

1 **Tetracycline resistance mediated by *tet(M)* has variable Integrative Conjugative Element**
2 **composition in *Mycoplasma hominis* isolated in the United Kingdom from 2005-2015.**

3 Victoria J Chalker^a, Martin G. Sharratt^b, Christopher Rees^b, Oliver H. Bell^b, Edward Portal^b, Kirsty
4 Sands^{b,c}, Matthew S. Payne^d, Lucy C. Jones^{b,e} and Owen B. Spiller^{a,b,#}

5 ^aNational Infection Service, Public Health England, London, UK

6 ^bDivision of Infection and Immunity, School of Medicine, Cardiff University, Wales, UK

7 ^cDepartment of Zoology, University of Oxford, UK

8 ^dSchool of Medicine, Division of Obstetrics and Gynaecology, The University of Western
9 Australia, Crawley, Australia.

10 ^eDepartment of Integrated Sexual Health, Dewi Sant Hospital, Cwm Taf Morgannwg University
11 Health Board, Pontypridd, UK

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13 #Correspondence: Dr Owen B. Spiller, Division of Infection and Immunity School of Medicine,
14 Cardiff University, Cardiff, UK CF14 4XN; email: spillerb@cardiff.ac.uk.

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18

19 **ABSTRACT**

20 A minimal genome and absent bacterial cell wall renders *Mycoplasma hominis* inherently
21 resistant to most antimicrobials except lincosamides, tetracyclines and fluoroquinolones.
22 Often dismissed as a commensal (except where linked to preterm birth), it causes septic
23 arthritis in immunodeficient patients and is increasingly associated with transplant failure
24 (particularly lung) accompanying immunosuppression. We examined antimicrobial
25 susceptibility (AST) on strains archived between 2005-2015 submitted to the Public Health
26 England reference laboratory and determined the underlying mechanism of resistance by
27 whole genome sequencing (WGS). Archived *M. hominis* strains included 32/115 from invasive
28 infection (sepsis, CSF, peritoneal and pleural fluid) over the 10-year period (6.4% of all
29 samples submitted between 2010-2015 were positive). No clindamycin resistance was
30 detected, while two strains were resistant to moxifloxacin and levofloxacin (resistance
31 mutations: S83L or E87G in *gyrA* and S81I or E84V in *parC*). One of these strains and 11
32 additional strains were tetracycline resistant, mediated by *tet(M)* carried within an integrative
33 conjugative element (ICE) consistently integrated at the somatic *rumA* gene; however, the
34 ICEs varied widely in 5-19 associated accessory genes. WGS analysis showed *tet(M)*-carrying
35 strains were not clonal, refuting previous speculation that the ICE was broken and immobile.
36 We found *tet(M)*-positive and -negative strains (including the multi-resistant 2015 strain) to
37 be equally susceptible to tigecycline and josamycin, however, the British National Formulary
38 does not include guidance for these. Continued *M. hominis* investigation and AST surveillance
39 (especially immunocompromised patients) is warranted, and expansion of the limited
40 therapeutics needs to be expanded in the UK.

41

42

44 **Introduction**

45 The bacterial species *Mycoplasma hominis* has a minimal genome and cell wall composition
46 more closely resembling eukaryotic cells (deficient in both peptidoglycan and
47 lipopolysaccharide) and therefore outside classification by Gram staining.(1) *Mycoplasma*
48 *hominis* is an opportunistic pathogen, normally found as a commensal on the mucosal
49 membranes of human urogenital tracts, more commonly in women.(2) As a pathogen, *M.*
50 *hominis* has been associated with endometritis and/or pelvic inflammatory disease(3) and
51 bacterial vaginosis(4). Infections in pregnant women can be serious, potentially causing
52 adverse pregnancy outcomes and vertical transmission to neonates with significant
53 sequelae.(5) However, *M. hominis* has also been associated with extragenital disease
54 particularly in immunocompromised patients. *M. hominis* was implicated in lung transplant
55 failure in some patients due to the combination of immunosuppression and inherent
56 resistance to traditional post-surgical prophylactic antimicrobials,(6, 7) as well as following
57 endocarditis associated with systemic infection(8) and in kidney transplant patients.(9)
58 However, *M. hominis* is largely a neglected pathogen as it does not generate a substantial
59 colony on any commonly used bacterial culture medium.

60 Treatment options are limited, the lack of a typical bacterial cell wall renders drugs such as β -
61 lactams (e.g. penicillin) or polymyxins (e.g. colistin) ineffective, and the nucleotide scavenging
62 of *M. hominis* excludes antifolates and trimethoprim.(10) *M. hominis* is inherently resistant
63 to rifampin due to amino acid substitutions in the beta-subunit of their RNA polymerase
64 complexes and resistant to 14-/15-membered ring (but not 16-membered ring) macrolides
65 due to sequence polymorphisms in their 23S rRNA gene.(11) The remaining effective
66 therapeutics include lincosamides, ketolides, tetracyclines and fluoroquinolones, which
67 inhibit the protein synthesis mechanisms or DNA replication of the bacteria, some of which
68 are inappropriate for use during pregnancy.(12)

69 The potential for increasing resistance rates to tetracyclines via the acquisition of the *tet(M)*
70 gene, which acts by displacing tetracycline and modifying the 16S rRNA subunit, is of
71 concern(13). The gene is inherited primarily by horizontal gene transfer (HGT) via transposons
72 and/or plasmids, with transposon *tn916* most commonly associated with its dissemination,
73 previously reported in 2015 by Calcutt and Foecking (14). However, the absence of *tn916*

74 conjugation genes led to speculation as to whether the *tet(M)* element retained mobility or
75 not.(15)

76

77 We undertook an analysis of 96 archived *M. hominis* strains originating from 81 separate
78 patient specimens submitted to Public Health England (PHE) between 2005 and 2015. Agar-
79 based antibiotic susceptibility testing (AST) was performed for tetracycline, clindamycin and
80 fluoroquinolones, and MICs for josamycin and tigecycline were determined by microbroth
81 dilution for a subset of tetracycline-susceptible and -resistant strains. All antibiotic-resistant
82 strains and an equivalent number of susceptible control strains were analysed by whole
83 genome sequencing to determine the underlying mechanisms of resistance observed and
84 bioinformatic interrogation was undertaken to fully characterise the variability in the *tet(M)*-
85 containing ICE.

86

87 **Methods**

88 **Clinical Samples**

89 *M. hominis* strains investigated were derived from clinical specimens referred to the Public
90 Health England (PHE) reference laboratory for diagnostic investigation or antimicrobial
91 susceptibility testing (from 21st February 2005 to 9th October 2015). Isolated strains were
92 archived at -80°C as agar cubes in liquid media and/or liquid cultures (with or without beads)
93 until investigated for this study. Samples submitted were derived from a range of clinical
94 specimens including neonatal, obstetric and sexual health as well as invasive infections (blood
95 culture, CSF, heart valve, ascitic fluid and pleural fluid; for details see Supplementary table
96 S1). Four vaginal reference strains from Australia were also included for comparative analysis
97 (3 from the same patient (AH3) collected at 20, 28 and 36 weeks gestation, as well as a single
98 strain from a separate patient (AH58).(16) Prior to July 2010, PHE used 16S RNA gene-based
99 PCR(17) and culture for *M. hominis* detection; from July 2010; however PHE later transitioned
100 to using an adapted PCR method to amplify the gap gene(18) (from January 2014-January
101 2015), which was then replaced by a superior qPCR method targeting the *yidC* gene January
102 2015-current.(19)

103 **Ethics Considerations**

104 This work was classified as service evaluation and clinical investigation, and so was exempted
105 from NHS Research Ethics Committee Review. Original ethical approval covering the

106 reference strains included from Australia were approved by the Human Research Ethics
107 Committee of Western Australian Department of Health, Women and Newborn Health
108 Service (2056/EW).

109 ***M. hominis* culture and antimicrobial screening**

110 Recovery from frozen archives was performed by resuspension in Mycoplasma selective
111 medium purchased from Mycoplasma Experience Limited (Reigate, UK). Plates were sealed
112 with clear adhesive film in a humidified chamber and incubated at 37°C for up to 5 days.
113 Cultures and plates were checked daily and growth recorded. “Fried-egg” colonies
114 characteristic of *M. hominis* were counted using a stereo microscope and growth in broth
115 culture was visualised as a yellow to red colour change in the absence of turbidity.
116 Antimicrobial screening was performed as outlined by CLSI guidelines (20) (the methodology
117 and data underlying these guidelines has been fully published in (21)) using defined resistance
118 thresholds: 2 mg/L levofloxacin, 0.5 mg/L moxifloxacin, 0.5 mg/L clindamycin, and 8 mg/L
119 tetracycline. Mycoplasma selective agar was purchased from Mycoplasma experience
120 Limited and Mycoplasma selective medium was prepared by CPM SAS (Rome, Italy). While no
121 resistance thresholds have been determined for josamycin and tigecycline, MICs were
122 determined under the same parameters. 134 *M. hominis* strains from 81 patients were
123 analysed. Initial screening for *tet(M)* presence was performed by traditional PCR (AppTaq
124 RedMix (Appleton Woods, UK), 40 cycles, T_m=56°C, extension 30 sec) visualising the expected
125 419 bp product by transilluminated ethidium bromide containing 1% w/v agarose gel
126 electrophoresis using PCR primers (designed in this study) tetM1338F 5’-
127 TATCTGTATCACCGCTCCG-3’ and tetM1758R 5’-AATACACCGAGCAGGGATTT-3’.

128 **Whole genome sequencing and bioinformatics.**

129 For the subset of strains to be examined by whole genome sequencing, individual colonies
130 were grown in 30mL Mycoplasma selective medium, pelleted at 13,000xg for 3h and
131 resuspended in 400 µL of sterile distilled water as the first step of DNA extraction using the
132 Qiagen EZ1 Advanced XL automated extractor utilizing the EZ1 DSP Virus Kit as per
133 manufacturer’s instructions. DNA yields were between 1-8 ng/µL (Qubit 4.0, Life
134 Technologies). Genomic sequencing was undertaken using a Nextera XTv2 library preparation
135 kit with V3 chemistry on an Illumina MiSeq platform. QC pipeline to validate & trim the raw
136 sequence reads, whole genome assembly and mapping as well as whole genome annotation
137 and profiling of genetic determinants were performed as previously published.(22)

138 Assembled contigs were further analysed utilising Geneious sequence analysis software
139 (BioMatters Ltd. New Zealand) and aligned and assessed against reference sequences (Sprott,
140 accession number NZ_CP011538; and PL5, accession number NZ_JRXA01000009) for the
141 identification of genetic elements and presence of point mutations and gene rearrangements
142 using Snippy v4.4.5 and Roary v3.12.0(23, 24). Multi-locus sequence typing was performed *in*
143 *silico* using the targets we have previously reported.(25) Whole Genome Shotgun project for
144 MH15-3 has been deposited at DDBJ/ENA/GenBank under the accession JACXZU000000000.
145 The version described in this paper is version JACXZU010000000 and the strain can be
146 obtained from the National Collection of Type Cultures (UK) (strain NCTC14456). The
147 remainder of the resistant strains can be found submitted to BioProject PRJNA675754.
148 Further phylogenetic analysis was performed by comparing a standardised 10kb DNA
149 segment upstream and downstream (separately) of the ICE insertion site for *tet(M)*-positive
150 and -negative control strains using Geneious software using the Jukes-Cantor distance model
151 and Neighbor-Joining tree building method to identify relatedness of the two groups.

152

153 **Results**

154 **Reference culture and PCR review**

155 Following inclusion of molecular methods in July 2010, records show 6.4% of samples
156 submitted for investigation of *M. hominis* infection were found to be positive (Table 1). The
157 type of clinical specimens submitted for these archived positive strains are shown in
158 Supplementary Table S1.

159

160 **Antibiotic susceptibility testing evaluation on recovered viable strains.**

161 A total of 96 of 134 archived strains originating from 81 separate patient specimens were
162 revived. These were investigated in parallel for growth on inoculated plates containing a final
163 concentration of either 2 mg/L Levofloxacin, 0.5 mg/L Moxifloxacin, 0.5 mg/L Clindamycin or
164 8 mg/L Tetracycline, representing the CLSI resistance breakpoints. No strains were resistant
165 to clindamycin. One strain (MH10-9) from 2010 showed resistance to two separate
166 fluoroquinolones (MIC= 8 mg/L for moxifloxacin and 16 mg/L for levofloxacin), and an
167 additional strain (MH15-3) from 2015 showed multi-drug resistance to both fluoroquinolones
168 tested (MIC= 16 mg/L for moxifloxacin and 32 mg/L for levofloxacin) as well as tetracycline

169 (MIC=16 mg/L). In total, 12/81 (14.8%) showed resistance to tetracycline distributed
170 sporadically and uniformly across the 10-year period (Table 1).

171

172 **Mechanisms of antimicrobial resistance.**

173 Genomic sequencing of the two fluoroquinolone-resistant strains identified mutations in both
174 the *gyrA* gene (S83L for MH15-3 and E87G for MH10-9) and the *parC* gene (S81I for MH15-3
175 and E84V for MH10-9). PCR screening of all strains identified the presence of the *tet(M)*
176 resistance gene only in the 12 tetracycline-resistant strains. The *tet(M)*-positive strains were
177 also the only ones that grew in the presence of 8 mg/L of tetracycline. PCR results were also
178 subsequently confirmed by whole genome sequence analysis. ICE regions uniformly showed
179 insertion at the 3' end of the *rumA* gene and ended at the hypothetical protein (Figure 1; ORF
180 MHOMSp_RS02665 in reference strain Sprott, accession number NZ_CP011538). Five groups
181 (Group I to V) characterised by genetic composition between all ICE regions were observed.
182 Group I contained the six largest ICE regions (PL5, Sprott, MH13-7, MH10-4, MH06-11, MH12-
183 9 and MH10-15); however, only PL5 contained a full set of uninterrupted genes with the full-
184 length ICE, while all the others had at least one ORF disruption by presence of a premature
185 stop codon. Group II ICE had lost 4 ORFs preceding the serine recombinase with further
186 mutation derived truncation of 1 or more ORFs. Group III contained the three reference
187 strains with different isolation dates from a single Australian patient (AH3) (16), as well as
188 MH06-1, all of which had lost 7 ORFs from the 3' end including the serine recombinase relative
189 to group I. Due to premature ORF termination by mutation, all three AH3 strains also had a
190 disrupted *tn916* integrase in addition to loss of the serine recombinase. Group IV had lost all
191 non-*tn916* ORFs from Group I, with the exception of the serine recombinase, but the *tn916*
192 excisase and integrase were lost, co-incident with 80% identity degeneration of ORF7 relative
193 to the other groups. Group V consisted solely of MH05-14, which had lost all ORFs between
194 the *tet(M)* gene and the insertion point at ORF MHOMSp_RS02665, as well as disrupted
195 ORF15 and 16, resulting in the loss of all genes that could facilitate transfer to another
196 genome. Irrespective of truncations and/or deletions in ICE gene composition, all strains
197 retained resistance to tetracycline.

198

199 **Sequence veracity and genetic drift**

200 A high frequency of ORF truncation mediated by stop codons arising by SNP were observed
201 in the ICE analysis. Veracity and repeatability of sequencing were investigated to ensure these
202 stop codons were stable and not a result of sequencing error. Sequences generated
203 independently on three separate occasions for the resistant strain MH06-12 showed no
204 sequence variation within the ICE and 2 SNPs external to the ICE on the contig were found (a
205 G to T transition synonymous mutation located 21.9 kb 5' and an additional T added to a poly-
206 T intergenic stretch 22 kb 3' of the ICE element). This demonstrates high reproducibility of the
207 SNPs identified within the ICE between strains.

208

209 The sequence variation from Australian antenatal screening strains taken at 20-, 28- and 36-
210 weeks' gestation from the same patient were additionally analysed to assess sequence
211 veracity but also determine temporal accumulation of mutations in this region. No SNPs were
212 observed within the ICE and 5 SNPs were observed within the entire 114,794 bp contig
213 containing *tet(M)*: A31,843G altering Ile642 in the exodeoxyribonuclease V subunit alpha (20
214 week specimen) to Val642 (28 and 36 week specimens); C53,551A truncates a hypothetical
215 open reading frame position 181 of 284aa hypothetical protein (36 week specimen); variation
216 in an intergenic poly-T region from 19 T and 21 T (28 week), 20 T (36 week); and T114,552C
217 resulting in synonymous codon polymorphism for hypothetical protein MHOMSp_RS02740.
218 Therefore, the rate of genetic drift for *M. hominis* over 16 weeks was found to be very low
219 and not found within the ICE region of the contig.

220

221 **Anecdotal evidence for ICE Mobility**

222 The initial description of the *tet(M)*-carrying ICE in *M. hominis* (Calcutt and Foecking)(15)
223 suggested the absence of the *tn916* conjugation ORFs 18-24 (which include the Ftsk
224 translocase, ArdA superfamily protein and Cro/C1 family initiation replicator ORF) potentially
225 resulted in lack of essential elements and therefore *tet(M)* gene mobility. This was supported
226 by the lack of homology between the ICE found in *M. hominis* and any other bacteria in the
227 genomic database, other than a single group B Streptococcus strain (GB00555, accession
228 number NZ_ALTN01000021), which did retain ORFs 18-24. We undertook genomic analysis of
229 the major surface protein (variable adherence antigen or VAA) type for all *tet(M)* carrying ICE
230 and other UK and Australian *M. hominis* strains to determine if *tet(M)* was restricted to a
231 single VAA type (Supplementary Table S2; listed as coloured circles in Figure 3). A more

232 intensive examination of ICE(+) strain clustering compared to ICE(-) strains was performed by
233 neighbour-joining tree construction of concatenated multi-locus sequence typing loci (Figure
234 3) using gene targets we have previously defined.(25)

235 ICE-carrying strains spanned three separate VAA types, suggesting potential to move between
236 lineages or loss from specific lineages. Most notably the three strains lacking the serine
237 recombinase were present in two different VAA types (MH05-14 and AH3, VAA-1; MH06-1,
238 VAA-2). Group IV (retaining serine recombinase, but missing *tn916* excisase and integrase)
239 were also present in two different VAA types (MH15-3 and MH13-4, VAA-2; MH13-5, VAA-
240 4b). While the majority of ICE(+) strains coincided with VAA-2, there was enough distribution
241 to suggest mobility or loss from the lineage: they distributed across the entire tree co-locating
242 to each other rather than to ICE(-) VAA-2 strains or to ICE(+) strains of alternative VAA types.
243 Early branching of fluoroquinolones and tetracycline resistant MH15-3 was noted suggesting
244 dual resistance may have arisen early in the strain's evolution.

245

246 Mardassi *et al.*,(26) noted the existence of *tet(M)* "sequence types" based on conserved SNPs
247 within the resistance gene at nt positions 593, G789A, T807C, C819A, G825A and G831A. We
248 found 6/13 of our *tet(M)* genes to match the proposed sequence type (typeA *tet(M)*) by these
249 authors (Supplementary figure S1) and while phylogenetic analysis of the individual *tet(M)*
250 genes confirmed that they clustered separately from type B *tet(M)* (Supplementary figure S2),
251 they did not co-locate to a single VAA type; however all but one (MH13-5) were found in the
252 top half of the MLST phylogenetic tree (Figure 3). Within the *tet(M)* genes lacking these
253 conserved SNPs (type B *tet(M)*), a subtype with the conserved single SNP at nt position C839T
254 (type B *tet(M)*) all clustered together on the gene phylogenetic tree. Of interest, all Type A
255 *tet(M)* containing strains co-located to the top half of the MLST phylogenetic tree (Figure 3),
256 while all type B1 *tet(M)* containing strains collocated to the bottom half of the MLST
257 phylogenetic tree. However, type B *tet(M)* were distributed equally across the tree. Identical
258 grouping of strains from the same patient (i.e. AH3-20, 28 and 36) was noted. Overall, no
259 defined lineage or common ancestry was observed accounting for the prevalence of *tet(M)*
260 relative to the *tet(M)*-negative strains. A lack of defined lineage within *tet(M)*-positive strains
261 was further confirmed by more in depth phylogenetic analysis comparing the adjacent 10kb
262 upstream and downstream of the insertion site (presuming better co-conservation and co-
263 evolution of proximal genetic markers between strains if no mobility was occurring)

264 (Supplementary Figures S3 and S4). Furthermore, no geographic relationships could be
265 identified comparing strain characteristics to origin of specimen (Supplementary Table S3).

266 **Alternative therapies for tetracycline and multi-resistant strains.**

267 Agar-based resistance screening identified 12 tetracycline-resistant strains and two
268 fluoroquinolone-resistant strains (one strain had combined resistance). We performed
269 antimicrobial susceptibility testing on these 13 strains and 17 susceptible strains and graphed
270 their susceptibility separated by *tet(M)* carriage, as the most common resistance determinant
271 (Figure 4). The CLSI resistance breakpoints are shown as dotted lines and the fluoroquinolone
272 strains (MH10-9 and MH15-3) are indicated on the levofloxacin and moxifloxacin graphs. In
273 particular, strain MH15-3 was only susceptible to clindamycin. To examine alternative
274 therapeutics, we also determined the MIC for these strains against tigecycline, a third
275 generation (glycyl)tetracycline to determine if *tet(M)* mediated an elevated MIC for this
276 antimicrobial (Figure 5). No difference in MIC was observed for strains with or without *tet(M)*.
277 Furthermore, despite *M. hominis* inherent resistance to 14- and 15-membered macrolides,
278 we also examined the susceptibility to josamycin (a 16-membered ring macrolide commonly
279 used to treat infections in France, Italy, Spain and Russia, Figure 5). Despite the ability of all
280 strains to grow in broth culture containing 16 mg/L azithromycin (data not shown), the MIC
281 for josamycin was 0.25 +/- 0.14 mg/L irrespective of *tet(M)* presence (as anticipated).
282 Therefore, therapeutic options beyond clindamycin are available (i.e. josamycin and
283 tigecycline) for multi-resistant *M. hominis* strains such as MH15-3.

284

285 **Discussion**

286 **Rates of Resistance**

287 Lincosamide, tetracycline and fluoroquinolone susceptibility testing of recovered strains from
288 2005-2015 (67% of the total received) showed tetracycline resistance was the most common
289 (12/81; 14.8%) followed by fluoroquinolone (2/81; 2.4%). One strain present in the archive,
290 MH15-3, was resistant to both tetracycline and fluoroquinolones, leaving only clindamycin as
291 a therapeutic option for this strain isolated in 2015. It is difficult to compare resistance rates
292 across countries; CLSI international guidelines were only published in 2011,(20) and a number
293 of published studies (presented in supplementary table S4) have been completed prior to this.
294 Reports of tetracycline resistance in the international literature range from 0-58%,

295 fluoroquinolone resistance from 0-94% and clindamycin resistance from 0-30%. We found
296 that all tetracycline resistance was mediated by the resistance gene *tet(M)*, and did not find
297 any strains with *tet(M)* that were tetracycline-susceptible as has been reported for rare *M.*
298 *hominis* strains elsewhere.(27)

299 In this study we identified mutations in the QRDR (quinolone-resistance determining
300 region)(28) for both *gyrA* of the gyrase complex and *parC* of the topoisomerase complex.
301 Mutation in the *parC* gene alone is a common occurrence in *Ureaplasma* spp., but has been
302 found consistently to retain susceptibility to moxifloxacin.(29, 30) Moxifloxacin has been
303 shown to be equally balanced for increased MIC when experimental mutation was induced in
304 either *parC* or *gyrA* separately, and significant moxifloxacin resistance has only been observed
305 when both genes were mutated cumulatively in *Streptococcus pneumoniae*.(31) Our results
306 also suggest that mutation of the serine residue in both genes (MH15-3; MIC=16mg/L)
307 induces greater resistance than mutation of the asparagine residues in both genes (10-9;
308 MIC=8mg/L). The availability of characterised *M. hominis* strains with defined antimicrobial
309 resistance would greatly benefit the accuracy of development of future commercial assays
310 and benefit researchers performing large retrospective cohorts, therefore we have deposited
311 MH15-3 in the National Culture Type Collection (NCTC 14456) and Genbank (BioSample
312 accession JACXZU010000000) as an open access reference resistance type strain for future
313 studies.

314

315 **Variation between strains, sequence veracity and temporal drift.**

316 When developing MLST schemes for *M. hominis* previously, we noted that inter-strain
317 diversity was unusually high – to the point where each strain had a unique ST unless it was
318 isolated from the same patient specimen.(25) It was based on this observation, that the
319 source of failed lung transplants caused by *M. hominis* infection in a clinical cohort were able
320 to be traced back to the original asymptomatic donor(6) who had right and left lobes
321 transplanted into different recipients – both of which failed due to *M. hominis* infection.
322 More recent MLST schemes have extended to including surface antigens *vaa*, *p120'*, *p60*,
323 *Imp1* and *Imp3* to segregate strains isolated from individuals with infertility from strains from
324 patients with gynaecological infections,(32) but still show wide varieties of individual ST
325 assignments. To account for this wide range of inter-strain sequence diversity, one would

326 expect that SNP acquisition rate is high. To that end, we determined the rate of change in a
327 strain that was separately extracted, sequenced, and assembled on three independent
328 occasions to determine the rate of SNP (also sequencing veracity) accumulation through short
329 passage difference (i.e. scaling up for sequencing). Comparison of the 115kb contig containing
330 the ICE showed only two SNPs – one variation of a single T in an intergenic poly-T region and
331 one synonymous mutation in an open reading frame. Therefore, short differences in passage
332 history *in vitro* do not result in significant sequence variation. Moreover, the changes in
333 sequence from strains obtained from specimens taken 16 weeks apart during antenatal
334 screening also only showed five SNPs over 112kb, two in intergenic mono-polynucleotide
335 stretches and three additional SNPs. Comparison of the five longest contigs between the AH3
336 strains collected at 20 weeks' and 36 weeks' gestation covered 537,152 nt and showed 18
337 genomic variations. Ten of these were variances in the number of nucleotides in non-coding
338 intergenic poly T or G regions, four resulted in non-synonymous mutations, one SNP resulted
339 in introduction of a stop codon, an ORF truncation and a deletion of a G from a polyG region,
340 resulting in extension of the ORF by 197 additional aa (data not shown). This would account
341 for approximately 76 SNPs per 700 kb genome per year, which would not account for the wide
342 diversity in MLST profiles observed between individual strains. Therefore, sporadic high
343 genetic alteration caused by antimicrobial exposure (fluoroquinolones are known to induce
344 SOS-mediated rapid genomic mutation,(33) immune pressure or adaptation during initial
345 infection following transmission(34, 35)) may be responsible for the high diversity.

346

347 **Variation of *tet(M)* ICE region**

348 The first observation made when undertaking an analysis of ICE regions of *tet(M)* positive
349 strains was the degree of gene content disparity between strains. Strains ranged from having
350 ICE regions consistent with the reference Sprott strain to having highly truncated regions,
351 with almost all the strains (except 13-7) containing SNP-mediated truncation of at least one
352 ORF. Surprisingly, the reference strain Sprott was observed to contain a SNP in ORF16 leading
353 to a truncation. PL5, the only other reference strain available; did not have any truncations
354 and had the same ICE gene contingent as originally reported for Sprott. The overall lack of
355 homology between *tet(M)* positive and negative strains indicates a lineage-agnostic method
356 of *tet(M)* acquisition, with horizontal gene transfer (HGT) being the most likely avenue.
357 Meygret *et al.*, recently reported the presence of other mycoplasma-specific ICEs (MICE) in

358 *M. hominis*,⁽³⁶⁾ although the essential consensus sequence SSLSDFDKTPTPKLDSKVINEYN is
359 missing from all our *tet(M)* carrying ICE and were not present anywhere in the rest of the
360 genome for any of the *tet(M)* positive strains. Furthermore, Meygret *et al.* also reported that
361 there were no antimicrobial resistance genes associated with these reported MICE. In related
362 research, mini-transposons have been used, albeit artificially, to deliver tetracycline
363 resistance genes.⁽³⁷⁾ This is further backed by the frequent occurrence of HGT that occurs in
364 mollicutes and prokaryotes in general ^(38, 39) and the phenomenon's ability to confer
365 antimicrobial resistance,^(34, 40) as well as the presence of genes in the ICE region of *M.*
366 *hominis* such as the aforementioned serine recombinase and *tn916* integrase, and including
367 *ArdA*, a known facilitator of gene mobility.⁽⁴¹⁾

368
369 Integrases are a mechanism for horizontal gene transfer, whereby this family of genes can
370 regulate not just the insertion but also the excision of gene cassettes.⁽⁴²⁾ Suspected to
371 originate from genomic insertions by bacteriophage,⁽⁴³⁾ they are a common facilitator of
372 genetic adaptation and evolution in a wide range of pathogens.⁽⁴⁴⁾ In particular, the integrase
373 in the *tet(M)* ICE region detailed here is part of *tn916*, a categorised transposon cassette, but
374 notably lacks ORFs 18-24 (but notably retains critical *VirB4* and transmembrane segregation-
375 mediating ORFs), which have been found to mediate the conjugation of the transposon.⁽⁴⁵⁾
376 Further, the authors noted the presence of tetracyclines stimulated the expression of these
377 ORFs, which encoded for the self-excision of *tn916*. Genetic drift in sub-inhibitory levels of
378 tetracycline could explain the loss of these genes, as well as genetic damage during or after
379 insertion.

380 The other main component of the ICE region previously reported by Calcutt and Foeking⁽¹⁵⁾
381 is a serine recombinase common to the ICESp2905 (originally described as a *tet(O)*- and
382 *erm(Tr)*-carrying ICE) identified in *Streptococcus pyogenes*, but identified as a common
383 ancestor to ICEs across Streptococci.⁽⁴⁶⁾ Serine recombinase elements in Streptococci
384 mediate the expected site-specific insertion into the 3' end of the *rumA* gene observed in *M.*
385 *hominis* here, consistent with most mobile genetic elements targeting specific hotspots of
386 bacterial genomes.^(47, 48)

387

388 **Antibiotics for Resistant Strains**

389 Finally, as shown in our antimicrobial resistance susceptibility analysis, MH15-3 harboured
390 inherent resistance to both 14 and 15-membered macrolides, in addition to resistance to
391 levofloxacin, moxifloxacin and tetracycline, but could be inhibited by josamycin (a 16-
392 membered macrolide) and tigecycline (a third generation (glycyl)tetracycline previously
393 reported to overcome *tet(M)* in *M. hominis* as GAR-936(49)). In fact, indistinguishable MICs
394 for tigecycline were observed between *tet(M)*-positive and -negative strains (Figure 5). There
395 are issues with the practical application of both these drugs. Currently there is no guidance in
396 the British National Formulary(50) for dosage and approved use of josamycin in the UK, while
397 it is routinely used in other countries such as France(51) or Russia (Clinical practice guidelines
398 for management of patients with sexually transmitted infections and reproductive tract
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400 Referenced in (52)). Tigecycline is available for use in a UK clinical setting, but currently can
401 only be administered IV in a hospital,(53) sharply limiting its utility. Even when pathogens are
402 susceptible to a wider range of tetracyclines, this is with the provision that their well-known
403 chelating properties prevent their administration to pregnant women and neonates two of
404 the demographics most commonly identified for treatment of *M. hominis* infections.(54, 55)
405 Neonatal, pregnancy and sexual health samples accounted for 50/115 of our specimens
406 investigated (Supplementary Table S1); however, there were a significant number of surgical
407 complication (four wound drains, four wound swabs, two surgical wounds, one heart valve
408 and four tissue samples) and invasive infection (two knee aspirates, three pleural fluids, two
409 peritoneal fluids, one ascitic fluid, five blood cultures, seven CSFs and two cerebral samples)
410 where IV therapeutics would be possible as part of standard care. In light of these invasive
411 infections, including multi-drug resistant strain MH15-3 that was derived from an adult blood
412 culture, it is imperative to further our understanding of the mechanisms behind the
413 development of AMR, and continue surveillance to monitor AMR prevalence. In light of the
414 resistance documented in this and other studies, provision of up to date guidance from NICE
415 and the British National Formulary on the use of antibiotics for invasive *M. hominis* infection
416 would be beneficial for neonatal patients and those invasive infection (including those with
417 post-operative infection and for immunocompromised patients).

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561

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Table 1 – Sample numbers per year, detailing detection of *M. hominis* by PCR and culture and associated antimicrobial resistance.

Year	Culture Positive (Including referred isolates)	PCR Positive, Culture Negative	PCR Negative	PCR Positive	Total Samples	Total MH Detected (% total)	Resistance Detected
2005	9	N/A	N/A	N/A	N/A	9 ^a	1 x tet ^R
2006	11	N/A	N/A	N/A	N/A	11 ^a	3 x tet ^R
2007	3	N/A	N/A	N/A	N/A	3 ^a	
2008	8	N/A	N/A	N/A	N/A	8 ^a	1 x tet ^R
2009	15	N/A	N/A	N/A	N/A	15 ^a	
2010	16	N/A	94	0	110	16 (14.5%) ^a	2 x tet ^R 1 x moxi ^{Rb}
2011	8	1	174	3	177	9 (5.0%)	
2012	9	1	200	10	210	10 (4.7%)	1 x tet ^R
2013	15	0	233	15	248	15 (6.0%)	3 x tet ^R
2014	7	3	185	10	195	10 (5.1%)	
2015	12	2	196	14	210	14 (6.7%)	1 x tet ^R /moxi ^{Rb}
Total	113	7	1082	52	1150	120	11 x tet ^R 1 x moxi ^R 1 x tet ^R /moxi ^R

569 ^a Prior to July 2010 culture positive samples were recorded and post-July 2010 samples submitted for *M. hominis*
570 investigation tested by molecular diagnostics were included. ^b Those isolates resistant to moxifloxacin (moxi^R) were also
571 resistant to levofloxacin.

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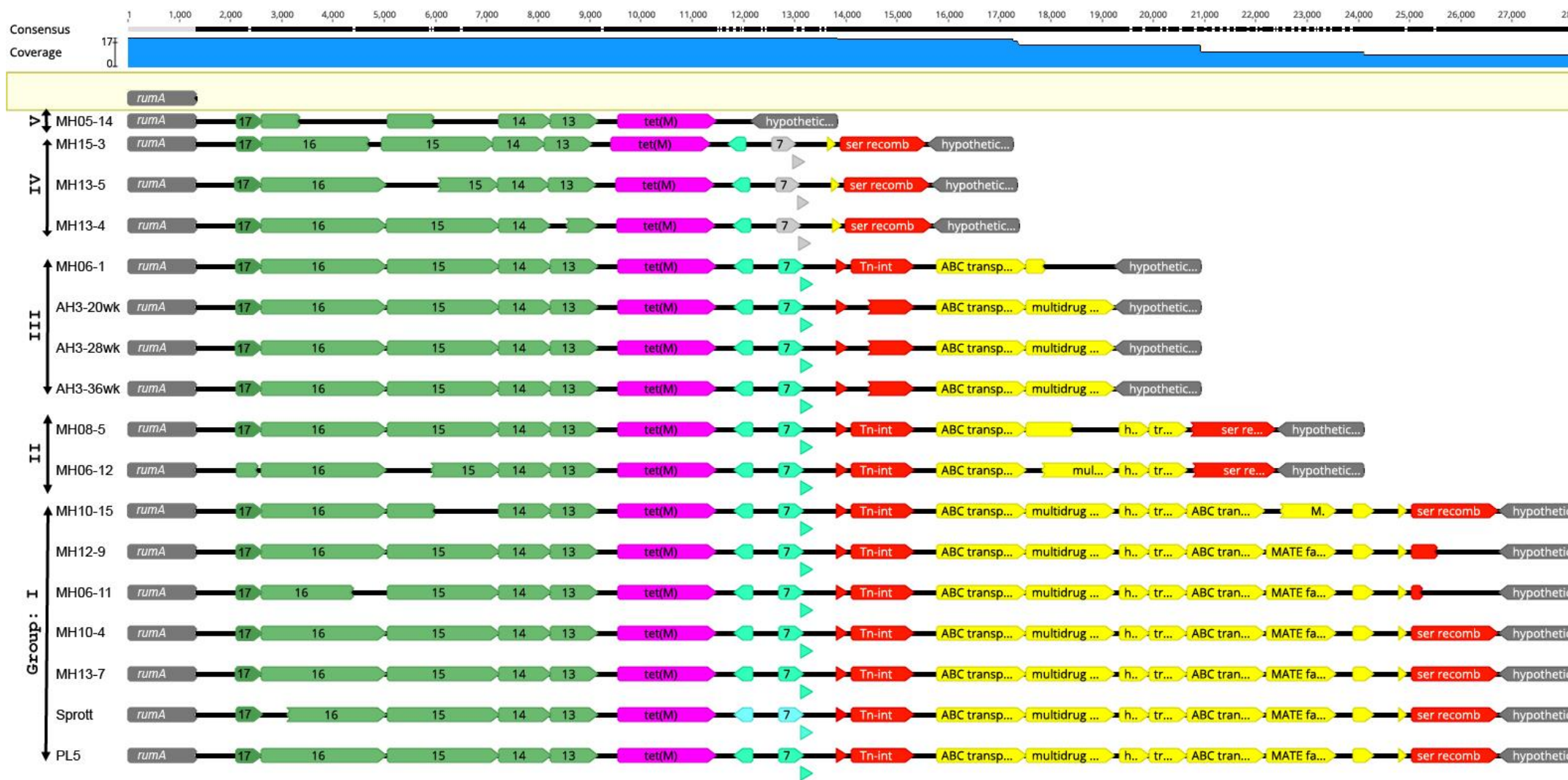


Figure 1. Alignment of ICE elements carrying the *tet(M)* gene, aligned relative to their insertion at the 3' end of the *rumA* gene. Open reading frames for PL5 reference gene consist of *rumA*; *tn916* conjugation genes (green) ORF17, ORF16, ORF15, ORF14, ORF13; *tet(M)* resistance gene (pink); *tn916* regulation genes (blue, or grey at 80% homology) ORF9, ORF7; *tn916* excisase and integrase genes (red); accessory transporter genes from ICESpy2905 (accession number FR691055; yellow) which also includes the serine recombinase (red) at the end of the mobile genetic element.

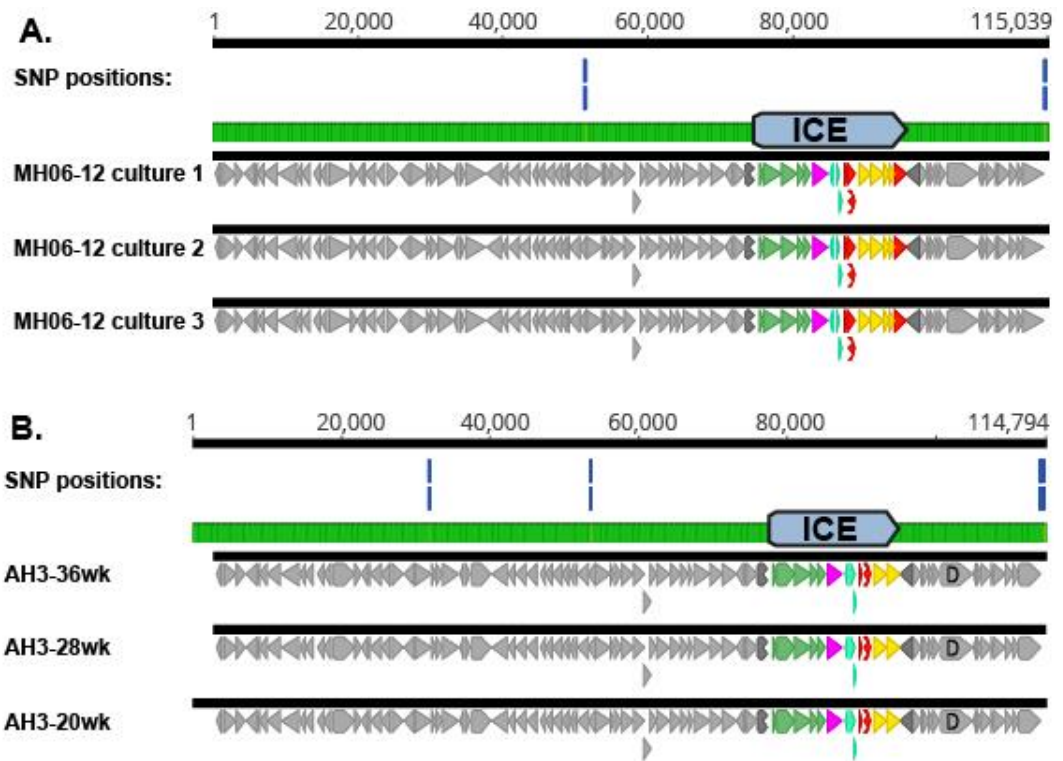


Figure 2. Nucleotide alignments for the contigs containing the *tet(M)* gene showing SNP locations identified when sequencing the same strain three independent times (A) and three independent isolations of *M. hominis* from the same patient at 20, 28 and 36 weeks' gestation (B).

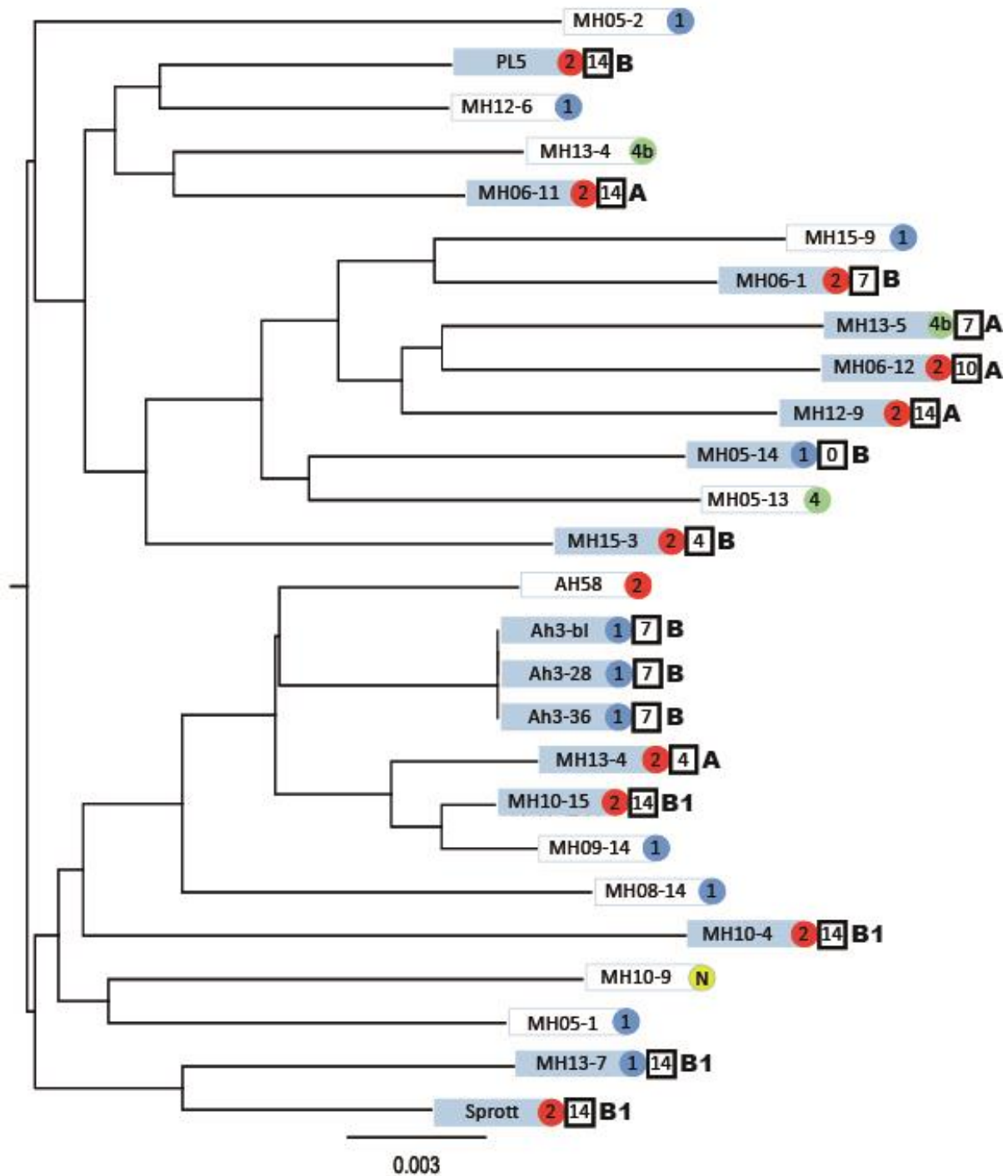


Figure 3. Neighbour joining phylogenetic analysis of MLST genes for strains with *tet(M)* (light blue box) relative to strains without (white box). Additionally, the typing of major surface antigen (VAA) is shown next to each isolate with the VAA type (blue circle = type 1, red circle = type 2, green circle = type 4 or 4b (due to 1 or 2 copies of module III, respectively), and yellow circle for novel VAA type) and the total number of ICE genes (excluding *tet(M)*) included in the ICE are shown in the square at the end of the isolate identifier. Conserved SNP variation previously identified by Mardassi *et al.*(26) is indicated as the last entry per line for type “A”, “B” and sub-variant “B1”.

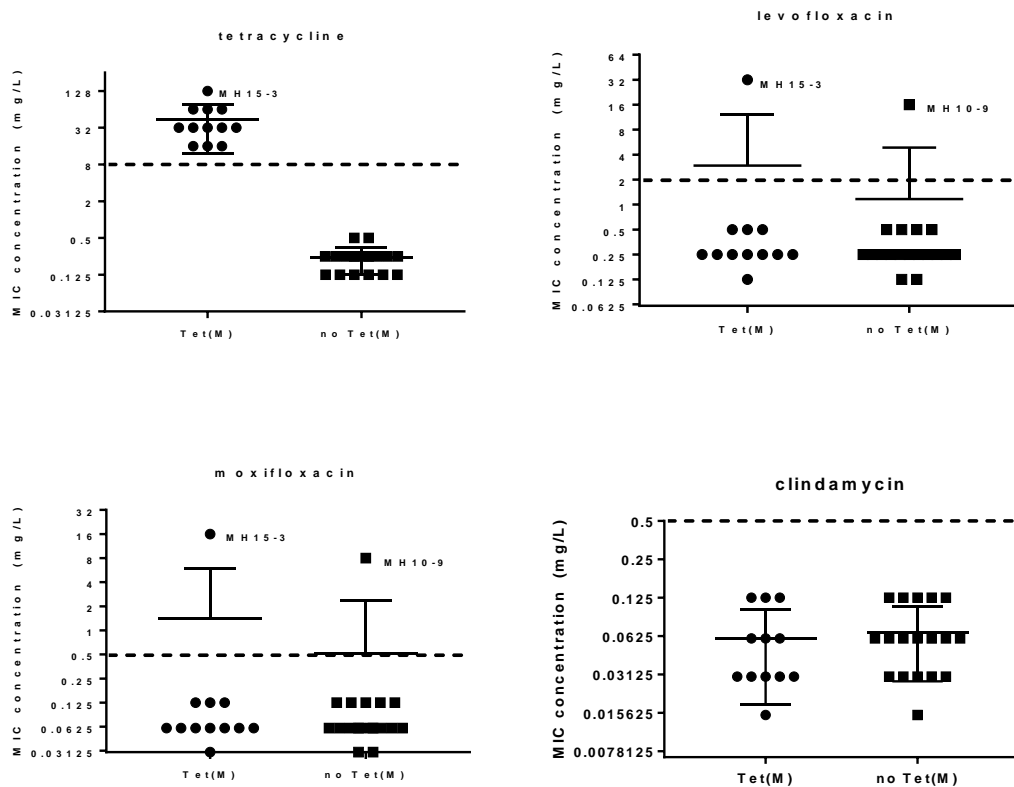


Figure 4. Antimicrobial susceptibility testing for 40 isolates (13 *tet(M)*-carrying and 27 susceptible controls) for antibiotics with CLSI-defined resistance thresholds.

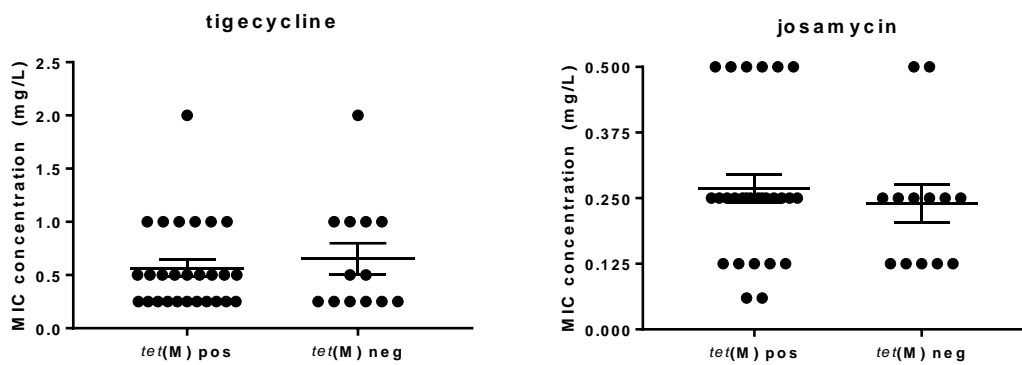


Figure 5. Antimicrobial susceptibility testing for 40 isolates (13 *tet(M)*-carrying and 27 randomly selected susceptible controls) for glycyctetracycline tigecycline and macrolide josamycin. Note that to date no breakpoints have been assigned for these antimicrobials.