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Evidence for Multidrug Resistance in Nonpathogenic *Mycoplasma* Species Isolated from South African Poultry

Amanda Beylefeld,[a] Pamela Wambulawaye,[b] Dauda Garba Bwala,[a] Johannes Jacobus Gouws,[b] Obed Mooki Lukhele,[c] Daniel Barend Rudolph Wandrag,[a] Celia Abolnik[a]

[a]Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[b]Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Onderstepoort, South Africa

[c]Astral Foods Ltd., Centurion, South Africa

ABSTRACT One hundred seventy-eight mycoplasma strains isolated from South African poultry flocks between 2003 and 2015 were identified by full-genome sequencing and phylogenetic analysis of the 16S rRNA gene and were classified as follows: *Mycoplasma gallisepticum* (25%), *M. gallinarum* (25%), *M. gallinaceum*, (23%), *M. pullorum* (14%), *M. synoviae* (10%), and *M. iners* (3%), as well as one *Acheoplasma laidlawii* strain (1%). MIC testing was performed on the axenic samples, and numerous strains of each species were resistant to either chlortetracycline or tylosin or both, with variable sensitivity to enrofloxacin. The strains of all species tested remained sensitive to tiamulin, except for one *M. gallinaceum* sample that demonstrated intermediate sensitivity. The mutation of A to G at position 2059 (A2059G) in the 23S rRNA gene, which is associated with macrolide resistance, was found in the South African *M. gallisepticum* and *M. synoviae* strains, as well as a clear correlation between macrolide resistance in *M. gallinarum* and *M. gallinaceum* and mutations G354A and G748A in the L4 ribosomal protein and 23S rRNA gene, respectively. No correlation between resistance and point mutations in the genes studied could be found for *M. pullorum*. Only a few strains were resistant to enrofloxacin, apart from one *M. synoviae* strain with point mutation D420N, which has been associated with quinolone resistance, and no other known markers for quinolone resistance were found in this study. Proportionally more antimicrobial-resistant strains were detected in *M. gallinaceum*, *M. gallinarum*, and *M. pullorum* than in *M. gallisepticum* and *M. synoviae*. Of concern, three *M. gallinaceum* strains showed multidrug resistance to chlortetracycline, tylosin, and oxytetracycline.

IMPORTANCE Nonpathogenic poultry *Mycoplasma* species are often overlooked due to their lesser impact on poultry health and production compared to the OIE-listed pathogenic strains *M. gallisepticum* and *M. synoviae*. The use of antimicrobials as in-feed growth promoters and for the control of mycoplasmosis is common in poultry production across the world. Here, we provide evidence that certain nonpathogenic *Mycoplasma* species are acquiring multidrug resistance traits. This would have significant implications if these species, for which no vaccines are applied, are able to transfer their antibiotic resistance genes to other mycoplasmas and bacteria that may enter the human food chain.

KEYWORDS *Mycoplasma*, MIC, poultry, 16S rRNA, antibiotic resistance, 23S rRNA, DNA gyrase, DNA topoisomerase

Mycoplasmas are the smallest free-living organisms and unique among prokaryotes in that they lack a cell wall. More than 23 different mycoplasma species are known to infect avian species, four of which are considered pathogenic and of

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Address correspondence to Amanda Beylefeld, amanda.beylefeld@gmail.com.

economic importance to poultry production, namely, *Mycoplasma gallisepticum*, *M. synoviae*, *M. meleagridis*, and *M. iowae* (1). *M. gallisepticum* and *M. synoviae* are of particular importance and are listed by the World Organization for Animal Health (OIE) as notifiable avian diseases. *M. gallisepticum* causes chronic respiratory disease in chickens, with signs that include coughing, sneezing, rales, and nasal discharge. More severe symptoms can occur when respiratory pathogens, such as Newcastle disease virus (NDV), infectious bronchitis virus (IBV), or *Escherichia coli*, are also present (2). Signs of *M. synoviae* infections include upper respiratory tract infection, lameness, a pale comb, retarded growth, and synovitis (3). As in most countries, *M. gallisepticum* and *M. synoviae* are considered to be the most important mycoplasma species affecting the South African poultry industry, causing increased mortality rates in broilers and higher rates of chick and embryo mortality on layer farms. Further economic losses are incurred through carcass downgrading and reduced egg production, weight gain, growth rate, and feed conversion, as well as an increase in management costs (4).

Optimal control of mycoplasma infection starts with a mycoplasma-free flock, followed by good biosecurity measures and a good monitoring program (5). Flocks are screened for exposure to mycoplasmas using serological tests like serum plate agglutination or enzyme-linked immunosorbent assays (ELISAs) (5). Culturing is still considered the gold standard for diagnosing mycoplasmas, which includes either a direct or indirect immunofluorescent test or a growth inhibition test for species identification. Since *M. gallisepticum* is slow growing (>72 to 96 h), it can easily be overgrown by faster-growing mycoplasmas, such as the prevalent nonpathogenic mycoplasma species *M. gallinarum* and *M. gallinaceum* (5). In recent years, DNA-based identification methods using *M. gallisepticum*- and *M. synoviae*-specific PCR or real-time PCR tests have replaced culture-based identification methods for their sensitivity and speed, since they can be used directly on clinical samples without the need for prior culture (6, 7).

As mycoplasmas are difficult to eradicate, medication and vaccines have been used with variable success. Mycoplasmas are resistant to penicillin and cephalosporins but, in general, remain sensitive to other classes of antimicrobials, including macrolides, tetracyclines, fluoroquinolones, and pleuromutilins (5). Macrolides and pleuromutilins inhibit protein synthesis by binding to 50S ribosomal proteins, tetracyclines inhibit protein synthesis by binding to 30S ribosomal proteins, and fluoroquinolones inhibit DNA synthesis by binding DNA gyrase and topoisomerase IV (8). The use of antimicrobials has been shown to reduce egg transmission of mycoplasmas, as well as the appearance of clinical signs and lesions (5). Antimicrobial resistance (AMR) is a global health threat, and indiscriminate antimicrobial usage in animal production, including poultry, is a contributing factor. In most countries, excessive amounts of a range of antimicrobials are administered to poultry in feed, not only to prevent and treat disease but also to enhance growth and productivity (9). AMR to macrolides and fluoroquinolones in both *M. gallisepticum* and *M. synoviae* has been linked to point mutations in the 23S rRNA genes and amino acid substitutions in the quinolone resistance-determining regions (QRDRs) of the DNA gyrase and topoisomerase IV genes, respectively (10–12). The mechanism of acquired tetracycline resistance has not yet been identified in poultry mycoplasmas.

Mycoplasmosis, presumably caused by *M. gallisepticum* and *M. synoviae*, remains a pervasive problem in many South African poultry flocks, and in-feed administration of medication has been a standard practice. In the work reported here, mycoplasmas isolated from poultry flocks between 2003 and 2015 were cultured and identified to species level by growth inhibition tests, full-genome sequencing, and DNA sequence analysis of the 16S rRNA gene. We then tested axenic samples for their MICs against four antimicrobial agents commonly used in-feed to treat mycoplasma-infected flocks in South Africa and analyzed the 23S rRNA, DNA gyrase, and topoisomerase IV genes for genetic markers for acquired AMR.

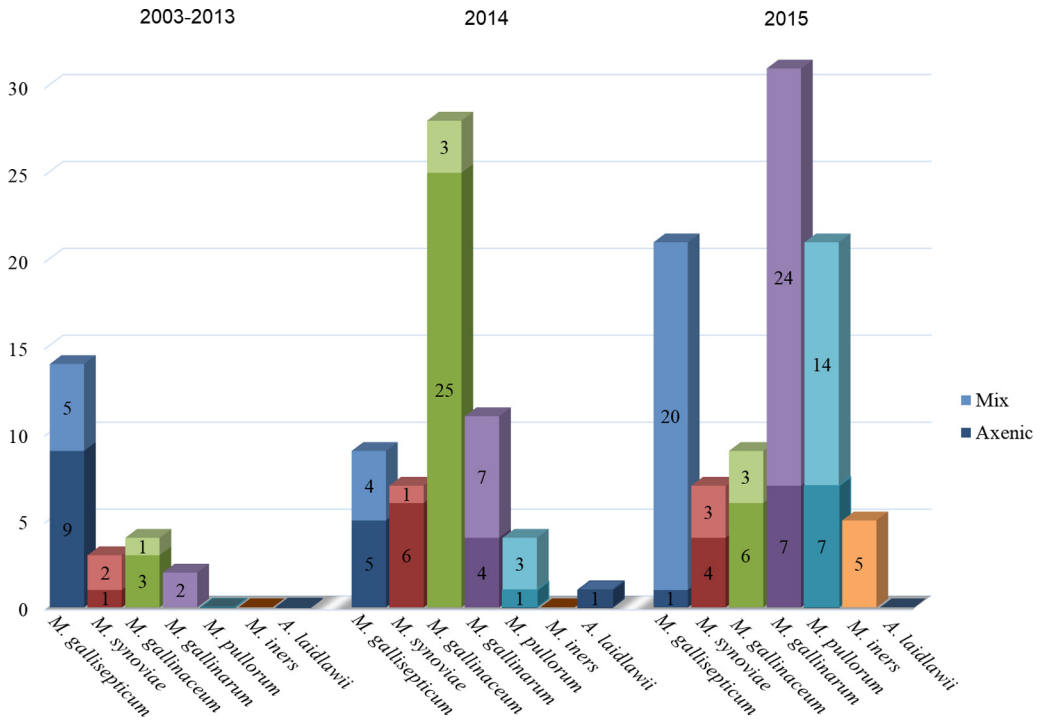

FIG 1 Total numbers of mycoplasma strains identified per year, based on 16S rRNA gene sequencing. The bottom number (dark color) in each bar is the number of strains identified in the axenic cultures, and the top number (light color) is the number of strains identified as part of mixed cultures.

RESULTS

Identification of *Mycoplasma* species. One hundred twenty-four mycoplasma strains were isolated from chickens displaying clinical signs typically associated with *M. gallisepticum* and *M. synoviae* infection. Growth inhibition tests identified 50/124 (40.32%) as *M. gallisepticum*, 15/124 (12.10%) as *M. synoviae*, and 59/124 (47.58%) as *Mycoplasma* spp. (see Table S1 in the supplemental material). Genomic DNA was extracted from the mycoplasma-positive samples and sequenced using next-generation sequencing methods, followed by phylogenetic identification based on the 16S rRNA gene sequence. The use of the 16S rRNA gene is considered the standard for bacterial identification and a useful tool for characterizing mycoplasma species (13).

IonGAP analysis indicated the presence of more than one mycoplasma species in 44/124 (35.48%) of the mycoplasma-positive samples (Table S1). In total, 178 mycoplasma strains were detected in the 124 samples. A 16S rRNA phylogenetic tree was constructed, using three species of the genus *Acholeplasma* as an outgroup (Fig. S1). 16S rRNA classification was as follows: 45/178 (25.28%) *M. gallisepticum*, 44/178 (24.72%) *M. gallinarum*, 41/178 (23.03%) *M. gallinaceum*, 25/178 (14.04%) *M. pullorum*, 17/178 (9.55%) *M. synoviae*, and 5/178 (2.81%) *M. iners*. One sample was identified as *Acholeplasma laidlawii*, and none of the *M. gallisepticum*-positive samples tested contained the putative transposase gene in the 16S-23S intergenic spacer region (IGSR) of *M. imitans*, which has been described as a method to distinguish these two species (data not shown) (14). One sample (B293-15-18) was not tested, as the read coverage was too low and the 16S-23S rRNA IGSR could not be assembled.

M. gallinaceum and *M. gallinarum* were isolated more frequently than other species in 2014 and 2015, respectively (Fig. 1), but *M. gallinarum* tended to occur more frequently in coinfections in 33/44 (75%) cases, whereas *M. gallinaceum* coinfections were only detected in 7/41 (17.07%) cases (Table S1). Both *M. gallinaceum* and *M. gallinarum* are fast-growing species, with growth on agar visible at 48 h, whereas growth of *M. gallisepticum* and *M. synoviae* is usually only visible after 72 to 96 h (1, 2).

It is standard laboratory procedure to incubate plates for longer to allow any small colonies that are possibly *M. gallisepticum* or *M. synoviae* to grow.

MIC testing and antimicrobial resistance genes. MIC analysis was performed on 64 of 80 axenic samples; the results are listed in Tables 1 and 2. The MICs for the remaining 16 axenic cultures could not be determined, because these strains were difficult to culture after prolonged freezing at minus 80°C. There are currently no international standards for *in vitro* susceptibility testing criteria for veterinary antimicrobial agents for mycoplasmas, and thus, the breakpoints provided by Hannan et al. as guidelines were used for oxytetracycline, tylosin, enrofloxacin, and tiamulin (Table 3) (15). No breakpoints are available for chlortetracycline; however, the microbiological activities of tetracycline, chlortetracycline, and oxytetracycline against mycoplasmas were determined to be comparable in *in vitro* MIC studies using human enteric isolates (16). The value for oxytetracycline is therefore used for chlortetracycline (Table 3). The MIC_{50} and MIC_{90} values were calculated but are only valid for sample sizes of 10 or more (Table 1) (15).

The *M. gallisepticum* strains showed variance in MICs for both chlortetracycline, with a range of 1 to 64 μg/ml, and tylosin, with a range of $<$0.01 to 16 μg/ml (Table 1). *M. gallisepticum* strains only showed resistance to chlortetracycline (2/10) and tylosin (6/10) and showed intermediate susceptibility to chlortetracycline (3/10) and enrofloxacin (2/10) (Fig. 2a and b; Table 2). Comparison of the 23S rRNA genes revealed the presence of a point mutation conferring a change of A to G at position 2059 (A2059G) (*E. coli* numbering is used throughout) on one or both of the 23S rRNA genes for 5/6 tylosin-resistant *M. gallisepticum* strains (Table 2). A comparison of the *rplD* and *rplV*, *gyrA* and *gyrB*, and *parC* and *parE* genes, which encode the L4 and L22 ribosomal proteins, DNA gyrase subunit A and B, and DNA topoisomerase IV subunit A and B proteins, respectively, showed no amino acid substitutions. The two chlortetracycline-resistant *M. gallisepticum* strains, B758-08 and B943-06, also showed resistance to tylosin and intermediate sensitivity to enrofloxacin (Table 2). Two of the three *M. gallisepticum* strains that showed intermediate susceptibility to chlortetracycline also showed resistance to tylosin. Four *M. gallisepticum* strains were resistant to one antimicrobial, and two were resistant to two antimicrobials.

The *M. synoviae* MICs for chlortetracycline ranged from 2 to 32 μg/ml, and for tylosin, the range was 0.02 to 10 μg/ml. *M. synoviae* strains displayed resistance to chlortetracycline (6/11), tylosin (3/11), or enrofloxacin (1/11), and 3/11 and 4/11 had acquired intermediate susceptibility to chlortetracycline and enrofloxacin, respectively (Fig. 2a and b; Table 1). All three tylosin-resistant *M. synoviae* strains had acquired an A2059G mutation in both 23S rRNA genes. Amino acid substitutions were observed at N89D and D461E of the topoisomerase IV subunit A and B proteins, respectively, but there was no observable correlation to enrofloxacin resistance (Table 2). Point mutation D420N was observed in the only enrofloxacin-resistant *M. synoviae* strain (Table 2). Only B1394-14-5 was resistant to both chlortetracycline and tylosin. Two other tylosin-resistant *M. synoviae* strains also showed intermediate sensitivity to chlortetracycline (Table 1). Eight *M. synoviae* strains were resistant to one antimicrobial, and one was resistant to two antimicrobials.

For *M. gallinarum*, resistance was observed to chlortetracycline (6/9) and tylosin (6/9), as well as intermediate susceptibility to chlortetracycline (3/9) and enrofloxacin (5/9) (Fig. 2a and b; Table 1). Genes of interest for strain B293-15-11 could not be retrieved from the data due to a combination of contaminating DNA and the lack of a reference genome and were thus not analyzed. All but one of the *M. gallinarum* strains tested had mutation G2059A, but only 5/8 of these strains were tylosin resistant. Three tylosin-resistant strains also had a G745A mutation in the 23S rRNA gene (Table 2), and H91K substitutions were observed in ribosomal protein L22 of three tylosin-resistant strains, as well as G354A in ribosomal protein L4 in five tylosin-resistant strains (Table 2). Three of the chlortetracycline-resistant strains, B293-15-6, B2772-15-1, and B2053-15-2, were also resistant to tylosin, with sample B2053-15-2 being intermediately

TABLE 1 MIC distribution of *Mycoplasma* strains

Drug	*Mycoplasma* species	No. of isolates with indicated MIC (μg/ml)																		MIC_{50}	MIC_{90}	% resistance
Chlortetracycline	0.5	1	1.25	2	2.5	4	5	8	10	16	20	>20	32	64								
	M. gallisepticum		2		1		2		1	2	1				1					4.000	16.00	20.00
	M. synoviae				1		1			3	1	3		2						16.00	0.640	54.55
	M. gallinarum								2	1	3	3										66.67
	M. pullorum		1				1				1			2	1							66.67
	M. gallinaceum	1		1	3	1		2	1	5	2	8	4							10.00	>20	50.00
Enrofloxacin		0.04	0.08	0.16	0.25	0.32	0.5	0.64	1	2	2.5	5	10	ND[a]								
	M. gallisepticum	1	1		6				2											0.250	1.000	0.00
	M. synoviae		1		3	1	1	4		1										0.500	0.640	9.09
	M. gallinarum				3	1		3	2													0.00
	M. pullorum				4				2													0.00
	M. gallinaceum		1	9	2	5		1	4	1	1	1	2	1						0.320	5.00	21.43
Tylosin		0.01	0.02	0.04	0.08	0.125	0.16	0.25	0.64	1	2	4	5	8	10	16	>16	20	>20			
	M. gallisepticum	1				3									1	5				10.00	16.00	60.00
	M. synoviae		1	2		5									3					0.125	10.00	27.27
	M. gallinarum			3													5		1			66.67
	M. pullorum					3		1				2										33.33
	M. gallinaceum									1	3	1	2	1	6	0	1	3	10	10.00	>20	85.71
Tiamulin		0.01	0.06	0.08	0.12	0.16	0.25	0.32	0.5	0.64	1	1.25	2	2.5	5	10						
	M. gallisepticum	1	3		1	1	2				1		1							0.120	1.000	0.00
	M. synoviae				2		2		1					6						2.500	2.500	0.00
	M. gallinarum						1		3		1	1		3								0.00
	M. pullorum		2				3		1													0.00
	M. gallinaceum							3	3	1	4	7		2	7	1				1.250	5.000	0.00

[a]ND, not determined.

TABLE 2 MICs and resistance mutations of *Mycoplasma* strains

Strain	*Mycoplasma* species	MIC (μg/ml) of[a]:				Point mutation in indicated gene associated with[b]:				
						Macrolide resistance			Quinolone resistance	
		Chlortetracycline[c]	Enrofloxacin	Tylosin	Tiamulin	23S rRNA	*rplD*	*rplV*	*parC*	*parE*
NCTC 10115 CONTROL	*M. gallisepticum*	1.250	0.160	0.160	0.160					
USDA 56 CONTROL	*M. gallisepticum*	2.500	0.160	0.160	0.160					
NCTC 11733 CONTROL	*M. imitans*	10	0.320	0.640	0.080					
B1102-03	*M. gallisepticum*	1	0.250	0.125	0.060					
B1102-06	*M. gallisepticum*	1	0.250	0.125	0.060					
B726-06	*M. gallisepticum*	4	0.250	16	0.250	A2059G[d]				
B943-06	*M. gallisepticum*	16	1	16	2					
B1028-07	*M. gallisepticum*	8	0.250	16	0.250	A2059G[d]				
B758-08	*M. gallisepticum*	64	1	16	1	A2059G[d]				
B2159-13	*M. gallisepticum*	4	0.250	16	0.120	A2059G[d]				
B1395-14-1	*M. gallisepticum*	10	0.080	10	0.160	A2059G				
B878-14-L3	*M. gallisepticum*	10	0.040	0.010	0.010					
B457-15-5	*M. gallisepticum*	2	0.250	0.125	0.060					
NCTC 10124 CONTROL	*M. synoviae*	5	2.500	0.080	2.500				N84D	D454E
ATCC 25204 CONTROL	*M. synoviae*	2.500	5	0.080	2.500	ND[e]	ND	ND	ND	ND
B2214-07	*M. synoviae*	2	2	0.125	0.500				N84D	D420N, D454E
B1064-14-H4	*M. synoviae*	10	0.640	0.020	2.500				N84D	D454E
B1064-14-H3	*M. synoviae*	20	0.640	0.040	2.500				N84D	D454E
B1064-14-H5	*M. synoviae*	20	0.640	0.040	2.500				N84D	D454E
B1394-14-2	*M. synoviae*	10	0.080	10	2.500	A2059G[d]			N84D	D454E
B1393-14-10	*M. synoviae*	10	0.320	10	2.500	A2059G[d]			N84D	D454E
B1394-14-5	*M. synoviae*	20	0.640	10	2.500	A2059G[d]			N84D	D454E
B458-15-1	*M. synoviae*	4	0.250	0.125	0.120				N84D	D454E
B458-15-5	*M. synoviae*	16	0.250	0.125	0.120				N84D	D454E
B458-15-6	*M. synoviae*	32	0.250	0.125	0.250				N84D	D454E
B458-15-11	*M. synoviae*	32	0.500	0.125	0.250				N84D	D454E
B1101-14-6	*M. gallinarum*	20	0.640	0.040	2.500	G2059A				
B1101-14-8	*M. gallinarum*	20	0.640	0.040	2.500					
B1101-14-9	*M. gallinarum*	20	0.640	0.040	2.500	G2059A				
B878-14-M3	*M. gallinarum*	10	0.320	>20	1.250	G745A, G2059A	G354A	H91K		
B2053-15-2	*M. gallinarum*	16	1	>16	0.500	G2059A	G354A			
B2772-15-1	*M. gallinarum*	16	0.250	>16	0.250	G2059A	G354A			
B293-15-10	*M. gallinarum*	8	0.250	>16	0.500	G745A, G2059A	G354A	H91K		
B293-15-11	*M. gallinarum*	8	1	>16	0.500	ND	ND	ND	ND	ND
B293-15-6	*M. gallinarum*	16	0.250	>16	1	G745A, G2059A	G354A	H91K		
B293-15-12	*M. pullorum*	64	0.250	4	0.250					
B293-15-15	*M. pullorum*	32	0.250	4	0.250					
B293-15-17	*M. pullorum*	16	0.250	0.250	0.060					
B359-15-5	*M. pullorum*	4	1	0.125	0.250					
B359-15-6	*M. pullorum*	1	1	0.125	0.500					
B540-15-2	*M. pullorum*	32	0.250	0.125	0.060	G748A			S81P	
B313-05	*M. gallinaceum*	16	0.250	>16	1	G748A[d]				
B733-05	*M. gallinaceum*	16	1	8	1	G748A[d]				
B1101-14-7	*M. gallinaceum*	20	10	>20	5	G748A[d]			S80L	
B1173-14-2a	*M. gallinaceum*	2.500	0.160	5	0.640	G748A[d]				
B1173-14-2b	*M. gallinaceum*	10	0.320	10	1.250	G748A[d]				
B1173-14-4a	*M. gallinaceum*	>20	0.320	>20	10	G748A[d]				
B1173-14-4b	*M. gallinaceum*	20	0.320	>20	5	G748A[d]				
B1173-14-5b	*M. gallinaceum*	20	0.320	>20	5	G748A[d]				
B1173-14-6b	*M. gallinaceum*	20	0.160	20	1.250	G748A[d]				
B1173-14-7b	*M. gallinaceum*	10	0.160	10	1.250	G748A[d]				
B1173-14-8b	*M. gallinaceum*	20	0.160	>20	5	G748A[d]				
B1342-14-10	*M. gallinaceum*	>20	10	20	2.500	G748A[d]				
B1342-14-13	*M. gallinaceum*	20	2.500	10	1.250	G748A[d]			E84G	
B1342-14-14	*M. gallinaceum*	10	5	10	5	G748A[d]				
B1342-14-8	*M. gallinaceum*	20	0.160	>20	5	G748A[d]				
B1395-14-2	*M. gallinaceum*	1.250	0.080	5	1.250	G748A[d]				
B1396-14-7	*M. gallinaceum*	10	0.160	20	1.250	G748A[d]				
B1396-14-8	*M. gallinaceum*	>20	0.160	10	1.250	G748A[d]				
B1396-14-9	*M. gallinaceum*	>20	0.160	10	5	G748A[d]				
B1414-14-1	*M. gallinaceum*	20	ND	>20	2.500	G748A[d]				
B878-14-M1	*M. gallinaceum*	5	0.160	>20	0.320	G748A[d]				
B878-14-M4	*M. gallinaceum*	5	0.640	>20	0.320	G748A[d]				
B878-14-M5	*M. gallinaceum*	10	0.320	>20	0.320	G748A[d]				

(Continued on next page)

TABLE 2 (Continued)

Strain	*Mycoplasma* species	MIC (μg/ml) of[a]:				Point mutation in indicated gene associated with[b]:				
						Macrolide resistance			Quinolone resistance	
		Chlortetracycline[c]	Enrofloxacin	Tylosin	Tiamulin	23S rRNA	*rplD*	*rplV*	*parC*	*parE*
B3381-15-1	*M. gallinaceum*	2	1	2	1					
B3381-15-2	*M. gallinaceum*	8	0.250	4	0.500					
B3381-15-3	*M. gallinaceum*	2	1	2	1					
B3381-15-4	*M. gallinaceum*	2	2	2	0.500					
B3381-15-5	*M. gallinaceum*	0.500	1	1	0.500					

[a]Breakpoints according to Hannan (Table 3) (15), with values indicative of resistant strains in boldface.
[b]*E. coli* numbering.
[c]No breakpoint available, so oxytetracycline values used (Table 3).
[d]Found on both 23S rRNA genes.
[e]ND, not determined.

susceptible to enrofloxacin. Two of the six *M. pullorum* strains, B293-15-12 and B293-15-15, were resistant to tylosin and chlortetracycline. A further two strains were resistant to chlortetracycline. One strain, B540-15-2, had a G748A point mutation in the 23S rRNA and S81P in *parC* of the QRDR, but no correlation with tylosin resistance was observed.

Proportionately more *M. gallinaceum* strains were isolated, and 28 were used for MIC analysis. MIC ranges were 0.5 to >20 μg/ml and 1 to >20 μg/ml for chlortetracycline and tylosin, respectively. The MIC_{50} for chlortetracycline of 10 μg/ml and MIC_{90} of >20 μg/ml indicate that the majority of *M. gallinaceum* strains were resistant to chlortetracycline; in fact, only six remained fully susceptible (Fig. 2a and b; Table 1). The MIC_{50} and MIC_{90} for tylosin were 10 and >20 μg/ml, respectively, with almost all the strains being resistant to tylosin (24/28). Only one strain was fully sensitive, and three samples had intermediate sensitivity to tylosin. The MICs for enrofloxacin ranged from 0.08 to 10 μg/ml, with a MIC_{50} of 0.32 and a MIC_{90} of 5 μg/ml. Six of the strains were resistant to enrofloxacin, and five more samples showed intermediate resistance. G748A was observed in both 23S rRNA genes of 23 tylosin-resistant strains (Table 2). Amino acid substitutions S81L and E84G in the topoisomerase subunit A protein were found in only two enrofloxacin-resistant strains, B1101-14-7 and B1342-14-13, respectively. Resistance to both chlortetracycline and tylosin was observed in 14 strains, and resistance to both enrofloxacin and either chlortetracycline or tylosin in two strains. Interestingly, *M. gallinaceum* strain B1173-14-4a showed intermediate sensitivity to tiamulin. Three strains, B1101-14-7, B1342-14-10, and B1342-14-13, showed resistance to chlortetracycline, enrofloxacin, and tylosin (Table 2).

Proportionally between species, only 20% of the *M. gallisepticum* strains were resistant to chlortetracycline, compared to 55%, 67%, 67%, and 50% observed for *M. synoviae*, *M. gallinarum*, *M. pullorum*, and *M. gallinaceum*, respectively (Fig. 2b; Table 1). For tylosin, only 27% of *M. synoviae* samples were resistant, compared to 60%, 67%, 33%, and 86% for *M. gallisepticum*, *M. gallinarum*, *M. pullorum*, and *M. gallinaceum*, respectively (Fig. 2b; Table 1). Enrofloxacin resistance was only detected in *M. gallinaceum* (21%) and *M. synoviae* (9%).

TABLE 3 MIC breakpoints

Class	Antibiotic	Breakpoint (μg/ml) for[a]:		
		Susceptible	Intermediate	Resistant
Tetracyclines	Chlortetracycline[b]	≤4	8	≥16
Fluoroquinolones	Enrofloxacin	≤0.5	1	≥2
Macrolides	Tylosin	≤1	2	≥4
Pleuromutilin	Tiamulin[a]	≤8		≥16

[a]Breakpoints according to Hannan (15).
[b]No chlortetracycline breakpoint available, so oxytetracycline values used.

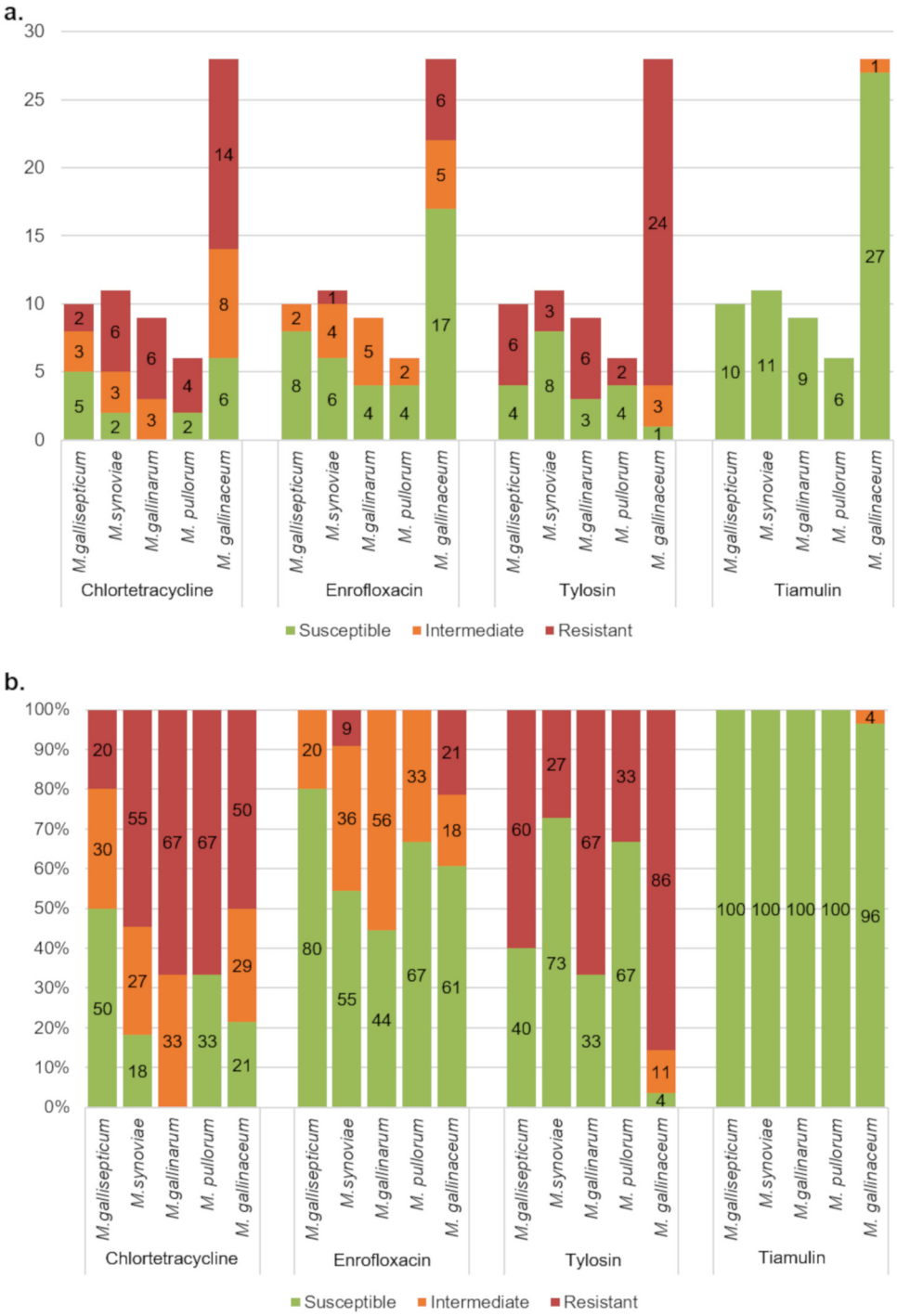

FIG 2 Antibiotic sensitivities of mycoplasma strains to chlortetracycline, enrofloxacin, tylosin, and tiamulin. (a) Total numbers of strains that were resistant, susceptible, or in the intermediate range. (b) Relative proportions of strains.

DISCUSSION

For several decades, culture followed by growth inhibition with hyperimmune sera was the only identification method for poultry mycoplasmas in South Africa, and ELISA and molecular detection methods were only recently implemented by diagnostic laboratories. The 16S rRNA gene sequences extracted from the complete genome confirmed various samples identified by growth inhibition tests to be *M. gallisepticum* and *M. synoviae* (Table S1 in the supplemental material), but we additionally identified

M. gallinaceum, *M. gallinarum*, *M. pullorum*, and *M. iners* in poultry samples collected between 2003 and 2015. Other poultry mycoplasma species reported globally were not detected, namely, *M. glycophilum*, *M. iowae*, *M. lipofaciens*, or *M. meleagridis*, the latter predictably because turkeys are not farmed in South Africa. Neither was *M. imitans* identified, which is difficult to distinguish from *M. gallisepticum* by serological methods.

M. gallinaceum, *M. gallinarum*, *M. pullorum*, and *M. iners* are considered nonpathogenic, but the effects of "nonpathogenic" mycoplasmas on production could be significant under certain circumstances, since they are known to exacerbate respiratory diseases in coinfections (17). *M. gallinaceum*, the most common axenic culture in 2014, has previously been associated with conjunctivitis in pheasants (18), and the role of *M. gallinaceum* in enhancing infectious bronchitis virus replication *in vivo* was demonstrated recently by Adeyemi and coworkers (19). *M. gallinarum* was the most prevalent species isolated in 2015, albeit mostly in coinfections with *M. gallisepticum*, and is known to cause airsacculitis in chickens (20). *M. pullorum* can cause an increase in embryo mortality, and *M. iners* can cause lesions in embryos (21, 22). The presence of these nonpathogenic species in flocks should therefore not be devalued.

AMR in poultry microbes is a growing global concern, yet few previous studies have determined MICs for poultry mycoplasma strains, and then only *M. gallisepticum* and *M. synoviae* were investigated (9). Here, we determined that resistance against chlortetracycline, enrofloxacin, or tylosin existed in some of the strains of all species cultured. The finding of relatively higher levels of chlortetracycline and tylosin resistance compared to the levels of enrofloxacin resistance for *M. gallisepticum* and *M. synoviae* was not unexpected, as long-term use of oxytetracycline, as practiced over decades in South Africa, is known to cause resistance (23, 24). Furthermore, *in vitro* studies have shown that tylosin resistance develops quickly, whereas enrofloxacin resistance develops slowly over time (25).

All of the strains tested were susceptible to tiamulin, except for one *M. gallinaceum* strain that had developed intermediate susceptibility. Tiamulin is a pleuromutilin that in general has been found to be effective in the treatment and control of *Mycoplasma* spp. *In vitro* studies demonstrated that resistance to tiamulin could not be acquired when *M. gallisepticum* and *M. synoviae* were passaged up to 10 times in the presence of subinhibitory concentrations of this drug, whereas the same process resulted in the emergence of resistance against other compounds (9). Continuing comparative genome analysis is expected to provide further insights into how and why this strain acquired intermediate resistance against tiamulin.

Mycoplasmas acquire AMR either by mutations in specific genes or through gene transfer between different species. Studies on acquired resistance to macrolides indicated that single point mutations in the 23S rRNA gene in both *M. gallisepticum* and *M. synoviae* are responsible (10, 12). As expected, all the *M. synoviae* strains and all but one of the *M. gallisepticum* strains had A2059G substitutions in one or both of the 23S rRNA genes. Point mutation G745A in the 23S rRNA gene and amino acid substitutions G354A and H91K were found in the L4 and L22 proteins, respectively, of *M. gallinarum*. Only the G354A mutation in the L4 protein was found in all tylosin-resistant *M. gallinarum* strains, and it could be the primary marker for macrolide resistance for this species. The mechanism of acquired macrolide resistance for *M. gallinaceum* is possibly G748A, as this mutation was present in all but one of the tylosin-resistant strains. Only one mutation was observed in the regions of interest for *M. pullorum*, but this was a susceptible strain, and as such, a mechanism of macrolide resistance could not be inferred. One *M. gallisepticum* and one *M. gallinaceum* strain did not contain the required mutation, A2059G and G748A, respectively, suggesting that other mechanisms of macrolide resistance are involved; therefore, further proteomic analysis is required (26).

Quinolone resistance in *M. synoviae* is acquired by point mutations in the QRDRs of the DNA gyrase and topoisomerase IV proteins (11, 25). All *M. gallisepticum*, *M. gallinarum*, and *M. pullorum* strains tested in this study were either sensitive or intermediately sensitive to enrofloxacin. No mutations in the *gyrA*, *gyrB*, *parC*, or *parE*

genes were observed except in one *M. pullorum* strain, which had point mutation S81P; however, this was not a resistant strain. In the case of *M. gallinaceum*, only 2 of the 6 resistant strains had a point mutation in the *parC*, but no other potential markers were observed. The single enrofloxacin-resistant strain of *M. synoviae* had a D420N mutation in the *parE* gene, which was suggested by Lysnyansky et al. as one of multiple possible markers for quinolone resistance in *M. synoviae* (11). All of the *M. synoviae* strains also contained D454E and N84D substitutions in the *parE* and *parC* genes, respectively. The latter have been shown to be associated with decreased susceptibility to quinolones, which could explain the intermediate susceptible phenotype. It is thus possible that point mutation D420N plays a larger role in determining resistance in *M. synoviae*, but further investigation is necessary. To our knowledge, this is the first time that a possible mechanism of acquired resistance to macrolides has been described for the avian mycoplasma species *M. gallinarum* and *M. gallinaceum*. Further investigation is required to identify the mechanisms of acquired resistance of *M. pullorum* to macrolides and all three of these species to quinolones.

Bacteria are considered to be multidrug resistant (MDR) if they have acquired nonsusceptibility to three or more antimicrobial classes (27). Three *M. gallinaceum* strains showed multidrug resistance to oxytetracycline (a tetracycline), tylosin (a macrolide), and enrofloxacin (a quinolone). Proportionately more *M. gallinaceum* strains were tested than for other mycoplasma species, and therefore, it is possible that other MDR mycoplasmas are circulating too. The *M. gallinarum*, *M. pullorum*, and *M. gallinaceum* samples showed proportionally more AMR than did *M. gallisepticum* and *M. synoviae* samples, and the frequent isolation from poultry flocks of nonpathogenic mycoplasma strains that have acquired AMR is a cause for concern, especially since they commonly occur in coinfections with *M. gallisepticum* and *M. synoviae* and no vaccines against these less-pathogenic species are available for their control.

It has been difficult to develop antibiotic resistance to oxytetracycline in *in vitro* studies, indicating that it is more likely due to the transfer of *tetM* from other species, as has been shown for *M. hominis*. This, however, has not yet been demonstrated in poultry mycoplasmas (25, 28). Although natural horizontal gene transfer (HGT) between mycoplasma species has not yet been reported, Dordet-Frisoni et al. recently demonstrated that conjugal transfer, a form of HGT, between mycoplasma species is possible if an integrative conjugative element is present (29). HGT has been put forward as a theory to explain the origin of the pMGA gene found in *M. gallisepticum*, which is closely related to the *vlhA* gene found in phylogenetically distant *M. synoviae* but not in other, phylogenetically close mycoplasma species (30, 31). Investigating the capability of poultry mycoplasmas for inter- and intraspecies AMR gene transfer or even the uptake or transfer of AMR genes between mycoplasmas and other bacterial species in the same environment should be prioritized.

MATERIALS AND METHODS

Sampling and culture. Suspected mycoplasma cases received at the University of Pretoria's (UP) Poultry Section for postmortem examination and tracheal and choanal cleft swabs taken from live chickens sampled from 2003 to 2015 were analyzed. Most samples originated from commercial layer chickens, but some were from breeder and broiler poultry farms in the Gauteng and Western Cape Provinces, two poultry-intensive farming regions in South Africa (Table S1 in the supplemental material).

The standard procedure at UP for mycoplasma identification has been culture followed by growth inhibition tests. Briefly, swab samples were plated directly onto modified Frey's agar medium (32) with NAD added, and the tip of each swab was thereafter swirled in a 5-ml tube of modified Frey's broth medium. The agar plates were incubated in 5% CO_2 atmosphere and examined daily under a stereomicroscope at ×40 magnification for the presence of colonies. Cultures were incubated for 21 days before being recorded as negative for *Mycoplasma*. Each morphologically distinct colony on a plate was subcultured by excising an agar plug with a single colony and rubbing it face-down on a new agar plate. These plates were incubated as described above and examined daily for the development of colonies. Pure morphologically distinct colonies were again harvested by excising an agar plug with a single colony and inoculating this in modified Frey's broth medium. The broth cultures were observed daily, and if a color change was observed, the broth too was plated out. Cultures were stored in the repository at −80°C.

Identification by growth inhibition. Species identification by growth inhibition tests on agar was as described by Clyde (33). Monospecific antisera were prepared by hyperimmunization of rabbits with

American Type Culture Collection (ATCC) cultures of *M. gallisepticum* strain NCTC10115 and *M. synoviae* strain ATCC 25204, using the method described by Ruhnke and Rosendal (34). An isolate was identified as a specific species when a clear zone of inhibition of growth was observed around a well in the agar filled with the homologous monospecific antiserum. When no zone of growth inhibition was observed, samples were reported as "*Mycoplasma* species." The ATCC *M. gallisepticum* strain NCTC10115 and *M. synoviae* strain ATCC 25204 were used as controls.

Production of hyperimmune serum in rabbits as a diagnostic reagent was approved by the University of Pretoria's Animal Ethics Committee.

MIC assays. Cultures were stored at −80°C before being thawed and subcultured for MIC analysis. MIC assays were performed according to the method published by Hannan et al. (35), with the exception that M broth was replaced by modified Frey's broth, both as culture medium and diluent. Glucose was used as the fermentation substrate, with phenol red as the pH indicator. The medium was adjusted to a final pH of 7.6 using 0.5 M sodium hydroxide. Tests were read when the positive test control had turned from red to yellow. The result was recorded as the lowest concentration of antibiotic where no color change occurred (36).

Genomic sequencing. Mycoplasma cells were harvested from 100-ml cultures by centrifugation at 10,500 rpm for 1 h at 4°C, and genomic DNA was isolated and purified using the PureLink genomic DNA minikit (Invitrogen, Carlsbad, CA, USA). DNA quality was verified using a Nanodrop 2000 (Thermo Scientific, Waltham, MA, USA). Samples collected from 2003 to 2014 were analyzed by Illumina MiSeq whole-genome sequencing by a commercial service provider (Inqaba Biotech [Pty.] Ltd., Pretoria, South Africa) and the full genomes for samples collected during 2015 were sequenced at the University of Pretoria's Ion Torrent Personal Genome Machine (PGM) sequencing facility. Whole-genome sequencing was performed to provide the necessary data for other studies under way at the University of Pretoria.

16S rRNA gene phylogeny. Paired-end MiSeq Illumina reads trimmed using the adapter Nextera library and Ion Torrent reads were *de novo* assembled into contigs using the default settings in CLC Genomics Workbench (version 8.5.1; CLC Bio-Qiagen, Aarhus, Denmark). The 16S rRNA genes were identified using the online RNAmmer 1.2 server (Department of Bio and Health Informatics, DTU Bioinformatics [http://www.cbs.dtu.dk/services/RNAmmer/]) (37). Digital normalization using Khmer (version 2.0; Lab for Data Intensive Biology, University of California, Davis) (38, 39) was performed to reduce the number of reads for submission to the IonGAP integrated genome analysis platform for Ion Torrent sequence data (http://iongap.hpc.iter.es/iongap), using the genome assembly and bacterial classification and annotation modules (40).

A 16S rRNA avian mycoplasma reference genome database was created using sequences retrieved from the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank/index.html) Nucleotide database. The 16S rRNA sequences of the samples were aligned with the 16S rRNA avian mycoplasma database using the online tool Multiple Alignment in Fast Fourier Transform (MAFFT) (version 7.304; http://mafft.cbrc.jp/alignment/server/) (41). The multiple-sequence alignment was edited and prepared for downstream analysis using BioEDIT Sequence Alignment Editor (version 7.0.5; Ibis Therapeutics [http://www.mbio.ncsu.edu/BioEdit/bioedit.html]).

The phylogenetic programs PAUP (version 4; Sinauer Associates, Inc.) (42), PhyML (version 3.0; ATGC Bioinformatics platform) (43), and MrBayes (version 3.2.6) (44) were used to perform parsimony, maximum-likelihood, and Bayesian inference analyses, respectively. The resulting phylogenetic trees, created in FigTree (version 1.4.3; Institute of Evolutionary Biology, Ashworth Laboratories), were used to infer the phylogenetic relationships between the samples and the reference strains to identify the species.

The 16S-23S rRNA intergenic spacer regions (IGSRs) of all samples that were identified as *M. gallisepticum* were extracted from the contigs using the 16S rRNA and 23S rRNA results obtained from the RNAmmer 1.2 server. The 16S-23S IGSRs of all *M. gallisepticum* samples were aligned to the reference 16S-23S IGSRs of *M. gallisepticum* and *M. imitans* to distinguish between these two species.

Antimicrobial resistance genes. The *de novo*-assembled contigs for samples with existing reference genomes were reconstructed using the CLC Genome Finishing Tool (version 1.5.4). The reference genomes used in this study were *M. gallisepticum* strain R(low) (accession number AE015450), *M. synoviae* strain 53 (accession no. AE017245), *M. pullorum* strain B359_6 (accession number CP017813), and *M. gallinaceum* strain B2096 8B (accession number CP011021). No published reference genome for *M. gallinarum* is available; therefore, *de novo*-assembled contigs of two samples from the present study were submitted to the RAST prokaryotic genome annotation server (http://rast.nmpdr.org) for annotation (45–47). The 23S rRNA, ribosomal protein L4 (*rplD*), ribosomal protein L22 (*rplV*), DNA gyrase subunit A (*gyrA*), DNA gyrase subunit B (*gyrB*), topoisomerase IV subunit A (*parC*), and topoisomerase IV subunit B (*parE*) genes were extracted, and the *de novo*-assembled contigs of the remaining *M. gallinarum* samples were aligned to the extracted genes. The 23S rRNA, *rplD*, *rplV gyrA*, *gyrB*, *parC*, and *parE* genes for each sample were aligned to the reference genes of their respective species using CLC genomic workbench (version 8.5.1) and compared. The nucleotide sequences of the *rplD*, *rplV*, *gyrA*, *gyrB*, *parC*, and *parE* genes were translated to the protein code for comparison in CLC Genomics Workbench (version 8.5.1). The reference genes were also aligned to the respective reference genes for *Escherichia coli* for numbering purposes (10–12).

Accession number(s). The 16S rRNA, 16S-23S rRNA IGSR, 23S rRNA, *rplD*, *rplV*, *gyrA*, *gyrB*, *parC*, and *parE* sequences determined in the study were deposited in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank/index.html) under accession numbers MH538971 to MH539148, MH571894 to MH571937, MH540196 to MH540321, MH548710 to MH548772, MH548647 to MH548709, MH548523 to

MH548584, MH548585 to MH548646, MH548834 to MH548895, and MH548773 to MH548833, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.01660-18.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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