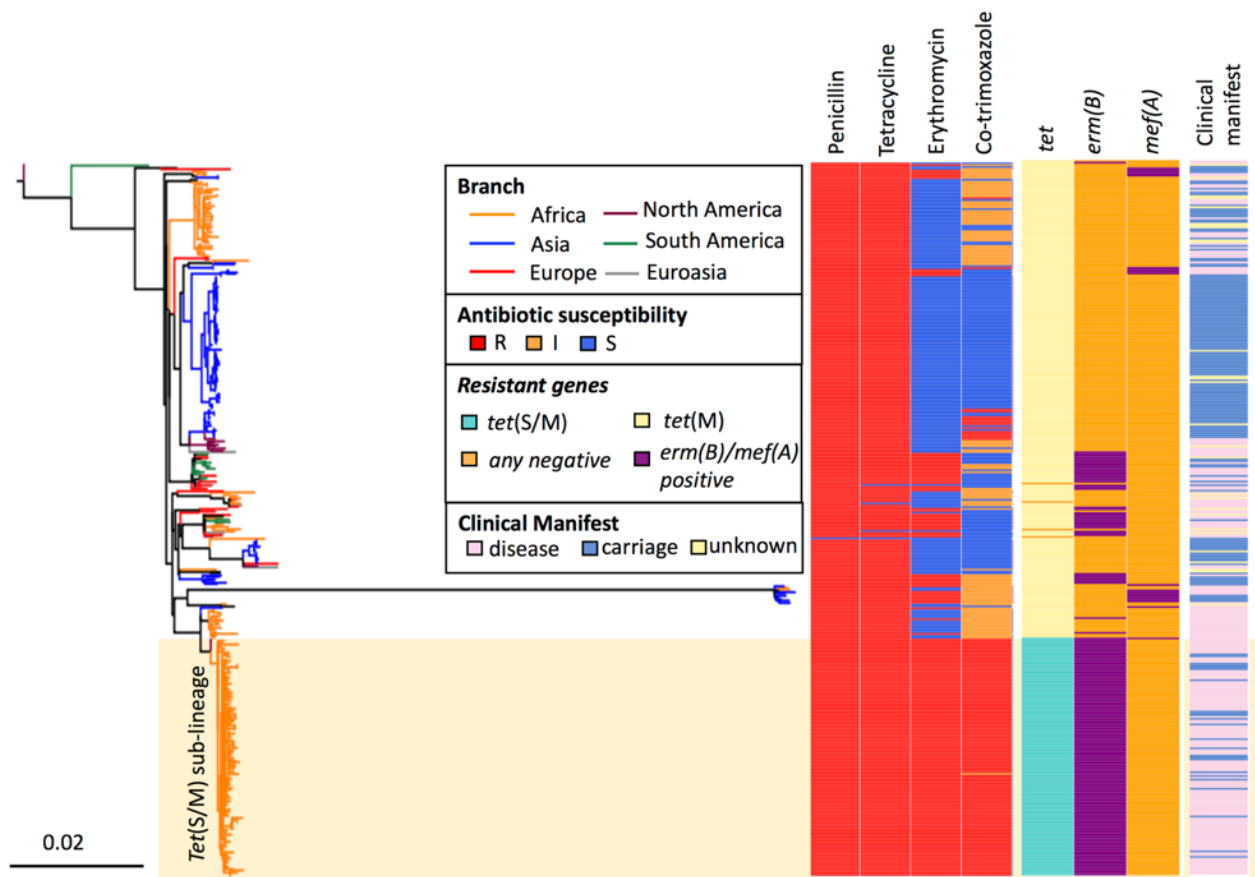
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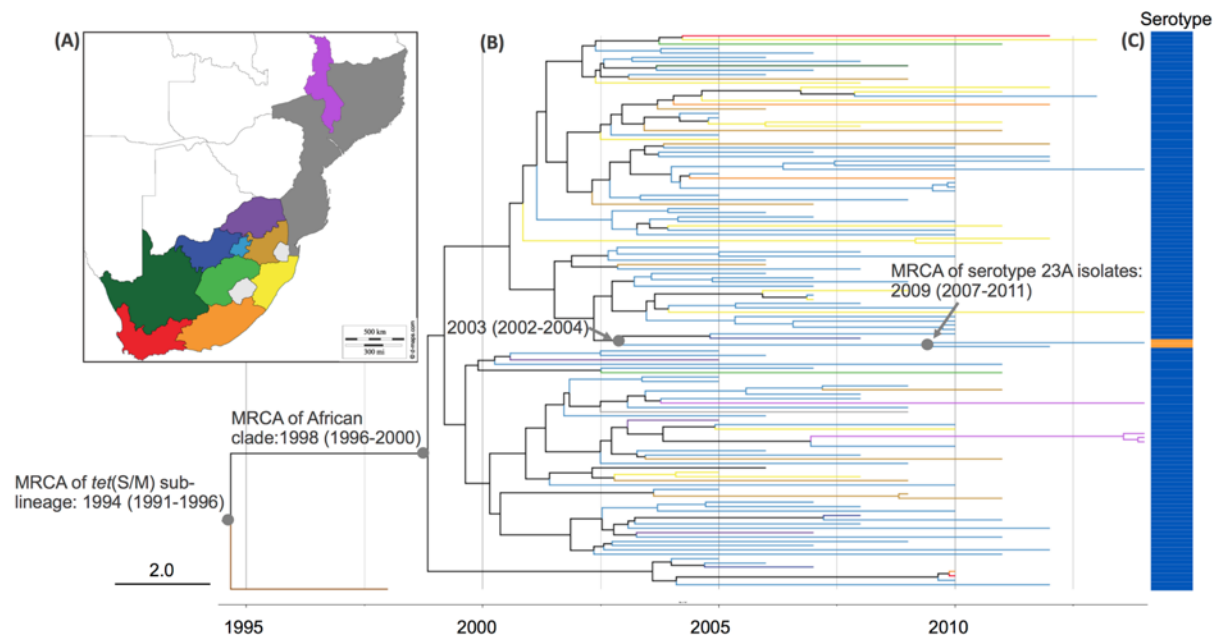


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**Figure 1.** Schematic representation of the mosaic structure of *tet(S/M)* alleles of the current and previous studies. The reference sequences for *tet(M)* and *tet(S)* were retrieved from NCBI Genbank using accession number EFU09422 and FN555436,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

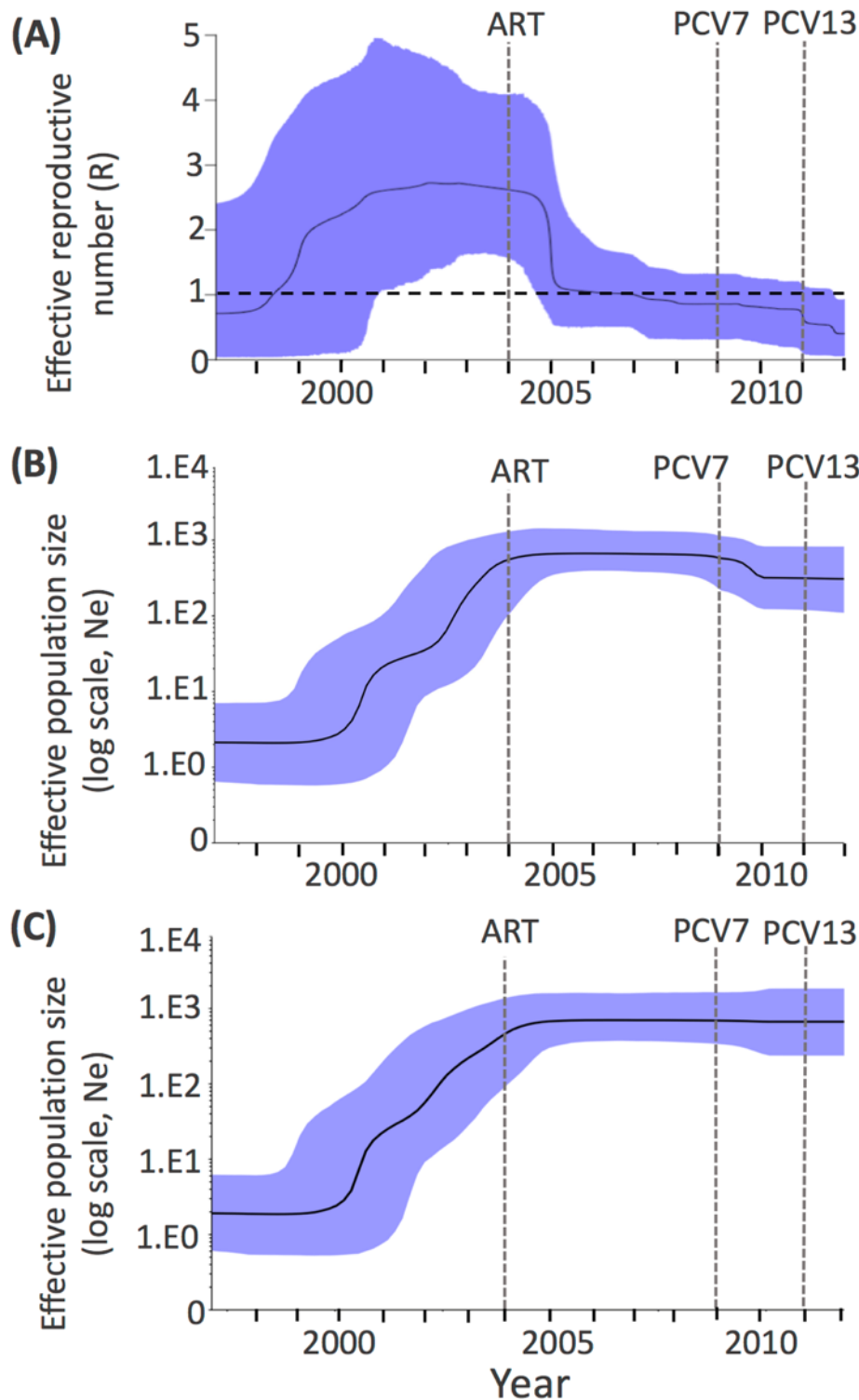


**Figure 2.** A SNP tree constructed with CC230 *tet(S/M)*-positive isolates (n=129) and *tet(S/M)*-negative carriage/disease isolates (n=260) collected from twenty countries. The tree was built based on 13,405 SNPs extracted from an alignment outside recombination regions, created by mapping reads of each isolate to the sequence of a ST230 reference strain, PMEN global clone Denmark<sup>14-32</sup>, 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.

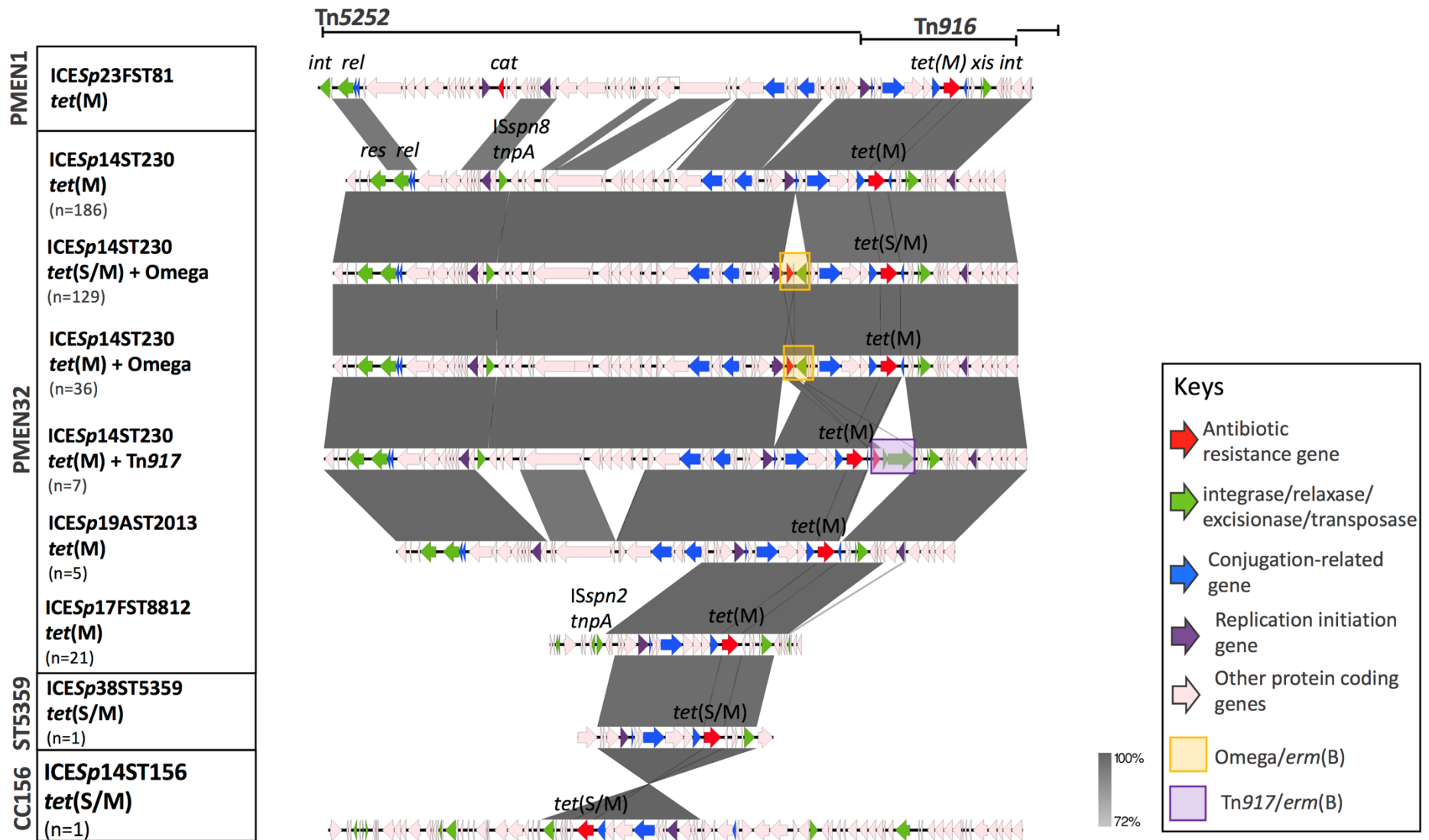


**Figure 3.** (A) Malawi, Mozambique and administration regions of South Africa (B) Timed phylogeny for *S. pneumoniae* tet(S/M) CC230 sub-lineage (n=129) reconstructed using BEAST. Tree branches are coloured according to the geographical locations in (A), except for the branch for an isolate collected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

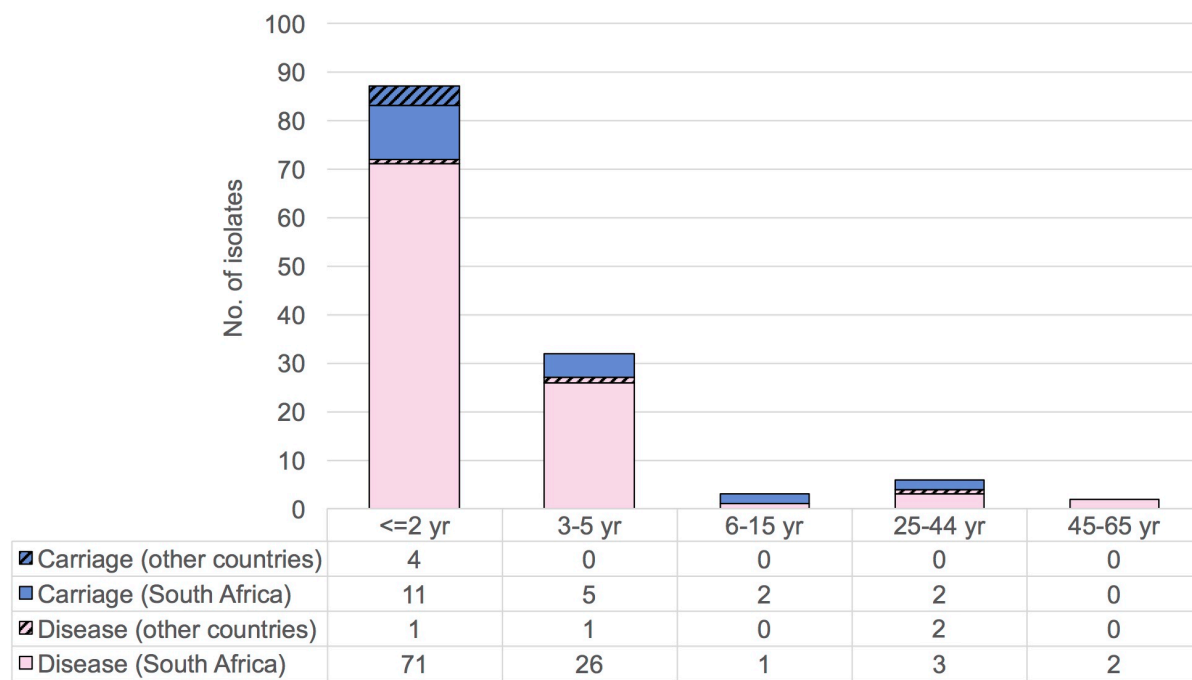




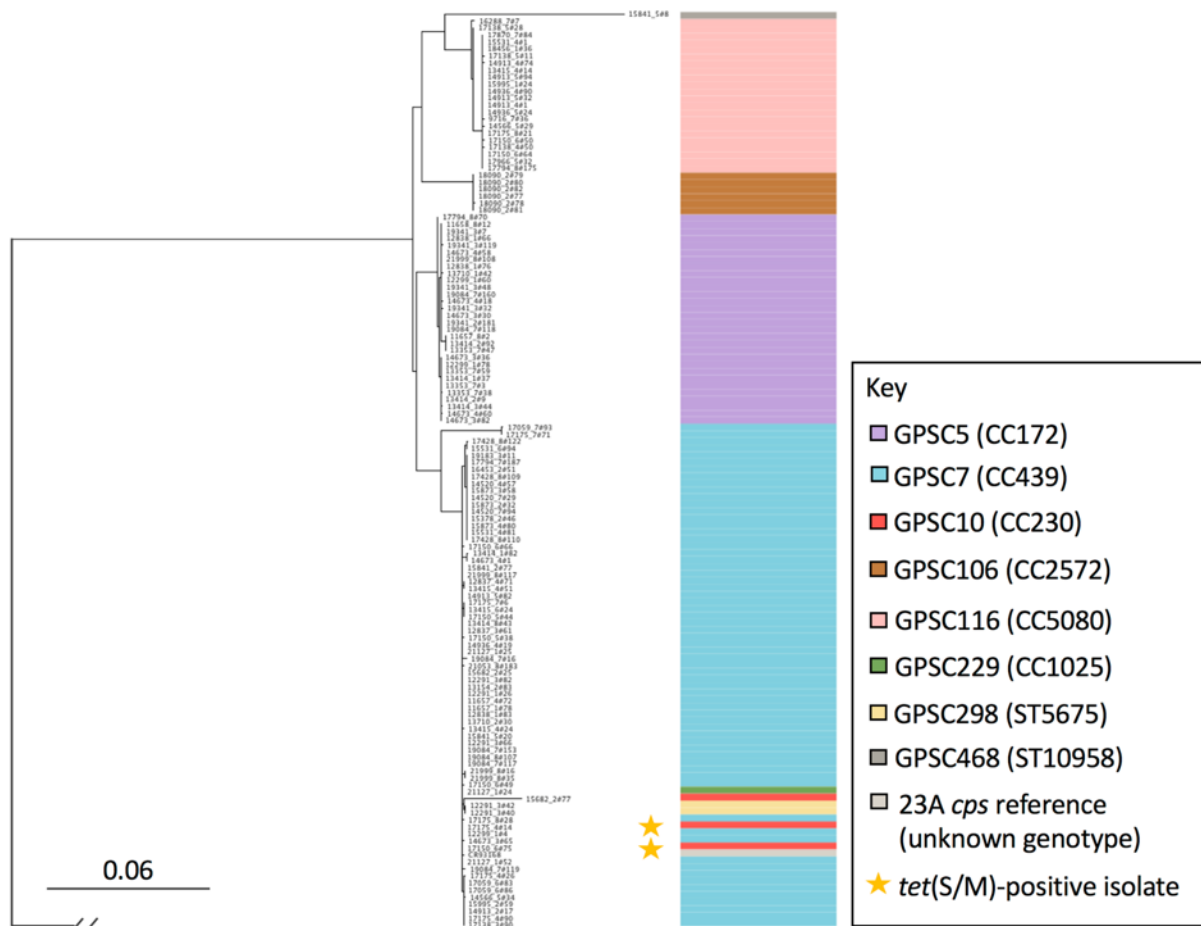
**Figure 4.** (A) Birth-death skyline plot of inferred changes in effective reproductive number ( $R$ ) of *S. pneumoniae tet(S/M) CC230* sub-lineage using IPD isolates ( $n=105$ ). (B) Coalescent-based skyline plot of inferred changes in the effective population size ( $N_e$ ) of *S. pneumoniae tet(S/M) 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.



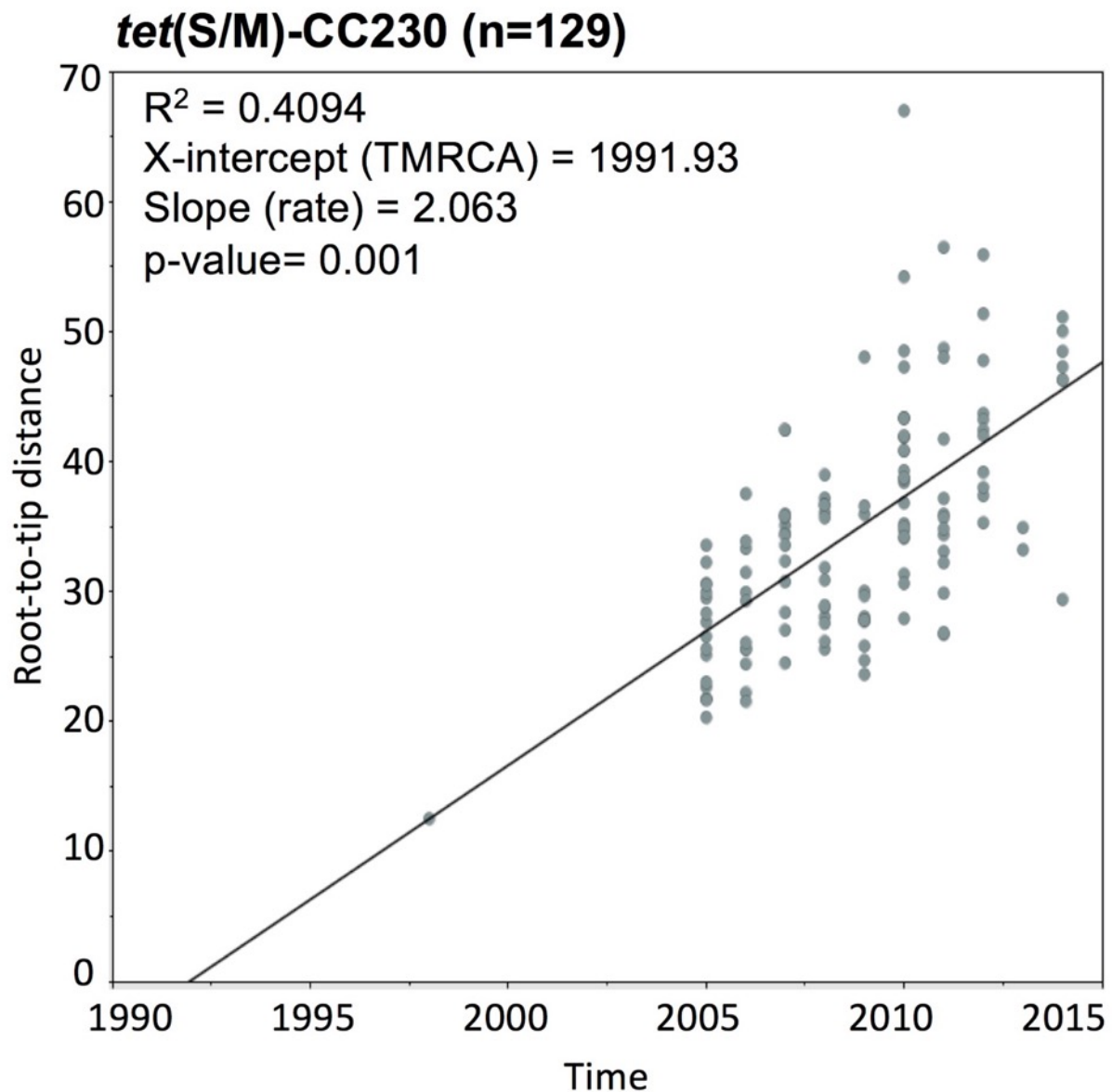
**Figure 5.** Comparison of integrative and conjugative element (ICE) identified in clonal complex (CC)230 with Spain<sup>23F</sup>-1 (PMEN1). Grey bands between the sequences indicate BLASTN matches.



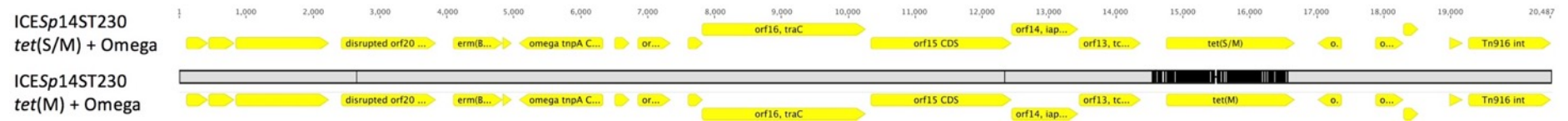
**Figure S1.** Number of *tet(S/M)*-positive isolates, by age and clinical manifest



**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.