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Antimicrobial susceptibility profiles of *Mycoplasma mycoides* subsp. *capri* field isolates from goats in India

ANUP N KALMEGH[1], ANBAZHAGAN S[1], RAJNEESH RANA[1]✉, ZUNJAR B DUBAL[1] and PALLAB CHAUDHURI[1]

ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh 243 122 India

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Mycoplasma mycoides subsp. *capri* (*Mmc*) is a cell-wall-less bacterium, belonging to the class *Mollicutes*. They are fastidious and slow-growing microorganisms (Nicholas and Ayling 2003). *Mmc* belongs to the *Mycoplasma mycoides* (*MM*) cluster, comprising 5 species including *M. mycoides* subsp. *mycoides* (*Mmm*), *M. mycoides* subsp. *capri* (*Mmc*), *M. capricolum* subsp. *capricolum* (*Mcc*), *M. capricolum* subsp. *capripneumoniae* (*Mccp*) and *M. leachii* (*Ml*). The organism causes caprine pleuropneumonia (CPP) in goats, which is characterized by mastitis, arthritis, pneumonia and septicaemia (Thiacourt *et al.* 2011). In India, CPP is widely prevalent in goat population (Rana *et al.* 2009). The disease spreads rapidly among animals through oculo-nasal discharge, excretion from urine and faeces and wounds etc. The caprine pleuropneumonia is associated with significant economic losses in the form of reduced production, increased treatment cost and high morbidity in goats in various states (OIE 2017). CPP can also be fairly treated with antibiotics like enrofloxacin, oxytetracycline, tylosin, erythromycin, gentamycin and kanamycin.

The microbial resistance against commonly used antibiotics is an emerging issue at national and international levels due to indiscriminate use of antibiotics in daily practice and in the field conditions (Ali *et al.* 2016). Keeping in view such situation, the present study was planned to determine the antibiotic sensitivity patterns of clinical isolates of *Mycoplasma mycoides* subsp. *capri* by using disc diffusion method. This will facilitate knowledge of judicious use of antibiotics under field conditions so as to minimize the treatment cost as well as to avoid the antimicrobial resistance (AMR) problem in host and ultimately in livestock sector.

A total of 10 freeze-dried isolates of *Mycoplasma mycoides* subsp. *capri* recovered from goats during 1982–2009 and maintained in the Mycoplasma Laboratory, Division of Bacteriology and Mycology, ICAR-IVRI, Izatnagar were used in the present study (Table 1). The cultures were revived by inoculation into 0.5 ml modified PPLO (Pleuropneumonea Like Organism) broth medium mainly containing PPLO broth (Difco) 30 g, distilled water 890 ml, yeast extract (Difco) 2.5 g, lactalbumin hydrolysate (Difco) 2.5 g, glucose 2 g, sodium pyruvate 2 g, phenol red (0.5% solution) 5 ml, thallous acetate (5%) 5 ml, penicillin 1000 IU/ml, DNA (0.2%) 50 mg/2L and horse serum (10%). The inoculated broth vials were incubated in micro-aerophillic conditions (5% CO_2 at 37°c) for 2–3 days. The vials were examined daily for the appearance of growth characterized by change in colour from red to yellow (acidic pH shift) and mild translucent growth. The vials showing positive growth were sub-cultured by transferring into PPLO agar plates using sterile Pasteur pipette while incubating at 37°C for the appearance of typical fried-egg colonies. *M. mycoides* subsp. *capri* PG3 (NCTC 10137) was used as reference strain in present study.

The genomic DNA of 10 isolates was extracted using QIAamp DNA Mini Kit (Qiagen, USA). The isolated DNA sample was used as template for *Mmc* group-specific and species-specific PCR. An amplified product of ~270 bp in size was obtained by group-specific PCR using the primers GPO3 (5´-GGG AGC AAA CAG GAT AGA TAC CCT-3´) and MGSO (5´-TGC ACC ATC TGT CAC TCT GTT AAC

Table 1. Details of *Mycoplasma mycoides* subsp. *capri* isolates

Isolate ID	Sample	Place of isolation	Year of isolation
A	Lung	Bhuj, Gujarat	1982
B	Lung	Bareilly, UP	1993
C	Lung	Bareilly, UP	1994
D	Lung	Bareilly, UP	2003
E	Lung	Mathura, UP	2008
F	Lung	Mathura, UP	2009
G	Synovial fluid	Bareilly, UP	2000
H	Synovial fluid	Mukteshwar, UK	2000
I	Lung	Mathura, UP	2009
J	Lung	Bareilly, UP	2002

UP, Uttar Pradesh; UK, Uttarakhand.

Present address: [1]Division of Bacteriology and Mycology; [2]Division of Veterinary Public Health, ICAR-Indian Veterinary Research Institute, Izatnagar, Uttar Pradesh. ✉Corresponding author email: rajneeshrana01@yahoo.com

CTC-3′) (Van Kuppeveld *et al.* 1992). Whereas species-specific PCR carried out by using primers P4 (5′-ACT GAG CAA TTC CTC TT-3′) and P6 (5′-TTA ATA AGT CTC TAT ATG AAT-3′) yielded the product of ~194 bp in size (Hotzel *et al.* 1996, Kumar Vijay *et al.* 2013).

Seventeen commercially available antibiotic discs provided by HiMedia® Laboratories Private Limited, Mumbai (India) with known concentration of antibiotic were used in the present study (Table 2). All isolates of *Mycoplasma mycoides* subsp. *capri* were subjected to *in vitro* antibiotic sensitivity testing by single disc diffusion method (Taylor Robinson and Bebear 1997). The clinical isolates were inoculated in to PPLO broth and incubated at 37°C under microaerophillic conditions for 24–48 h until colour change occurred from red to yellow due to acidic pH shift. The liquid culture was standardized for CFU so as 1 ml of log phase growth is having 1×10^8 organisms. A 200 µL of this *Mmc* culture was uniformly spread over separate PPLO agar plates using sterile swabs. The inoculum was allowed to adsorb over PPLO agar plate for 1–2 min and the antibiotic discs were placed onto the surface of medium at a distance of 3 cm apart by using sterile forceps. The plates were incubated using 5% CO_2 at 37°C for 24–48 h. The results were interpreted as zone of inhibitions in mm according to CLSI guidelines (CLSI 2002).

Table 2. Antibiotic discs used for susceptibility testing (concentrations in mcg)

Antibiotic group	Name of antibiotic	Code of antibiotic disc	Concentration per disc
Amphenicols	Chloramphenicol	C 30	30 mcg
Macrolides	Erythromycin	E 15	15 mcg
	Tylosin	TL 15	15 mcg
	Spiramycin	SR 30	30 mcg
Lincosamides	Clindamycin	CD 30	30 mcg
Tetracyclines	Tetracycline	TE 30	30 mcg
	Doxycycline	D 30	30 mcg
Fluoroquinolones	Enrofloxacin	ENO 5	5 mcg
	Levofloxacin	LVA 5	05 mcg
Cephalosporins	Cefotaxime	CTX 30	30 mcg
	Ceftriaxone	CRO 30	30 mcg
	Ceftazimide	CAZ 30	30 mcg
	Cefepime	FEP 30	30 mcg
Aminoglycosides	Streptomycin	S 10	10 mcg
	Gentamicin	GM 10	10 mcg
Penicillins	Oxacillin	OX 1	01 mcg
	Amoxycillin-Clavulalic Acid	AMC 30	30 mcg

*Antibiotic discs of penicillin and cephalosporin groups were used as negative control.

The results of antimicrobial susceptibility testing (Table 3) showed that erythromycin has the maximum activity against majority of the *Mmc* isolates with the largest zone of inhibition (33±0.8 mm), followed by tylosin (32±0.91 mm), doxycycline (32±0.5 mm), tetracycline (31±0.21 mm), enrofloxacin (29±0.59) and levofloxacin (29±0.25 mm). The isolates exhibited lower susceptibility to chloramphenicol (27±1.13 mm). Four out of 10 *Mmc* isolates displayed resistance to spiramycin and clindamycin, whereas all the isolates showed resistance against streptomycin and gentamicin (Aminoglycosides). As the cephalosporins and penicillins act on bacterial cell wall, which is lacking in Mollicutes; so these group of antibiotics did not show any zone of inhibition. However to prove this we kept them as control.

Mycoplasmosis is commonly treated with antibiotic agents such as macrolides, tetracyclines, fluoroquinolones and aminoglycosides (Antunes *et al.* 2007). However, tylosin is generally regarded as the drug of choice for the treatment of caprine, bovine and avian mycoplasmosis. The results of our study showed that macrolides (erythromycin

Table 3. Zones of inhibition of various antibiotic discs for *Mmc* isolates (in mm)

Antibiotic group	Antibiotic disc	Isolate ID										
		PG3	A	B	C	D	E	F	G	H	I	J
Amphenicols	Chloramphenicol	27	26	30	32	23	32	23	24	34	28	28
Fluoroquinolones	Enrofloxacin	30.5	25	31	31	21	36	25	29	35	32	31
	Levofloxacin	29.5	29	33	31	19	33	26	28	35	31	30
Tetracyclines	Tetracycline	27.5	29	38	31	28	35	27	30	37	35	30
	Doxycycline	28.5	29	36	33	30	34	23	32	36	36	32
Macrolides	Erythromycin	34	30	37	35	31	38	29	29	38	31	31
	Tylosin	33	28	39	39	26	39	24	35	33	28	38
	Spiramycin	32	–	–	–	30	36	–	31	37	33	32
Lincosamides	Clindamycin	25.5	–	–	–	24	31	–	27	36	31	28
Cephalosporins	Cefotaxime	–	–	–	–	–	–	–	–	–	–	–
	Ceftriaxone	–	–	–	–	–	–	–	–	–	–	–
	Ceftazimide	–	–	–	–	–	–	–	–	–	–	–
	Cefepime	–	–	–	–	–	–	–	–	–	–	–
Aminoglycosides	Streptomycin	–	–	–	–	–	–	–	–	–	–	–
	Gentamicin	–	–	–	–	–	–	–	–	–	–	–
Penicillins	Oxacillin	–	–	–	–	–	–	–	–	–	–	–
	Amoxycillin-Clavulanic Acid	–	–	–	–	–	–	–	–	–	–	–

and tylosin) were the most potent antimicrobial agents against Indian isolates of *Mmc.* Tetracyclines (doxycycline and tetracycline) were the second most effective antimicrobial agents followed by fluoroquinolones (enrofloxacin and levofloxacin). In contrast to our study, Shah *et al.* (2017) concluded that enrofloxacin (fluoroquinolones) to be the best drug of choice for 54 *Mmc* isolates in Pakistan. They also observed lower susceptibility to tylosin. The study also revealed that all the Pakistan isolates were resistant to oxytetracycline and ceftiofur. The prime probable reason may be due to the strain variation. Besides that, it may also be because; during the evolution of *Mycoplasma* spp., there is a role of horizontal gene transfer and genome exchange may occur (Citti and Blanchard 2013); which leads to the difference in genomics of these organisms and ultimately leads to the different behaviour to the antibiotic sensitivity pattern.

Amphenicols (chloramphenicol) can be graded as weaker drug of choice. However, lincosamide (clindamycin) was found to be least effective. The isolates A, B, C and F were found to be resistant to spiramycin and clindamycin. Surprisingly, all the isolates were found to be resistant to aminoglycosides (streptomycin and gentamicin). As penicillins and cephalosporins act over the bacterial cell wall by inhibition of peptidoglycan synthesis, none of them were found to be active as *Mycoplasma* spp. lack cell wall.

In Europe, antimicrobial resistance of *Mycoplasma* spp. has been reported against Tylosin and Oxytetracycline (Ayling *et al.* 2005). But in our case, these antibiotics were graded as drugs of choice. The probable reason for the resistance may be that they studied different *Mycoplasma* species, viz. *Mmm*SC (Now *Mmm*).

Our observations were in agreement with Al-Momani *et al.* (2006), who studied the effect of 6 antimicrobial agents on *M. mycoides subsp. mycoides* LC (presently known as *Mmm*). They found Macrolides (Tylosin and Erythromycin) to be the most potent and Amphenicols (Chloramphenicol) to be the least effective in vitro, by micro-broth dilution method. Our findings were also in agreement with Bebear *C. et al.* (1993), who could observe that Macrolides and tetracyclines were the most active antibiotics against *M. pneumoniae* in vitro.

SUMMARY

Erythromycin and Tylosin (Macrolides) followed by Tetracycline and Doxycycline (Tetracyclines) are found to be the best drugs of choice, under *in-vitro* antibiotic sensitivity test conducted with Disc diffusion method for the 10 Indian *Mmc* isolates recovered from goats. These relevant antibiotics may be recommended for use under field conditions. Although we have to keep in mind the gene exchange in *Mollicutes* along with other mechanisms, which may lead to the variation in their sensitivity to antibiotics in coming time.

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