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Antimicrobial resistance patterns of *Mycoplasma mycoides* subsp *Capri*, *M agalactiae* and *M arginini* isolates of caprine origin in India

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Abstract

In India *Mycoplasma mycoides* subsp. *capri* (*Mmc*) and *Mycoplasma agalactiae* cause caprine pleuropneumonia and contagious agalactia in goats. Unchecked therapeutic overuse of antimicrobials over the years has served as driving facet for the emergence of antimicrobial resistance (AMR) jeopardy in mollicutes. In the present study, antimicrobial susceptibility profiles of 30 caprine *Mycoplasma* spp. isolates (20 of *Mmc* and 5 each of *M. agalactiae* and *M. arginini*) of Indian origin were estimated against 15 antimicrobials. The minimum inhibitory concentration (MIC) results revealed species-specific dissimilarities in sensitivity patterns: *Mmc* and *M. arginini* exhibited maximum susceptibility for moxifloxacin, whereas doxycycline was the most potent antimicrobial against *M. agalactiae*. Multidrug fluoroquinolone-resistance was evident in one isolate each of *Mmc* and *M. arginini*. Moreover, two *M. arginini* isolates strikingly demonstrated complete and intermediate resistance against erythromycin and tylosin respectively. On sequence analysis, no mutations were detected in *Mmc* isolates at prescribed amino acid positions of QRDR subunits (GyrA, GyrB and ParE) ruling out the possibility of target gene mutational alteration in fluoroquinolone-resistant Indian mycoplasma isolates. However, earnest research focusing on alternative resistance mechanisms (efflux pumps, enzymatic inactivation, etc.) in mycoplasma isolates of Indian origin is further needed to safeguard livestock productivity and ultimately public health.

Keywords: *Mmc*, *Mycoplasma agalactiae*, AMR, *M. arginini*, MIC, QRDR

1. Introduction

India is an agrarian country with livestock sector holding 27% share of total agricultural GDP. According to the 20th livestock census, India ranks second and third in goat and sheep populations respectively. Goat, the “Poor man’s cow”, serves as a valuable livestock asset contributing 15.19% of total wool production, 13.74% of total meat production and 4% of the milk production in India ^[1, 2]. Infectious respiratory diseases share 5.6% of total diseases of goats and sheep, responsible for around 50% mortality in the animals ^[3]. Caprine mycoplasmosis, caused by the smallest cell wall-less bacteria belonging to the class *Mollicutes*, represents one of the major threat in the small ruminant sector associated with pertinent economic losses in terms of respiratory and systemic infections, significant morbidity and mortality, reduced production, retarded growth, high treatment costs and enhanced susceptibility to secondary infections ^[4, 5].

In India, *Mycoplasma mycoides* subsp. *capri* (*Mmc*) acts as an etiological agent of Caprine Pleuropneumonia (CPP), a clinical syndrome characterized by pneumonia, mastitis, arthritis, keratoconjunctivitis and septicemia. The disease is endemic in numerous states of the country and serves as a serious animal health concern for farmers and livestock holders ^[6]. Contagious agalactia (CA) is an OIE-listed pathogenic mycoplasmosis chiefly caused by *Mycoplasma agalactiae* (*Ma*), imparting substantial loss of milk production in goat and sheep population ^[7]. *M. arginini* is a commensal organism frequently isolated from the upper respiratory tract of ruminants ^[8]. However, the association of *M. arginini* with caprine keratoconjunctivitis

and pneumonic cases has been established recently [9, 10]. Frequent outbreaks of mycoplasmosis in small ruminant population necessitates the development of effective prophylactic measures for the prevention and control of infection [11]. However, antimicrobial chemotherapy is routinely preferred in order to regulate the spread of mycoplasmosis outbreak at field level owing to the lack of commercially available anti-mycoplasmal vaccines in India [12]. A vast range of the antimicrobials, viz. macrolides, fluoroquinolones, tetracyclines, lincosamides, etc. have been frequently used for the therapeutic resolution of mycoplasmosis [13]. Nevertheless, unregulated overuse and misuse of antibiotics in recent years has led to the development of antimicrobial resistance in mollicutes resulting in treatment failure and persistence of subclinical and chronic infections in animals thereby reducing their productive value [14-16]. Therefore, the present research work was conducted in order to evaluate the in-vitro antimicrobial sensitivity patterns of Indian *Mycoplasma* spp. of caprine origin isolated during 1982-2016.

2. Materials and Methods

2.1 Mycoplasma isolates

A total of 30 lyophilized caprine mycoplasma isolates including *Mmc* (n=20), *M. agalactiae* (n=5) and *M. arginini* (n=5) maintained in the Mycoplasma Laboratory, Division of Bacteriology & Mycology, ICAR-IVRI, Izatnagar (U.P., India) were investigated in the present study (Table 1). This *Mycoplasma* spp. were isolated from different states of Northern India, viz. Uttar Pradesh, Gujarat, Uttarakhand, Jammu & Kashmir and Rajasthan during 1982-2016.

The majority of the *Mycoplasma* spp. isolates (n=12) were collected from the goat lung samples showing gross pathological lesions of pneumonia, viz. consolidation and congestion, accumulation of thoracic fluid and fibrin deposition. In some cases, pleura were adhered with the thoracic cavity. The samples were collected during 1993-2016 from local abattoirs of Bareilly and Mathura districts of Uttar Pradesh and Ladakh region in Kashmir. The isolate no. 698 (*Mmc*) was collected from Bhuj, Gujarat in 1982 from caprine pneumonia outbreak. Nine mycoplasma isolates were obtained from the post-mortem cases of pneumonic goats from Mathura and Bareilly during 1996-2010. These goats were showing clinical history of pneumonia.

Six of the mycoplasma isolates were procured from clinical mastitis and acute arthritis cases of goats from Barielly and Mukteshwar (Uttarakhand) during 2000-2011. They were the antemortem cases attended in local clinics. The animals were exhibiting history of clinical mastitis with inflammatory swelling or atrophy in either one or both teats and cessation of milk as well as inflammation of joints. Two *Mycoplasma* spp. were isolated from ante-mortem caprine pneumonia cases after collection and processing of nasal swabs from pneumonic goats during 2010-2011 from Barielly and Avikanagar (Rajasthan). Additionally, reference strains of each spp., viz. *Mmc* PG3 (NCTC 10137), *M. agalactiae* PG2 (NCTC 10123) and *M. arginini* (NCTC 10129) were also involved in the current investigation as quality control strains.

The isolates were revived by culturing into modified Difco™ PPLO broth medium (Becton Dickinson India Pvt. Ltd.) for the appearance of mild translucent growth and colour change in the medium (due to pH shift). Subsequently, the positive samples were sub-cultured on Difco™ PPLO agar for the detection of typical 'fried-egg' like colonies. The pure cultures were derived by sub-culturing thrice and confirmed using growth inhibition test and PCR [17-20]. The concentrations of the inocula were estimated by microplate dilution method [21, 22] and diluted upto the desired concentration using sterile saline solution.

2.2 Microbroth dilution test

The antimicrobial susceptibility testing was performed employing the microdilution method for the determination of minimum inhibitory concentration (MIC) of each antimicrobial [22]. For this purpose, fifteen different commonly used antimicrobial agents (Table 2) were procured from HiMedia Laboratories Pvt. Ltd., Mumbai. Antimicrobial stock solutions with concentration of 1 mg/ml (except Norfloxacin) were prepared in sterile distilled water and sterilized by membrane filtration using 0.2 µm syringe filters (Axiva Biotech Pvt. Ltd.). However, 95% ethanol was used as a solvent in case of chloramphenicol and erythromycin, whereas, enrofloxacin and oxytetracycline were diluted using 0.1 M NaOH and 3N hydrochloric acid as solvents, respectively [23, 24]. The stock solutions were stored as sterile aliquots at -20°C till the future use. Serial two-fold dilutions of the antimicrobials were prepared within the concentration range set according to the previously published data [14-16, 25, 26].

Microbroth dilution method was conducted using sterile 96-well microtitre plates (Genetix Biotech Pvt. Ltd.). Initially, 150 µl of sterile PPLO medium containing 0.007% phenol red was added in each well, followed by the inclusion of 25.6 µl of each antimicrobial dilution and the standardized inocula (diluted to the final concentration of 10³-10⁵ CFU/ml). A well containing inoculum without antimicrobial (growth control) and a well without inoculum (sterility control) were incorporated for each antimicrobial under investigation. The plates were sealed aseptically and incubated at 37°C in 5% CO₂ atmosphere. MIC for each antimicrobial was determined as the lowest antimicrobial concentration inhibiting mycoplasmal growth thereby producing no colour change in corresponding wells in-vitro. The results were recorded when visible colour change in the medium was evident in the growth control wells. Furthermore, the concentrations of an antimicrobial required to inhibit the visible growth of 50% and 90% of the mycoplasma isolates under study were calculated as MIC₅₀ and MIC₉₀ respectively.

2.3 Epsilometric test (E-test)

E-test was employed for the determination of MIC in case of norfloxacin. For this, the inoculum concentration was standardized to 10⁷-10⁸ CFU/ml by microplate dilution [25]. Subsequently, 100 µl of the standardized culture was streaked on the 90 mm PPLO agar plate using sterile L-shaped spreader. The norfloxacin Ezy MIC™ strip (HiMedia Laboratories Pvt. Ltd.) was placed on the agar plate with the help of applicator following standard manufacturer's protocol. The plate was further incubated as described earlier. The MIC was interpreted as concentration at the point where the inhibition zone coincides with the graded MIC scale on the strip.

2.4 Fluoroquinolone resistance profiling of MMC isolates

The *Mmc* isolates showing high MIC values for the fluoroquinolone group of antimicrobials (ciprofloxacin, enrofloxacin, norfloxacin and moxifloxacin) were selected for the amplification of target genes encoding subunits of Quinolone Resistance Determining Regions (QRDR): *gyrA* and *gyrB* encoding DNA gyrase and *parE* encoding Topoisomerase IV. The PCR was performed in a thermocycler (Eppendorf Mastercycler®, USA) using the previously reported oligonucleotide primers and cyclic conditions [26]. The reaction mixture (25 µl/sample) consisted of 0.4 mM dNTPs, 3 mM MgCl₂, 1 mM of each forward and reverse primer, 1.24 units of Taq DNA polymerase and 3 µl DNA template. The PCR amplicons were resolved and evaluated by electrophoresis using 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml). The product size was determined, photographed and documented by UV gel documentation system (Alpha Innotech

Corp., USA). The amplicons were further subjected to bidirectional sequencing using the same PCR primers at Eurofins Genomics India Pvt. Ltd., Bengaluru. Sequence alignment and analysis was performed using MEGA 7.0 [27].

3. Results

3.1 Antimicrobial susceptibility patterns of mycoplasma isolates

Species-specific differences were noticed in the susceptibility patterns of *Mycoplasma* spp. under evaluation. Moxifloxacin ($MIC_{50}=0.03$ and $MIC_{90}=0.07$ µg/ml) followed by tylosin and doxycycline ($MIC_{50}=0.1$ and $MIC_{90}=0.2$ µg/ml) were proved to be the most potent antimicrobials against *M. mycoides* subsp. *capri*. On the contrary, *M. agalactiae* manifested the highest susceptibility for doxycycline, moxifloxacin and tylosin in-vitro with MIC values within the range of 0.1-0.2 µg/ml, 0.15-0.3125 µg/ml and 0.1-0.4 µg/ml respectively.

In the case of *Mmc* isolates, macrolides (tylosin and erythromycin) and fluoroquinolones (ciprofloxacin and enrofloxacin) displayed lower MIC in the range of 0.05-0.4 µg/ml and 0.15-0.625 µg/ml, respectively. A variable range of MIC values was recorded for norfloxacin (0.25-1.5 µg/ml). However, one *Mmc* isolate (No. 0) exhibited higher MIC values for ciprofloxacin (1.25 µg/ml), enrofloxacin (0.625 µg/ml) and norfloxacin (1.5 µg/ml) and hence can be categorized as fluoroquinolone-resistant according to the recommended breakpoints (Table 3). Another *Mmc* isolate, 135 was demonstrating norfloxacin-resistance and intermediate resistance against enrofloxacin with elevated MIC values of 1.5 µg/ml and 0.625 µg/ml respectively. Eight of the *Mmc* isolates under investigation were characterized by intermediate susceptibility against norfloxacin ($MIC=0.7$ µg/ml). Nonetheless, intermediate-level spiramycin resistance ($MIC=3.2$ µg/ml) was noticed in two *Mmc* isolates, viz. 21.6C and K. Majority of the *Mmc* strains (19 out of 20) exhibited susceptibility to clindamycin with MIC value within 0.1-0.4 µg/ml, whereas one of the isolates (21.6 C) was clindamycin-resistant having MIC of 0.8 µg/ml. Doxycycline was observed to be the most potent antimicrobial among the tetracyclines with MIC ranging from 0.1-0.4 µg/ml followed by tetracycline (0.1-0.8 µg/ml). Oxytetracycline was found to be active against *Mmc* at a comparatively higher MIC range of 0.4-3.2 µg/ml. Intermediate resistance against chloramphenicol ($MIC=8$ µg/ml) was evident in three of the *Mmc* isolates under investigation. A broad MIC range was detected for the aminoglycosides (16-256 µg/ml). Majority of the isolates (16 out of 20) were resistant against streptomycin; whereas all the strains possessed resistance for gentamicin and kanamycin. An intermediate degree of streptomycin resistance was recorded in four of the *Mmc* isolates in-vitro.

Tetracycline and ciprofloxacin were effective against the *M. agalactiae* isolates with MIC ranges of 0.2-0.4 µg/ml and 0.3125-0.625 µg/ml respectively. However, one isolate (216)

exhibited intermediate susceptibility against norfloxacin with MIC value of 0.7 µg/ml. Two of the *M. agalactiae* strains (216, 5/11 Milk) displayed intermediate-level resistance for enrofloxacin with MIC value exceeding 0.5 µg/ml (i.e., 0.625 µg/ml). MIC value in the range of 0.8-1.6 µg/ml was recorded for the two antimicrobial agents, viz. spiramycin and oxytetracycline. Four *M. agalactiae* isolates were categorized as intermediately resistant against chloramphenicol with MIC value of 8 µg/ml. However, all the isolates exhibited intermediate resistance for erythromycin (0.8 µg/ml) and complete resistance against the aminoglycoside group with MIC value ranging from 32-64 µg/ml.

MIC results for *M. arginini* suggested moxifloxacin as the drug of choice in-vitro with the lowest MIC values ranging from 0.01-0.625 µg/ml. One *M. arginini* isolate, 1/11 T2 displayed multidrug-resistance to fluoroquinolones, viz. ciprofloxacin, enrofloxacin and norfloxacin having higher MIC values of 1-1.25 µg/ml. Moreover, another isolate, 1/11 T6 showed ciprofloxacin-resistance with MIC of 1.25 µg/ml. Intermediate level of enrofloxacin resistance was evident in three isolates, viz. 1/11 T2, 1/11 T6 and 41E ($MIC=1.25$ µg/ml). Chloramphenicol was found to be effective against all five *M. arginini* isolates under the investigation having MIC range of 0.5-4 µg/ml. Tylosin susceptibility was evident in three isolates while two strains, 1/11 T2 and 1/11 T6 were showing intermediate-level resistance against the antimicrobial ($MIC=1.6$ µg/ml). Spiramycin was effective against all the studied *M. arginini* isolates having MIC within the range of 0.2-3.2 µg/ml. One isolate, 41E exhibited intermediate resistance for erythromycin with MIC of 0.8 µg/ml whereas three isolates, VP3B/05, 1/11 T2 and 1/11 T6 were erythromycin-resistant with high MIC values of 1.6-3.2 µg/ml. All the *M. arginini* isolates were susceptible to the tetracycline group of antimicrobials with MIC values lying within the range of 0.8-3.2 µg/ml. However, resistance against clindamycin and aminoglycosides was present in all the evaluated isolates with intermediate resistance to streptomycin and gentamicin in one (26/10) and two (1/11 T6 and 26/10) of the five isolates respectively.

3.2 Sequencing analysis study of the QRDR in MMC isolates

Genetic analysis study for the fluoroquinolone resistance profiling was performed on the reference strain (PG2), two *Mmc* isolates displaying elevated MIC for fluoroquinolones and a strain with normal MIC values for comparison. However, the sequencing results of *gyrA*, *gyrB* and *parE* revealed the absence of mutations at the specified amino acid positions [25]. This suggests that mutational alteration in the target sites, i.e., Quinolone Resistance Determining Regions (QRDR) subunits: GyrA, GyrB and ParE may not be the actual mechanism of acquisition of fluoroquinolone-resistance in studied Indian *Mmc* isolates recovered from goats.

Table 1: Epidemiological details of *Mycoplasma* spp. isolates used in the MIC study

Sr No.	Isolate ID	Host	Sample	Clinical presentation	Source	Year
<i>Mmc</i> Isolates						
1.	698	Goat	Lung	Goat flock pneumonia outbreak	Bhuj	1982
2.	135	Goat	Lung	Pneumonic pathology of Lung	Bareilly	1993
3.	137	Goat	Lung	Pneumonic pathology of Lung	Bareilly	1994
4.	VP2-L7/03	Goat	Lung	Pneumonic pathology of Lung	Bareilly	2003
5.	15.8B	Goat	Lung	Post mortem case of pneumonic goat	Mathura	2008
6.	A7E	Goat	Lung	Post mortem case of pneumonic goat	Bareilly	2008
7.	ii6F	Goat	Milk	Goat suffering with clinical mastitis	Bareilly	2009
8.	31.7H	Goat	Lung	Pneumonic pathology of Lung	Bareilly	2009
9.	48.J	Goat	Lung	Post mortem case of pneumonic goat	Mathura	2009
10.	No.0	Goat	Lung	Pneumonic pathology of Lung	Bareilly	2009
11.	39	Goat	Lung	Pneumonic pathology of Lung	Bareilly	1999
12.	19	Goat	Mediastinal Lymph Node	Post mortem case of pneumonic goat	Bareilly	1999
13.	2/99	Goat	Synovial fluid	Goat suffering with acute arthritis	Bareilly	2000
14.	7/2000	Goat	Synovial fluid	Goat suffering with acute arthritis	Mukteshwar	2001

15.	VP20-L9/02	Goat	Lung	Post Mortem case of pneumonic goat	Bareilly	2002
16.	26.7D	Goat	Lung	Post Mortem case of pneumonic goat	Mathura	2009
17.	21.6C	Goat	Milk	Goat suffering with clinical mastitis	Bareilly	2009
18.	K	Goat	Milk	Goat suffering with clinical mastitis	Bareilly	2009
19.	RLM 6/11	Goat	Lung	Pneumonic pathology of Lung	J & K	2011
20.	LC-goat	Goat	Lung	Pneumonic pathology of Lung	Mathura	2016
<i>M. agalactiae</i>						
21.	200 RNS	Goat	Lung	Post Mortem case of pneumonic goat	Bareilly	1996
22.	216 RPNS	Goat	Lung	Post Mortem case of pneumonic goat	Bareilly	1996
23.	VP20-L15/02	Goat	Lung	Pneumonic pathology of Lung	Bareilly	2002
24.	RLM 5/11 NS	Goat	Nasal swab	Goat suffering with pneumonia	Bareilly	2011
25.	RLM 5/11 Milk	Goat	Milk	Goat suffering with clinical mastitis	Bareilly	2011
<i>M. arginini</i>						
26.	VP3 B/05	Goat	Lung	Pneumonic pathology of Lung	Bareilly	2005
27.	RLM 01/11 T2	Goat	Lung	Pneumonic pathology of Lung	Ladakh	2011
28.	RLM 01/11 T6	Goat	Lung	Pneumonic pathology of Lung	Ladakh	2011
29.	41E	Goat	Nasal swab	Goat suffering with pneumonia	Avikanagar	2010
30.	26/10	Goat	Lung	Post Mortem case of pneumonic goat	Bareilly	2010

Isolate designation, host, source of isolation, clinical presentation, place and year of isolation of each isolate are mentioned in the table

Table 2: Antimicrobial agents used in MIC testing with concentration ranges

Sr No.	Antimicrobial Class	Antimicrobial	Presentation	Conc. Range (µg/mL)
1.	Aminoglycosides	Streptomycin	Powder	0.5-256
2.		Gentamicin	Powder	
3.		Kanamycin	Powder	
4.	Amphenicols	Chloramphenicol	Powder	0.06-16
5.	Fluoroquinolones	Ciprofloxacin	Powder	0.01-10
6.		Enrofloxacin	Powder	
7.		Moxifloxacin	Powder	
8.		Norfloxacin	Strip	
9.	Macrolides	Tylosin	Powder	0.006-12.8
10.		Tilmicosin	Powder	
11.		Erythromycin	Powder	
12.	Lincosamides	Clindamycin	Powder	0.006-12.8
13.	Tetracyclines	Doxycycline	Powder	0.006-12.8
14.		Oxytetracycline	Powder	
15.		Tetracycline	Powder	

Table 3: Minimum inhibitory concentration (MIC) range, MIC50 and MIC90 of *Mmc* Isolates for 15 antimicrobials against the Reference Strain (PG3)

Antimicrobial	MIC range (µg/ml)	MIC ₅₀ (µg/ml) N=20	MIC ₉₀ (µg/ml) N=20	Breakpoints: susceptible, intermediate, resistant (µg/ml) [References]	Reference strain PG3 (µg/ml)
Streptomycin	16-256	64	256	≤ 4, 8-16, ≥ 32 ^[45]	64
Gentamicin	32-256	128	256	ND	128
Kanamycin	32-256	128	256	ND	128
Chloramphenicol	2-8	4	8	≤ 4, ≤ 8, ≥ 16 ^[45]	2
Ciprofloxacin	0.15-1.25	0.3125	0.625	≤ 0.5, ND, ≥ 1 ^[16]	0.625
Enrofloxacin	0.15-0.625	0.15	0.3125	≤ 0.5, ≤ 1, ≥ 2 ^[43]	0.3125
Norfloxacin	0.25-1.5	0.5	0.7	≤ 0.5, > 0.5, ≥ 1 ^[16]	0.5
Moxifloxacin	0.01-0.15	0.03	0.07	≤ 0.25, ND, ≥ 0.5 ^[46]	0.03
Tylosin	0.1-0.25	0.1	0.2	≤ 1, ≤ 2, ≥ 4 ^[43]	0.25
Spiramycin	0.2-3.2	0.8	1.6	≤ 2, 2-8, > 8 ^[46]	1
Erythromycin	0.05-0.4	0.1	0.4	≤ 0.5, ND, ≥ 1 ^[46]	0.25
Clindamycin	0.1-0.8	0.4	0.4	≤ 0.25, ND, ≥ 0.5 ^[46]	0.4
Doxycycline	0.1-0.4	0.1	0.2	ND	0.2
Oxytetracycline	0.4-3.2	0.8	1.6	≤ 4, 8, ≥ 16 ^[43]	1.6
Tetracycline	0.1-0.8	0.2	0.4	ND	0.4

ND: No data

Table 4: Amino acid profiles of Quinolone resistance determining regions (QRDR) subunits of sequenced *Mmc* isolates

Isolate	MIC (µg/ml)			Amino acid profile						
				GyrA				GyrB	ParE	
	Ciprofloxacin	Enrofloxacin	Norfloxacin	47	83	84	87	445	458	459
PG3	0.625	0.3125	0.5	Arg	Ser	Ala	Glu	Pro	Asn	Glu
No. 0	1.25	0.625	1	-	-	-	-	-	-	-
135	0.625	0.625	1	-	-	-	-	-	-	-
ii6F	0.625	0.15	0.7	-	-	-	-	-	-	-

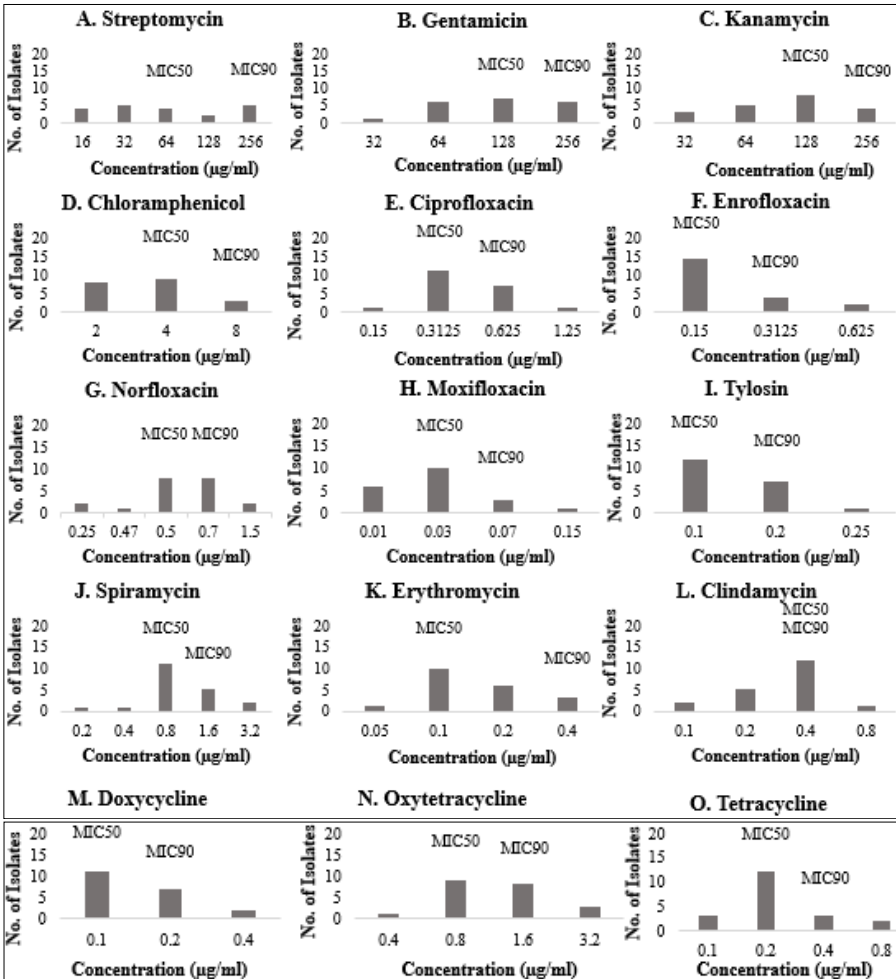


Fig 1: MIC distribution of Indian *Mmc* isolates for each antimicrobial under Evaluation

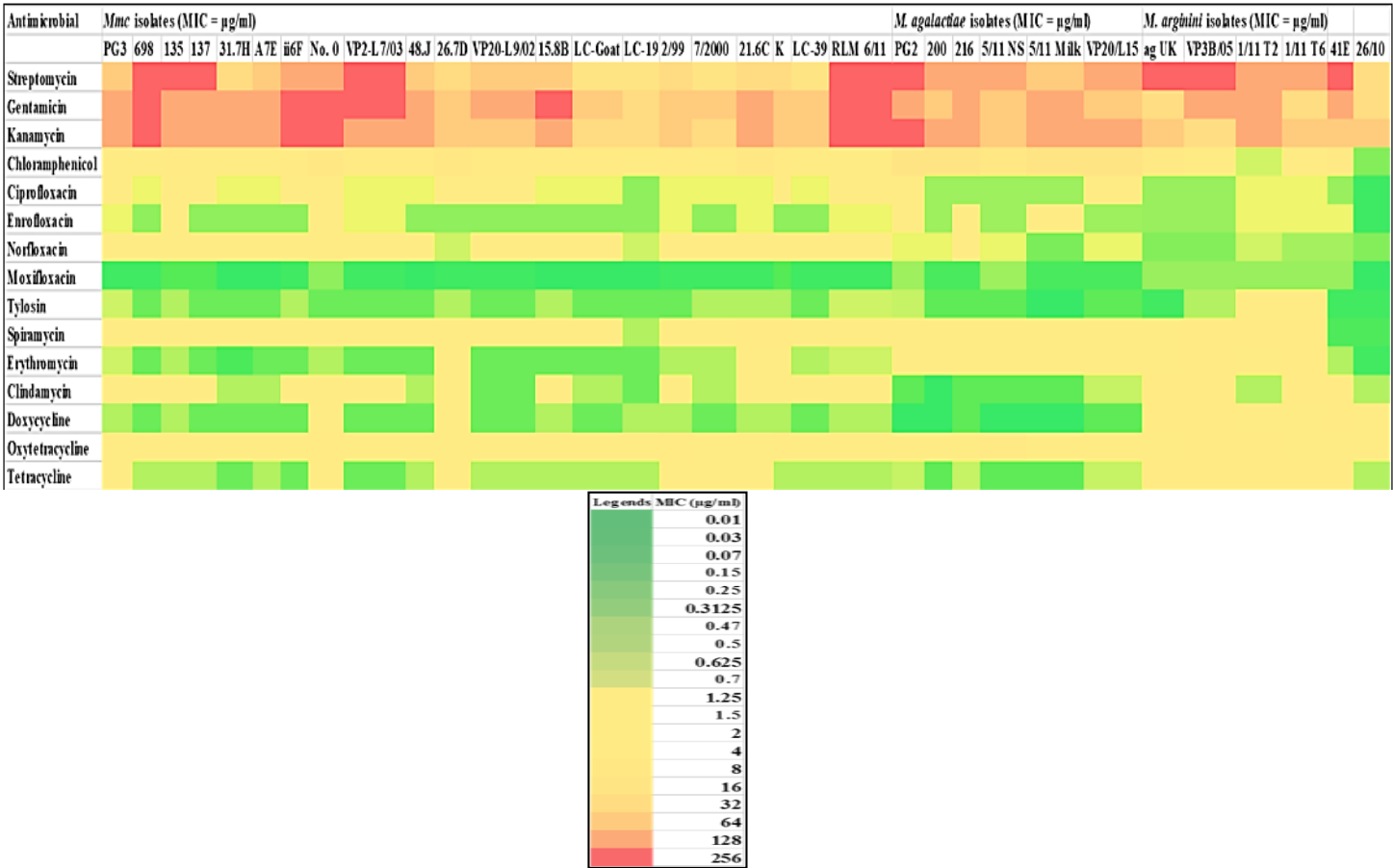


Fig 2: Heatmap showing MIC distribution of 30 caprine *Mycoplasma* spp. isolates in India

4. Discussion

Caprine mycoplasmosis, a cardinal pathogenesis in goat population is of paramount importance in the livestock sector in terms of pertinent economic losses. In the field conditions, certain factors such as fastidious nutrient requirements in culture media, long incubation period for growth, unavailability of proper diagnostic equipments for the isolation and identification act as lacunae in the proper diagnosis of mycoplasmal infections^[12]. Furthermore, due to the lack of effective anti-mycoplasmal vaccines in India, field veterinarians primarily rely on antimicrobial therapy for the purpose of treatment and control of mycoplasmosis. However, routine MIC testing of veterinary *Mycoplasma* spp. is seldomly performed due to the constraints such as variable media requirements for different species, lack of CLSI- approved guidelines, need of different quality control strains and longer incubation period for the interpretation of results^[22]. In the current investigation, the in-vitro effects of fifteen antimicrobial agents belonging to the various classes, viz. aminoglycosides (streptomycin, gentamicin, kanamycin), fluoroquinolones (ciprofloxacin, enrofloxacin, norfloxacin and moxifloxacin), amphenicols (chloramphenicol), macrolides (tylosin, spiramycin, erythromycin), lincosamides (clindamycin) and tetracyclines (doxycycline, oxytetracycline, tetracycline) were evaluated against 30 *Mycoplasma* spp. isolates, including 20 of *Mmc* and 5 each of *M. agalactiae* and *M. arginini*. To the best of our knowledge, this is the first antimicrobial susceptibility evaluation conducted on Indian mycoplasma strains recovered from goats for various antibiotic drugs used commonly at field levels. Fluoroquinolones possess mycoplasmacidal activity through suppression of the DNA replication machinery by interacting with type-II topoisomerases, namely DNA gyrase and topoisomerase IV^[30]. According to the MIC results, moxifloxacin displayed maximum in-vitro potency against *Mmc* and *M. arginini* isolates with the lowest MIC values ranging from 0.01-0.15 µg/ml and 0.01-0.625 µg/ml respectively. Enrofloxacin (0.15-0.625 µg/ml) and ciprofloxacin (0.15-1.25 µg/ml) were second and third respectively among the fluoroquinolone class in terms of efficacy for the *Mmc* strains under study. However, higher MIC value for ciprofloxacin (>1 µg/ml), exceeding breakpoints was recorded for one *Mmc* isolate, No. 0 and two *M. arginini* isolates, 1/11 T2 and 1/11 T6. Two isolates each of *Mmc* (No. 0 and 135), *M. agalactiae* (216, 5/11 Milk) and three *M. arginini* strains (1/11 T2, 1/11 T6 and 41E) were found to be intermediately susceptible against enrofloxacin. All the *M. agalactiae* isolates were inhibited by the low concentrations of moxifloxacin and ciprofloxacin with MIC ranges of 0.15-0.3125 µg/ml and 0.3125-0.625 µg/ml respectively. Norfloxacin was the least active fluoroquinolone drug with the highest MIC₅₀ (0.5 µg/ml) and MIC₉₀ (0.7 µg/ml) concentrations required to inhibit *Mmc* isolates in-vitro.

Tylosin essentially serves as the drug of choice for the therapeutic purpose in cases of caprine, bovine and avian mycoplasmosis. It acts by interfering with the peptidyl transferase activity of 50S ribosomal subunit with the resultant inhibition of protein synthesis^[31]. All the studied isolates were highly sensitive for tylosin with low MIC values in the range of 0.1-0.25 µg/ml. However, high MIC values exceeding breakpoints for intermediate tylosin-resistance (≤ 2 µg/ml) were recorded for two *M. arginini* isolates, 1/11 T2 and 1/11 T6, indicating an alarming threat of AMR emergence in the mollicute spp. Spiramycin showed a broad range of MIC for 20 *Mmc* and 5 *M. arginini* isolates: 0.2-3.2 µg/ml; whereas in the case of *M. agalactiae* strains the MIC was within the range of 0.8-1.6 µg/ml. Erythromycin inhibited all the *Mmc* isolates at low concentrations of 0.05-0.4 µg/ml. However, intermediate

erythromycin-resistance was evident in all the *M. agalactiae* isolates (MIC= 0.8 µg/ml). These findings were in agreement with earlier investigation which suggested the association between point mutations in 23S rRNA gene and intrinsic resistant against erythromycin in *M. hominis*^[32]. Three *M. arginini* isolates demonstrated complete resistance against erythromycin (MIC= 1.6-3.2 µg/ml). Tetracyclines are routinely utilized for the treatment of caprine respiratory tract infections including mycoplasmosis at the field level. They exert bacteriostatic activity through binding with the 30S ribosomal subunit, thereby preventing the addition of new amino acids to the nascent peptide chain, potentially interfering with bacterial protein synthesis^[33]. All the isolates under current investigation were detected as susceptible for tetracyclines. Doxycycline was found to be the most effective antimicrobial agent against *M. agalactiae* with the lowest MIC in the range of 0.1-0.2 µg/ml. *Mmc* isolates were also inhibited by the low concentrations of doxycycline in-vitro (0.1-0.4 µg/ml). Tetracycline exerted high inhibitory activity with MIC value ranging from 0.1-0.8 µg/ml for *Mmc* and 0.2-0.4 µg/ml in case of *M. agalactiae*. However, a comparatively wider range of MIC values was observed in the case of oxytetracycline (0.4-3.2 µg/ml). In the case of *M. arginini* isolates, tetracyclines were found to be effective at higher MIC concentrations ranging from 0.8-3.2 µg/ml. These results suggest that despite the unregulated, long-term usage of tetracyclines for the treatment of animal diseases at field levels; they possess significant anti-mycoplasmal activity in the Indian context. Amphenicols and lincosamides bind with 50S ribosomal subunit thereby hindering the transpeptidation with resultant suppression of protein synthesis in the bacterial cell^[34]. Chloramphenicol was proved to be effective against most of the *Mmc* isolates (17 out of 20) with low MIC range of 2-4 µg/ml. However, intermediate resistance against the drug was detected in 3 *Mmc* isolates having a MIC value of 8 µg/ml. All the *M. arginini* isolates under investigation displayed susceptibility to the antimicrobial drug with MIC in the range of 0.5-4 µg/ml. In contrast, majority of the studied *M. agalactiae* isolates (4 out of 5) were demonstrating intermediate chloramphenicol-resistance (MIC=8 µg/ml). Susceptibility to clindamycin had been observed in all the *Mmc* (except 21.6 C) and *M. agalactiae* strains under present investigation with MIC value between 0.1-0.4 µg/ml. However, the *M. arginini* isolates under present investigation were found to be clindamycin-resistant with MIC values exceeding 0.5 µg/ml. Aminoglycosides were found to be the least potent antimicrobial group against the *Mycoplasma* strains under study as per the results of in-vitro susceptibility testing. All the studied isolates were exhibiting resistance pattern for the group with high MIC values >16 µg/ml. Similar results were obtained in the antimicrobial sensitivity studies on *Mycoplasma* spp. performed by various investigators^[15, 35, 36]. These results support the proposed hypothesis indicating the presence of intrinsic aminoglycoside resistance in mycoplasmas^[36]. MIC results for *Mmc* isolates in the present research work were in accordance with the study conducted in Spain, which reported that moxifloxacin and doxycycline possessed the highest inhibitory effect against the 41 *Mmc* field isolates^[15]. Similar findings were obtained by the screening of 32 field isolates of *M. capricolum* subsp. *capricolum* (*Mcc*) in Italy with the lowest MIC values for moxifloxacin and doxycycline indicating their significant potency against *Mcc*^[16]. Our results were supported by the earlier pharmacokinetics study which concluded that moxifloxacin show effective milk penetration following intramuscular administration to the lactating goats^[37]. In another study, increased drug

concentration of doxycycline in caprine milk was reported after intravenous administration in female goats^[38]. Tylosin was concluded to be the drug of choice for *Mcc* and *M. mycoides* subsp. *mycoides* LC (*Mmm*LC) isolates from Jordan in an investigation^[25]. Another research work reported maximum efficacies for tylosin and erythromycin followed by ciprofloxacin and doxycycline for *Mmm*LC^[36]. AntibioGram testing performed on 54 *Mmc* isolates from Pakistan detected maximum sensitivity for enrofloxacin and gentamicin^[39]. Disc diffusion assay conducted earlier on 10 *Mmc* isolates manifested the highest susceptibility against macrolides and tetracyclines^[40]. One of the earliest reports of MIC testing on *Mmc* isolates dates back to the year 1971 when potent anti-mycoplasmal effect was observed for the antimicrobials: tylosin, tetracycline, spiramycin, lincomycin and chlortetracycline^[41]. In the case of *M. agalactiae*, our findings revealed doxycycline as the most potent antimicrobial agent in-vitro followed by tylosin and clindamycin. Similar results were obtained in screening performed on 24 *M. agalactiae* isolates from Italy with highest efficacy for enrofloxacin, tylosin and tetracycline^[27]. In another study, maximum susceptibility of *M. agalactiae* has been detected for spiramycin, followed by tetracycline and tylosin^[42]. In contrast, enrofloxacin was concluded to be the most effective in-vitro drug against *M. agalactiae* followed by ciprofloxacin and spectinomycin^[14]. Another investigation reported high susceptibility pattern of *M. agalactiae* for tiamulin, enrofloxacin and oxytetracycline^[43]. An investigation conducted on Spanish *M. agalactiae* isolates revealed that clindamycin, norfloxacin and danofloxacin were the effective antimicrobials in decreasing order^[44]. According to the sequencing analysis study, no mutations were detected in fluoroquinolone-resistant *Mmc* isolates at the specified regions of QRDR subunits, viz. GyrA, GyrB and ParE. This indicates that the Indian *Mmc* isolates under investigation may have probably acquired resistance against the antimicrobial group by an alternative mechanism different from the alteration of target site by mutations. The involvement of ABC (ATP-binding cassette) efflux pumps in the acquisition of fluoroquinolone resistance in *Mmc* had been suggested earlier, which could serve as the actual mechanism in the studied Indian *Mmc* isolates^[28]. Moreover, intensive research regarding the role of efflux pump as AMR mechanism in the Indian perspective on furthermore number of mycoplasma isolates is the need of the hour so as to gain better knowledge regarding the mechanism of AMR development and ultimately to combat the future emerging antimicrobial resistance hazard and to safeguard the livestock and public health.

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