Tetracycline Resistance Mediated by *tet(M)* Has Variable Integrative Conjugative Element Composition in *Mycoplasma hominis* Strains Isolated in the United Kingdom from 2005 to 2015

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ABSTRACT A minimal genome and absent bacterial cell wall render *Mycoplasma hominis* inherently resistant to most antimicrobials except lincosamides, tetracyclines, and fluoroquinolones. 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 from 2005 to 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, cerebrospinal [CSF], peritoneal, and pleural fluid) over the 10-year period (6.4% of all samples submitted from 2010 to 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 to 19 associated accessory genes. WGS analysis showed that *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 multiresistant 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 the limited number of therapeutics needs to be expanded in the United Kingdom.

KEYWORDS *Mycoplasma hominis*, epidemiology, United Kingdom, antimicrobial resistance, genomics, *Mycoplasma*, antibiotic resistance, antimicrobial activity, genome analysis, integral conjugative element, tetracyclines

The bacterial species *Mycoplasma hominis* has a minimal genome and cell wall composition closely resembling that of eukaryotic cells (deficient in both peptidoglycan and lipopolysaccharide) (1). *Mycoplasma hominis* is an opportunistic pathogen, normally found as a commensal on the mucosal membranes of human urogenital tracts, most 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

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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 patients due to immunosuppression and inherent resistance to traditional postsurgical prophylactic antimicrobials (6, 7), as well as endocarditis associated with systemic infection (8) and kidney transplant patients (9). It has been isolated and detected in neonates with respiratory or systemic infection but remains a neglected pathogen.

Treatment options are limited; the lack of a typical bacterial cell wall renders drugs such as β -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 is resistant to 14- and 15-membered ring (but not 16-membered ring) macrolides and ketolides due to sequence polymorphisms in their 23S rRNA gene (11). The remaining effective therapeutics include lincosamides, 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 rates of resistance 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 Tn916 most commonly associated with its dissemination, previously reported in 2015 by Calcutt and Foecking (14). However, the absence of Tn916 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) (2005 to 2015). Agar-based antibiotic susceptibility testing (AST) was performed for tetracycline, clindamycin, and fluoroquinolones, and MICs for josamycin and tigecycline were determined by broth microdilution for a subset of tetracycline-susceptible and -resistant strains. All antibiotic-resistant strains and an equivalent number of susceptible control strains were analyzed by whole-genome sequencing to determine the underlying mechanisms of resistance observed, and bioinformatic interrogation was undertaken to fully characterize the variability in the *tet(M)*-containing integrative conjugative element (ICE).

RESULTS

Reference culture and PCR review. Following inclusion of molecular methods in July 2010, records show that 6.4% of samples submitted for investigation of *M. hominis* infection were found to be positive (Table 1). The types of clinical specimens submitted for these archived positive strains are shown in Table S1 in the supplemental material.

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/liter levofloxacin, 0.5 mg/liter moxifloxacin, 0.5 mg/liter clindamycin, or 8 mg/liter tetracycline, representing the CLSI resistance break-points. No strains were resistant to clindamycin. One strain (MH10-9) from 2010 showed resistance to two separate fluoroquinolones (MIC, 8 mg/liter for moxifloxacin and 16 mg/liter for levofloxacin), and an additional strain (MH15-3) from 2015 showed multidrug resistance to both fluoroquinolones tested (MIC, 16 mg/liter for moxifloxacin and 32 mg/liter for levofloxacin) as well as tetracycline (MIC, 16 mg/liter). 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

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

Yr	No. of samples found to be:				Total no. of samples	Total <i>M. hominis</i> detected (% total)	Resistance detected
	Culture positive (including referred isolates)	PCR positive, culture negative	PCR negative	PCR positive			
2005	9	NA	NA	NA	NA	9 ^b	1 × tet ^R
2006	11	NA	NA	NA	NA	11 ^b	3 × tet ^R
2007	3	NA	NA	NA	NA	3 ^b	
2008	8	NA	NA	NA	NA	8 ^b	1 × tet ^R
2009	15	NA	NA	NA	NA	15 ^b	
2010	16	NA	94	0	110	16 (14.5) ^b	2 × tet ^R 1 × moxi ^{Rc}
2011	8	1	174	3	177	9 (5.0)	
2012	9	1	200	10	210	10 (4.7)	1 × tet ^R
2013	15	0	233	15	248	15 (6.0)	3 × tet ^R
2014	7	3	185	10	195	10 (5.1)	
2015	12	2	196	14	210	14 (6.7)	1 × tet ^R /moxi ^{Rc}
Total	113	7	1,082	52	1,150	120	11 × tet ^R 1 × moxi ^R 1 × tet ^R /moxi ^R

^aNA, not applicable.^bPrior to July 2010, culture positive samples were recorded, and post-July 2010, samples submitted for *M. hominis* investigation tested by molecular diagnostics were included.^cThose isolates resistant to moxifloxacin (moxi^R) were also resistant to levofloxacin.

that grew in the presence of 8 mg/liter 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 (Fig. 1; open reading frame [ORF] MHOMSp_RS02665 in reference strain Sprott, GenBank accession number [NZ_CP011538](#)). Five groups (groups I to V) characterized by genetic composition between all ICE regions were observed.

Group I contained the seven largest ICE regions (PL5, Sprott, MH13-7, MH10-4, MH06-11, MH12-9, and MH10-15); however, only PL5 contained a full set of

FIG 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 the PL5 reference gene consist of *rumA*; Tn916 conjugation genes (green) ORF17, ORF16, ORF15, ORF14, and ORF13; the *tet(M)* resistance gene (pink); Tn916 regulation genes (blue, or gray at 80% homology) ORF9 and ORF7; Tn916 excisase and integrase genes (red), and accessory transporter genes from ICESpy2905 (GenBank accession number [FR691055](#) [yellow]), which also includes the serine recombinase (red) at the end of the mobile genetic element.

uninterrupted genes with the full-length ICE, while all the others had at least one ORF disruption by the presence of a premature stop codon. Group II ICE had lost four ORFs preceding the serine recombinase, with further mutation-derived truncation of one 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 seven 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 Tn916 integrase in addition to a loss of the serine recombinase. Group IV had lost all non-Tn916 ORFs from group I, with the exception of the serine recombinase, but the Tn916 excisase and integrase were lost, coincident 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 ORF16, 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 single-nucleotide polymorphism (SNP) were observed in the ICE analysis. Veracity and repeatability of sequencing were investigated to ensure that 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 two 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 of gestation from the same patient were additionally analyzed to assess sequence veracity but also to 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 truncating a hypothetical open reading frame position 181 of a 284-amino acid (aa) hypothetical protein (36-week specimen); variation in an intergenic poly-T region from 19 T and 21 T (28 weeks) and 20 T (36 weeks); and T114,552C, resulting in synonymous codon polymorphism for hypothetical protein MHOMSp_RS02740 (Fig. 2). Therefore, the rate of genetic drift for *M. hominis* over 16 weeks was found to be very low and was 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 Tn916 conjugation ORFs 18 to 24 (which include the FtsK translocase, ArdA superfamily protein, and Cro/C1 family initiation replicator ORF), potentially resulting in the 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, GenBank accession number [NZ_ALTN01000021](#)), which did retain ORFs 18 to 24. We undertook genomic analysis of the major surface protein (variable adherence antigen [VAA]) type for all *tet(M)* carrying ICE and other U.K. and Australian *M. hominis* strains to determine if *tet(M)* was restricted to a single VAA type (Table S2; shown as colored circles in Fig. 3). A more intensive examination of ICE (+) strain clustering compared to ICE(−) strains was performed by neighbor-joining tree construction of concatenated multilocus sequence typing loci (Fig. 3) using gene targets that we previously defined (17).

ICE-carrying strains spanned three separate VAA types, suggesting the 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 Tn916 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

FIG 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).

with VAA-2, there was enough distribution to suggest mobility or loss from the lineage; they distributed across the entire tree collocating 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 that dual resistance may have arisen early in the strain's evolution.

Mardassi et al. (18) noted the existence of *tet(M)* “sequence types” based on conserved SNPs within the resistance gene at nucleotide positions 593, G789A, T807C, C819A, G825A, and G831A. We found 6/13 of our *tet(M)* genes to match the proposed sequence type [type A *tet(M)*] by these authors (Fig. S1), and while phylogenetic analysis of the individual *tet(M)* genes confirmed that they clustered separately from type B *tet(M)* (Fig. S2), they did not collocate to a single VAA type; however, all but one (MH13-5) were found in the top half of the multilocus sequence type (MLST) phylogenetic tree (Fig. 3). Within the *tet(M)* genes lacking these conserved SNPs [type B *tet(M)*], a subtype with the conserved single SNP at nucleotide position C839T [type B *tet(M)*] all clustered together on the gene phylogenetic tree. Of interest, all type A *tet(M)*-containing strains collocated to the top half of the MLST phylogenetic tree (Fig. 3), while all type B1 *tet(M)*-containing strains collocated to the bottom half of the MLST phylogenetic tree. However, type B *tet(M)* was 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)*-positive 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 10 kb upstream and downstream of the insertion site (presuming better coconservation and coevolution of proximal genetic markers between strains if no mobility was occurring) (Fig. S3 and S4). Furthermore, no geographic relationships were identified comparing strain characteristics to origin of specimen (Table S3). Finally, phylogeny was examined by identifying the core genome (304 conserved genes from a total of 1,931 identified across all strains) using Roary (Fig. 4), and no clustering for the 13 *tet(M)*-positive strains (except for related AH3 strains) was noted relative to 8 *tet(M)*-negative strains that represented the highest-quality sequencing reads.

FIG 3 Neighbor-joining phylogenetic analysis of MLST genes for strains with *tet(M)* (light blue boxes) relative to strains without (white boxes). 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 is shown in the square at the end of the isolate identifier. Conserved SNP variation previously identified by Mardassi et al. (18) is indicated as the last entry per line for types A and B and subvariant B1.

Alternative therapies for tetracycline- and multiresistant strains. Agar-based resistance screening identified 12 tetracycline-resistant strains and two fluoroquinolone-resistant strains (1 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 (Fig. 5). 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 broad-spectrum (glycyl) tetracycline to determine if *tet(M)* mediated an elevated MIC for this antimicrobial (Fig. 6). No difference in MIC was observed for strains with (MIC, 0.56 ± 0.41 mg/liter) or without (MIC, 0.65 ± 0.52 mg/liter) *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; Fig. 6). Despite the ability of all strains to grow in broth culture containing 16 mg/

FIG 4 Neighbor-joining phylogenetic analysis for the core genome (304 genes) identified by Roary analysis of the assembled contigs following annotation using the Sprott genome (which defined a total of 1,931 genes) across all genomes. Even distribution of positive and negative strains supports ICE mobility. Furthermore, no clustering of the associated number of accessory genes [number in box, or *tet(M)*-SNP type (A, B or B1)] was seen, further confirming no clonal origins of ICE subtypes. The closest association continues to be observed between serially isolated strains AH3-20, AH3-28, and AH3-36 (the latter 2 of which were identical). Low contig length and anomalous large genome deletions required the removal of *tet(M)*-positive strains MH08-5 and MH10-15, as well as *tet(M)*-negative strains MH15-9 and MH05-13, from the analysis (otherwise, the core genome dropped significantly to 128 genes with poor discriminatory power).

liter azithromycin (data not shown), the MIC for josamycin was 0.25 ± 0.14 mg/liter irrespective of the presence of *tet(M)* (as anticipated). Therefore, therapeutic options beyond clindamycin are available (i.e., josamycin and tigecycline) for multiresistant *M. hominis* strains such as MH15-3.

DISCUSSION

Rates of resistance. Lincosamide, tetracycline, and fluoroquinolone susceptibility testing of recovered strains from 2005 to 2015 (67% of the total received) showed that 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 (19), and a number of published studies (presented in Table S4) were completed prior to this. Reports of tetracycline resistance in the international literature range from 0 to 58%, fluoroquinolone resistance from 0 to 94%, and clindamycin resistance from 0 to 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 *M. hominis* strains elsewhere (20).

In this study, we identified mutations in the QRDR (quinolone resistance-

FIG 5 Antimicrobial susceptibility testing for 40 isolates [13 *tet(M)*-carrying and 27 susceptible controls] for antibiotics with CLSI-defined resistance thresholds.

determining region) (21) 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 (22, 23). 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* (24). Our results also suggest that mutation of the serine residue in both genes (MH15-3; MIC, 16 mg/liter) induces greater resistance than mutation of the asparagine residues in both genes (10-9; MIC, 8 mg/liter). The availability of characterized *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 deposited MH15-3 in the National Culture Type Collection (NCTC 14456) and GenBank (BioSample accession [JACXZU010000000](https://www.ncbi.nlm.nih.gov/biosample/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 interstrain diversity was unusually high—to the point where each strain had a unique sequence type (ST)

FIG 6 Antimicrobial susceptibility testing for 40 isolates [13 *tet(M)*-carrying and 27 randomly selected susceptible controls] for glycy/tetracycline tigecycline and macrolide josamycin. Note that, to date, no breakpoints have been assigned for these antimicrobials.

unless it was isolated from the same patient specimen (17). It was based on this observation that the source of failed lung transplants caused by *M. hominis* infection in a clinical cohort was 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 the surface antigens vaa, p120', p60, Imp1, and Imp3 to segregate strains isolated from individuals with infertility from strains from patients with gynecological infections (25) but still show wide varieties of individual ST assignments. To account for this wide range of interstrain sequence diversity, one would expect that the 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 115-kb 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 112 kb, 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 of gestation covered 537,152 nucleotides (nt) and showed 18 genomic variations. Ten of these were variances in the number of nucleotides in noncoding intergenic poly T or G regions, four resulted in nonsynonymous mutations, and 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 [26], immune pressure, or adaptation during initial infection following transmission [27, 28]) may be responsible for the high diversity.

Variation of the 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 MH13-7) 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 ICE (MICE) in *M. hominis* (29), although the essential consensus sequence SSLSDFDKTPKLDKSKVINEYN 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, minitransposons have been used, albeit artificially, to deliver tetracycline resistance genes (30). This is further backed by the frequent occurrence of HGT that occurs in mollicutes and prokaryotes in general (31, 32) and the phenomenon's ability to confer antimicrobial resistance (27, 33), 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 (34).

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 (35). Suspected to originate from genomic insertions by bacteriophages (36), they are a

common facilitator of genetic adaptation and evolution in a wide range of pathogens (37). In particular, the integrase in the *tet(M)* ICE region detailed here is part of Tn916, a categorized transposon cassette, but notably lacks ORFs 18 to 24 (but notably retains critical VirB4 and transmembrane segregation-mediating ORFs), which have been found to mediate the conjugation of the transposon (38). Further, the authors noted that the presence of tetracyclines stimulated the expression of these ORFs, which encoded the self-excision of Tn916. Genetic drift in subinhibitory 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 previously 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 (39). 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 hot spots of bacterial genomes (40, 41).

In conclusion, the presence of a recent *M. hominis* strain resistant to all antimicrobials with CLSI-validated resistance thresholds, except clindamycin, highlights the need to expand the available guidelines, especially as a significant number of our isolates originated from invasive *M. hominis* infection (Table S1; including five blood cultures, seven CSF samples, and two cerebral samples). We confirm the capacity of tigecycline (previously reported as GAR-936 [42]) to overcome *tet(M)*-mediated resistance and recommend formal determination of threshold resistance guidance for josamycin in *M. hominis*, particularly as Europe and Russia frequently use josamycin as a therapeutic, but unfortunately is it currently omitted from the British National Formulary (43).

MATERIALS AND METHODS

Clinical samples. The *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 21 February 2005 to 9 October 2015). Isolated strains were archived at -80°C as agar cubes in liquid medium and/or liquid cultures (with or without beads) until investigated for this study. The 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 Table S1). Four vaginal reference strains from Australia were also included for comparative analysis (3 from patient AH3 collected at 20, 28, and 36 weeks) of gestation, as well as a single strain from a separate patient (AH58) (16). Prior to July 2010, PHE used 16S RNA gene-based PCR (44) and culture for *M. hominis* detection; in July 2010, however, PHE transitioned to using an adapted PCR method to amplify the gap gene (45) (from January 2014 to January 2015), which was then replaced by a superior quantitative PCR (qPCR) method targeting the *yidC* gene (January 2015 to present) (46).

Ethics considerations. Antimicrobial resistance evaluation of bacterial isolates does not require NHS Research Ethics Committee review. Original ethical approval covering the reference strains included from Australia was granted 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 was recorded. "Fried-egg" colonies characteristic of *M. hominis* were counted using a stereo microscope, and growth in broth culture was visualized as a yellow to red color change in the absence of turbidity. Antimicrobial screening was performed as outlined by CLSI guidelines (19) (the methodology and data underlying these guidelines is fully published in reference 47) using the following defined resistance thresholds: 2 mg/liter levofloxacin, 0.5 mg/liter moxifloxacin, 0.5 mg/liter clindamycin, and 8 mg/liter 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. A total of 134 *M. hominis* strains from 81 patients were analyzed. Initial screening for the presence of *tet(M)* was performed by traditional PCR (AppTaq RedMix [Appleton Woods, UK], 40 cycles, melting temperature [T_m] of 56°C ; extension, 30 sec) visualizing the expected 419-bp product by transilluminated ethidium bromide containing 1% (wt/vol) agarose gel electrophoresis using PCR primers (designed in this study) *tetM*1338F 5'-TATCTGTATCACCGCTTCCG-3' and *tetM*1758R 5'-AATACACCGAGCAGGGATT-3'.

Whole-genome sequencing and bioinformatics. For the subset of strains to be examined by whole-genome sequencing, individual colonies were grown in 30 ml *Mycoplasma* selective medium, pelleted at $13,000 \times g$ for 3 h, 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

the manufacturer's instructions. DNA yields were between 1 and 8 ng/ μ l (Qubit 4.0; Life Technologies). Genomic sequencing was undertaken using a Nextera XTv2 library preparation kit with V3 chemistry on an Illumina MiSeq platform. A quality control (QC) pipeline to validate and 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 (2). Assembled contigs were further analyzed utilizing Geneious sequence analysis software (BioMatters Ltd., New Zealand) and aligned and assessed against reference sequences (Sprott, GenBank accession number [NZ_CP011538](#); PL5, GenBank accession number [NZ_JRXA01000009](#)) for the identification of genetic elements and the presence of point mutations and gene rearrangements using Snippy v4.4.5 and Roary v3.12.0 (48, 49). Multilocus sequence typing was performed *in silico* using the targets we previously reported (17). The whole-genome shotgun project for MH15-3 has been deposited at DDBJ/ENA/GenBank under the accession number [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 have been submitted under BioProject number [PRJNA675754](#). Further phylogenetic analysis was performed by comparing a standardized 10-kb DNA segments 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 the neighbor-joining tree building method to identify the relatedness of the two groups.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.8 MB.

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We have no conflicts of interest to declare.

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