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

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ABSTRACT

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

Introduction

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

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

The potential for increasing resistance rates to tetracyclines via the acquisition of the *tet*(M) gene, which acts by displacing tetracycline and modifying the 16S rRNA subunit, is of concern(13). The gene is inherited primarily by horizontal gene transfer (HGT) via transposons and/or plasmids, with transposon *tn*916 most commonly associated with its dissemination, previously reported in 2015 by Calcutt and Foecking (14). However, the absence of *tn*916

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

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

Methods

Clinical Samples

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

Ethics Considerations

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

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

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

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

Whole genome sequencing and bioinformatics.

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

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

Results

Reference culture and PCR review

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

Antibiotic susceptibility testing evaluation on recovered viable strains.

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

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

Mechanisms of antimicrobial resistance.

Genomic sequencing of the two fluoroquinolone-resistant strains identified mutations in both the *gyrA* gene (S83L for MH15-3 and E87G for MH10-9) and the *parC* gene (S81I for MH15-3 and E84V for MH10-9). PCR screening of all strains identified the presence of the *tet*(M) resistance gene only in the 12 tetracycline-resistant strains. The *tet*(M)-positive strains were also the only ones that grew in the presence of 8 mg/L of tetracycline. PCR results were also subsequently confirmed by whole genome sequence analysis. ICE regions uniformly showed insertion at the 3' end of the *rumA* gene and ended at the hypothetical protein (Figure 1; ORF MHOMSp_RS02665 in reference strain Sprott, accession number NZ_CP011538). Five groups (Group I to V) characterised by genetic composition between all ICE regions were observed. Group I contained the six largest ICE regions (PL5, Sprott, MH13-7, MH10-4, MH06-11, MH12-9 and MH10-15); however, only PL5 contained a full set of uninterrupted genes with the full-length ICE, while all the others had at least one ORF disruption by presence of a premature stop codon. Group II ICE had lost 4 ORFs preceding the serine recombinase with further mutation derived truncation of 1 or more ORFs. Group III contained the three reference strains with different isolation dates from a single Australian patient (AH3) (16), as well as MH06-1, all of which had lost 7 ORFs from the 3' end including the serine recombinase relative to group I. Due to premature ORF termination by mutation, all three AH3 strains also had a disrupted *tn*916 integrase in addition to loss of the serine recombinase. Group IV had lost all non-*tn*916 ORFs from Group I, with the exception of the serine recombinase, but the *tn*916 excisase and integrase were lost, co-incident with 80% identity degeneration of ORF7 relative to the other groups. Group V consisted solely of MH05-14, which had lost all ORFs between the *tet*(M) gene and the insertion point at ORF MHOMSp_RS02665, as well as disrupted ORF15 and 16, resulting in the loss of all genes that could facilitate transfer to another genome. Irrespective of truncations and/or deletions in ICE gene composition, all strains retained resistance to tetracycline.

Sequence veracity and genetic drift

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

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

Anecdotal evidence for ICE Mobility

The initial description of the *tet*(M)-carrying ICE in *M. hominis* (Calcutt and Foecking)(15) suggested the absence of the *tn*916 conjugation ORFs 18-24 (which include the Ftsk translocase, ArdA superfamily protein and Cro/Cl family initiation replicator ORF) potentially resulted in lack of essential elements and therefore *tet*(M) gene mobility. This was supported by the lack of homology between the ICE found in *M. hominis* and any other bacteria in the genomic database, other than a single group B Streptococcus strain (GB00555, accession number NZ_ALTN01000021), which did retain ORFs 18-24. We undertook genomic analysis of the major surface protein (variable adherence antigen or VAA) type for all *tet*(M) carrying ICE and other UK and Australian *M. hominis* strains to determine if *tet*(M) was restricted to a single VAA type (Supplementary Table S2; listed as coloured circles in Figure 3). A more

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

ICE-carrying strains spanned three separate VAA types, suggesting potential to move between lineages or loss from specific lineages. Most notably the three strains lacking the serine recombinase were present in two different VAA types (MH05-14 and AH3, VAA-1; MH06-1, VAA-2). Group IV (retaining serine recombinase, but missing *tn*916 excisase and integrase) were also present in two different VAA types (MH15-3 and MH13-4, VAA-2; MH13-5, VAA-4b). While the majority of ICE(+) strains coincided with VAA-2, there was enough distribution to suggest mobility or loss from the lineage: they distributed across the entire tree co-locating to each other rather than to ICE(-) VAA-2 strains or to ICE(+) strains of alternative VAA types. Early branching of fluoroquinolones and tetracycline resistant MH15-3 was noted suggesting dual resistance may have arisen early in the strain's evolution.

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

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

Alternative therapies for tetracycline and multi-resistant strains.

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

Discussion

Rates of Resistance

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

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

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

Variation between strains, sequence veracity and temporal drift.

When developing MLST schemes for *M. hominis* previously, we noted that inter-strain diversity was unusually high – to the point where each strain had a unique ST unless it was isolated from the same patient specimen.(25) It was based on this observation, that the source of failed lung transplants caused by *M. hominis* infection in a clinical cohort were able to be traced back to the original asymptomatic donor(6) who had right and left lobes transplanted into different recipients – both of which failed due to *M. hominis* infection. More recent MLST schemes have extended to including surface antigens vaa, p120', p60, lmp1 and lmp3 to segregate strains isolated from individuals with infertility from strains from patients with gynaecological infections,(32) but still show wide varieties of individual ST assignments. To account for this wide range of inter-strain sequence diversity, one would

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

Variation of *tet*(M) ICE region

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

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

Integrases are a mechanism for horizontal gene transfer, whereby this family of genes can regulate not just the insertion but also the excision of gene cassettes.(42) Suspected to originate from genomic insertions by bacteriophage,(43) they are a common facilitator of genetic adaptation and evolution in a wide range of pathogens.(44) In particular, the integrase in the *tet*(M) ICE region detailed here is part of *tn*916, a categorised transposon cassette, but notably lacks ORFs 18-24 (but notably retains critical VirB4 and transmembrane segregation-mediating ORFs), which have been found to mediate the conjugation of the transposon.(45) Further, the authors noted the presence of tetracyclines stimulated the expression of these ORFs, which encoded for the self-excision of *tn*916. Genetic drift in sub-inhibitory levels of tetracycline could explain the loss of these genes, as well as genetic damage during or after insertion.

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

Antibiotics for Resistant Strains

Finally, as shown in our antimicrobial resistance susceptibility analysis, MH15-3 harboured inherent resistance to both 14 and 15-membered macrolides, in addition to resistance to levofloxacin, moxifloxacin and tetracycline, but could be inhibited by josamycin (a 16-membered macrolide) and tigecycline (a third generation (glycyl)tetracycline previously reported to overcome *tet*(M) in *M. hominis* as GAR-936(49)). In fact, indistinguishable MICs for tigecycline were observed between *tet*(M)-positive and -negative strains (Figure 5). There are issues with the practical application of both these drugs. Currently there is no guidance in the British National Formulary(50) for dosage and approved use of josamycin in the UK, while it is routinely used in other countries such as France(51) or Russia (Clinical practice guidelines for management of patients with sexually transmitted infections and reproductive tract infections // Moscow, Russia, 2012. Izdatel'skiy dom Delovoy ekspress. - 112 S (In Russian). Referenced in (52)). Tigecycline is available for use in a UK clinical setting, but currently can only be administered IV in a hospital,(53) sharply limiting its utility. Even when pathogens are susceptible to a wider range of tetracyclines, this is with the provision that their well-known chelating properties prevent their administration to pregnant women and neonates two of the demographics most commonly identified for treatment of *M. hominis* infections.(54, 55) Neonatal, pregnancy and sexual health samples accounted for 50/115 of our specimens investigated (Supplementary Table S1); however, there were a significant number of surgical complication (four wound drains, four wound swabs, two surgical wounds, one heart valve and four tissue samples) and invasive infection (two knee aspirates, three pleural fluids, two peritoneal fluids, one ascitic fluid, five blood cultures, seven CSFs and two cerebral samples) where IV therapeutics would be possible as part of standard care. In light of these invasive infections, including multi-drug resistant strain MH15-3 that was derived from an adult blood culture, it is imperative to further our understanding of the mechanisms behind the development of AMR, and continue surveillance to monitor AMR prevalence. In light of the resistance documented in this and other studies, provision of up to date guidance from NICE and the British National Formulary on the use of antibiotics for invasive *M. hominis* infection would be beneficial for neonatal patients and those invasive infection (including those with post-operative infection and for immunocompromised patients).

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561

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565

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 $moxi^{R\,b}$
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/$moxi^{R\,b}$
Total	113	7	1082	52	1150	120	11 x tet^R 1 x $moxi^R$ 1 x tet^R/$moxi^R$

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

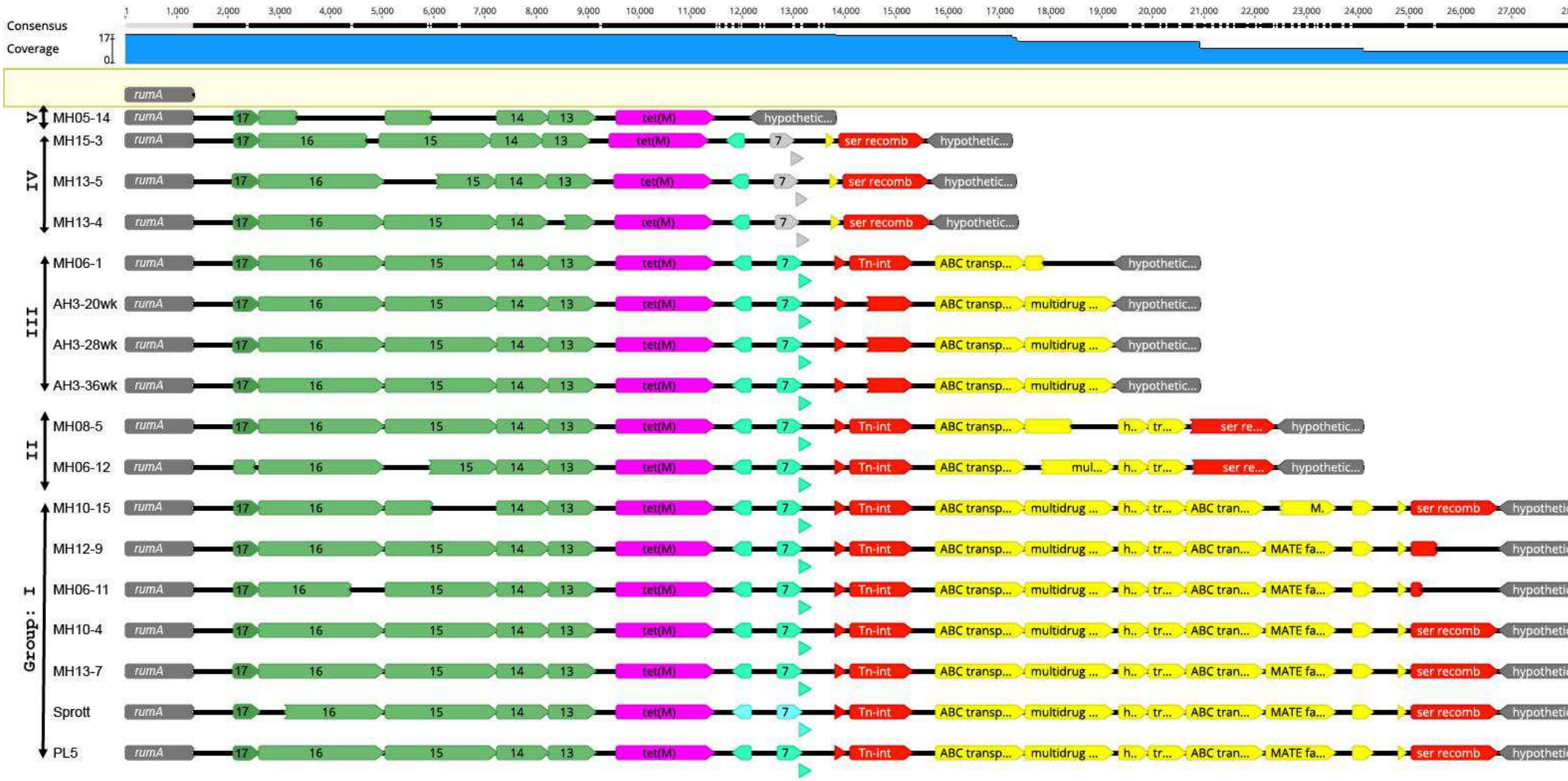

Figure 1. Alignment of ICE elements carrying the tet(M) gene, aligned relative to their insertion at the 3' end of the *rumA* gene. Open reading frames for PL5 reference gene consist of *rumA*; *tn*916 conjugation genes (green) ORF17, ORF16, ORF15, ORF14, ORF13; *tet*(M) resistance gene (pink); *tn*916 regulation genes (blue, or grey at 80% homology) ORF9, ORF7; *tn*916 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.

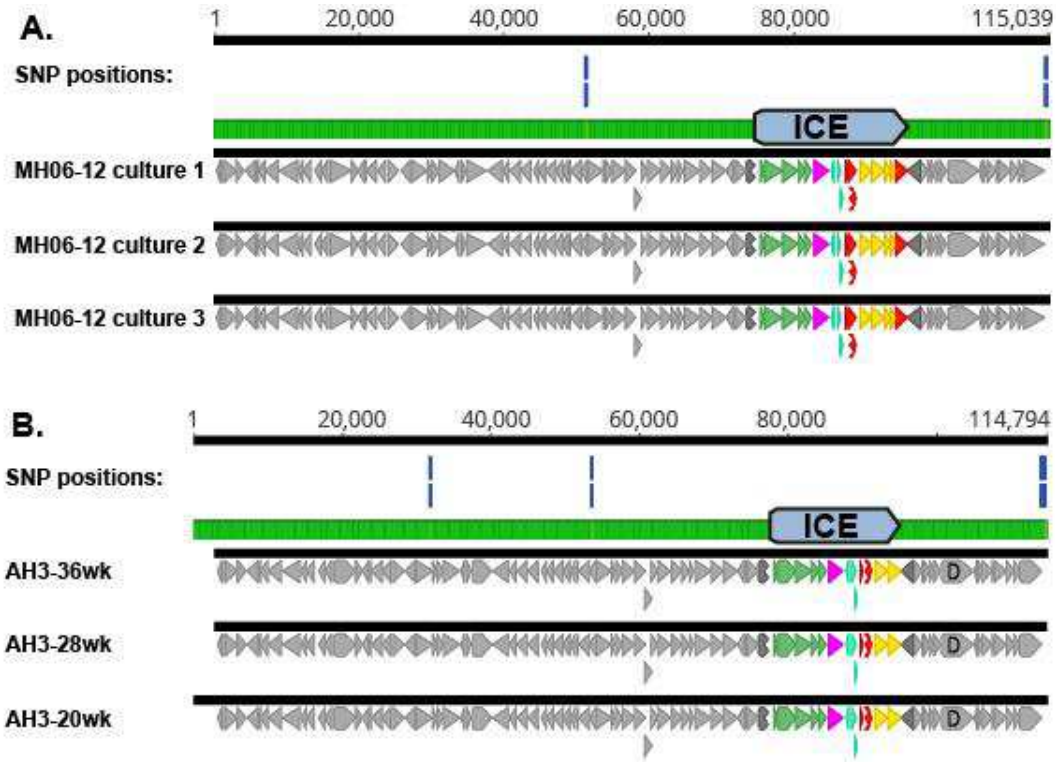

Figure 2. Nucleotide alignments for the contigs containing the *tet*(M) gene showing SNP locations identified when sequencing the same strain three independent times (A) and three independent isolations of *M. hominis* from the same patient at 20, 28 and 36 weeks' gestation (B).

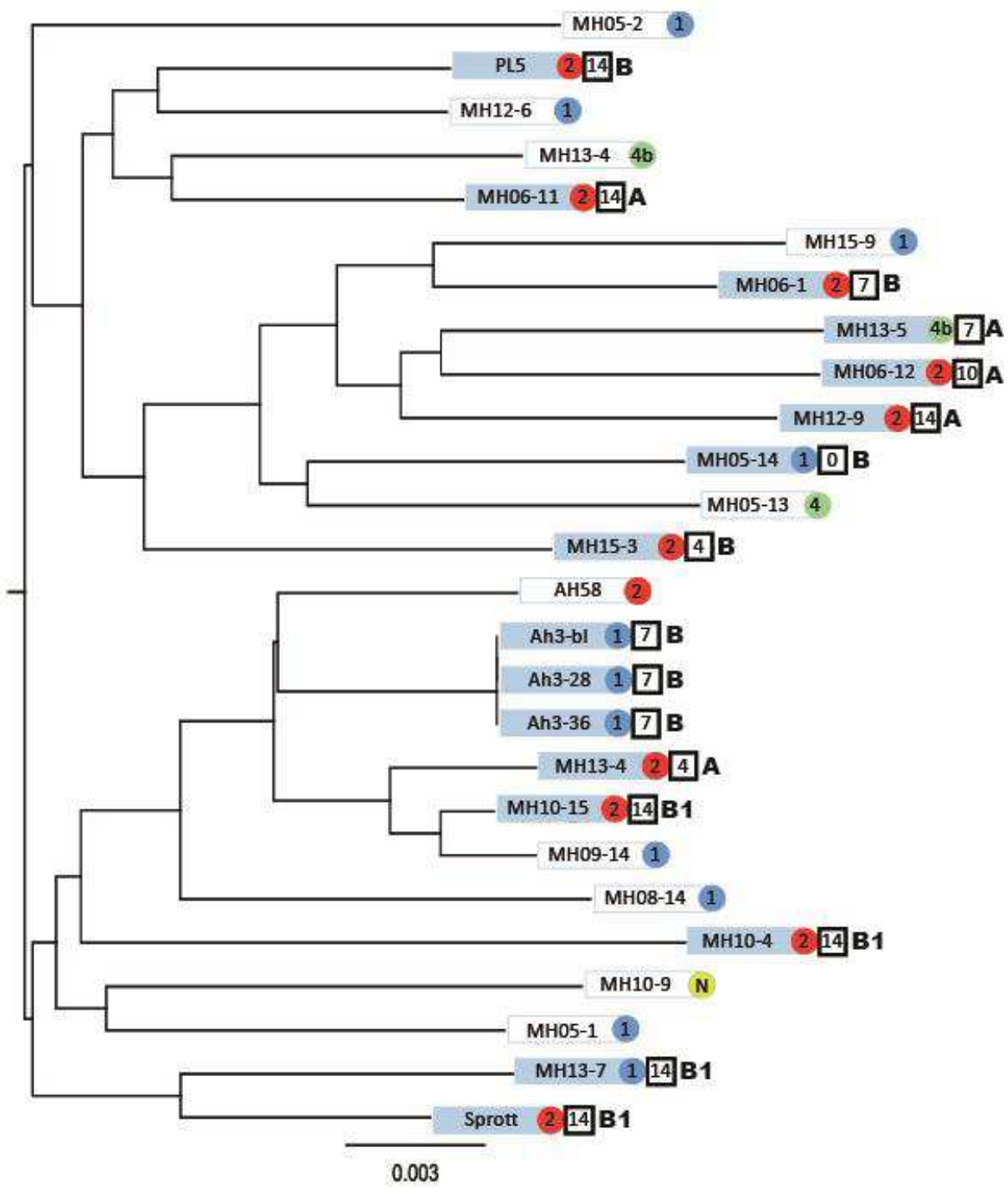

Figure 3. Neighbour joining phylogenetic analysis of MLST genes for strains with *tet*(M) (light blue box) relative to strains without (white box). Additionally, the typing of major surface antigen (VAA) is shown next to each isolate with the VAA type (blue circle = type 1, red circle = type 2, green 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".

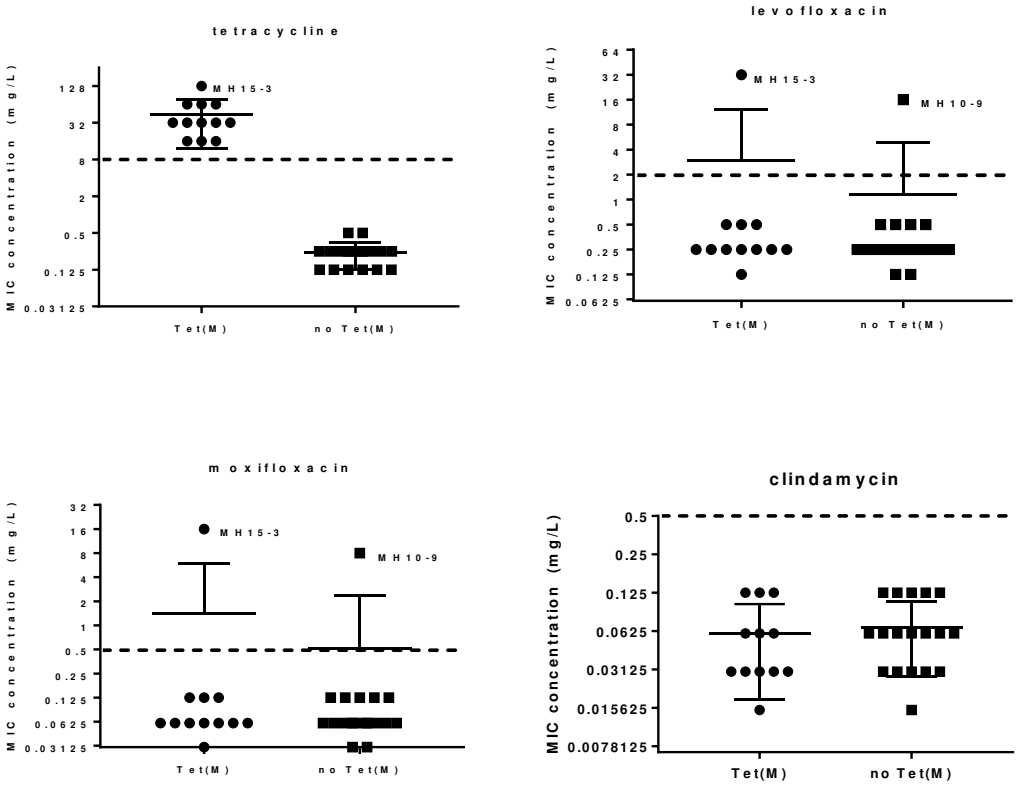

Figure 4. Antimicrobial susceptibility testing for 40 isolates (13 *tet*(M)-carrying and 27 susceptible controls) for antibiotics with CLSI-defined resistance thresholds.

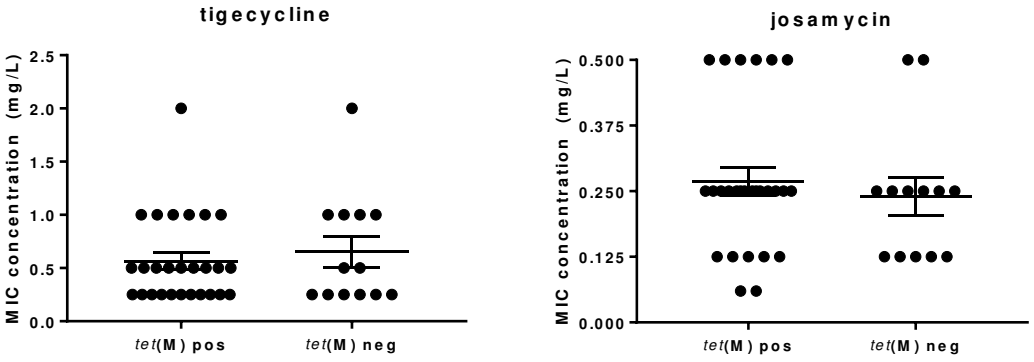

Figure 5. Antimicrobial susceptibility testing for 40 isolates (13 *tet*(M)-carrying and 27 randomly selected susceptible controls) for glycyltetracyline tigecycline and macrolide josamycin. Note that to date no breakpoints have been assigned for these antimicrobials.