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RESEARCH ARTICLE

In vitro Quinolones Susceptibility Analysis of Chinese *Mycoplasma bovis* Isolates and their Phylogenetic Scenarios based upon QRDRs of DNA Topoisomerases Revealing a Unique Transition in ParC

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ABSTRACT

Mycoplasma bovis can cause different systemic problems in cattle, and recently has been resulted in huge economic losses in China. *In vitro* susceptibilities of 26 twice sub-cultured Chinese *M. bovis* field isolates were determined at physiological pH including PG45 through broth micro-dilution method. Except Huanggang isolate, all isolates and PG45 were in the sensitive range for levofloxacin, lomefloxacin and ciprofloxacin, whereas, for norfloxacin and nalidixic acid, they had shown intermediate resistant and complete resistant patterns, respectively. The multiple sequence analysis revealed point mutations in QRDRs of *gyrA* and *parC* genes of Huanggang isolate resulting in amino acid substitutions at positions 83 (S-F) in GyrA (*E. coli* numbering) and 80 (S-I) in ParC proteins, the latter is reported for first time in *M. bovis*. Conclusively, fluoroquinolones are the potential veterinary therapeutic agents for mycoplasmosis in China and resistance to these agents comes through point mutations in QRDRs of g*yrA* and *parC* genes with ParC and GyrA mutation orientation.

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INTRODUCTION

Mycoplasma species are the smallest self-replicating organisms that lack cell wall. Among mycoplasma, mainly *Mycoplasma bovis* (*M. bovis)* is primarily involved in bovine pneumonia, mastitis, arthritis, otitis, meningitis, infertility and abortion leading to worldly massive economic losses for cattle industry (Pfützner, 1990; Nicholas and Ayling, 2003; Arcangioli *et al.,* 2012). It has also horizontal transmission ability in cattle herds with the highest sero-prevalence in May, June and August (Nicholas *et al.,* 2008).

The intrinsic resistance to β-lactamases, while acquired resistance to sulphonamides and aminogly-cosides had resulted in the clinical in-effectiveness of chemotherapy guidelines. *M. bovis* has also ability to disseminate extensively in the host that further limits their effective chemotherapy. Furthermore, biofilm production by mycoplasma may enable their survival in the host reducing their antimicrobial susceptibility (Kornspan *et al.,* 2011).

Due to the lull of effective available vaccine for *M. bovis* control, its prophylaxis is exclusively reliant on first-rate husbandry practices and chemotherapy. Following *M. bovis* isolation in 1961 from USA, its worldwide spread to many countries comes through animal movements. In China, *M. bovis* was first reported by this laboratory in 2008 (Lei *et al.,* 2008).

In vitro antimicrobial susceptibilities of *M. bovis* had already been reported from other countries (Nicholas *et al.,* 2008) but nothing was reported about the Chinese *M. bovis* strains. Since China imports animals from different countries, so, it was quite worthy to know the antimicrobial susceptibilities of Chinese *M. bovis* isolates.

Because quinolones were the most commonly used veterinary chemotherapeutic agents in China against mycoplasmosis and other infectious diseases, we decided to test their susceptibilities. We have determined the

minimum inhibitory concentrations (MICs) of *M. bovis* isolates and reference type strain *Mycoplasma bovis* ATCC® 25523™ (PG45), against five quinolones through broth micro-dilution susceptibility method. Following scrutinization, quinolones-resistant determining regions (QRDRs) of *gyrA, gyrB, parC* and *parE* genes of these isolates were amplified and mutation evidence was measured by multiple sequence alignments (MSA) and phylogeny of their respective proteins.

MATERIALS AND METHODS

Culture of *M. bovis*: *M. bovis* isolates (n=26) from different beef and dairy farms were collected from 2008 to 2011. Isolates as *M. bovis* were confirmed by PCR using primers to *uvrC* gene and 16s rRNA as previously reported (Lei *et al.,* 2008). The PCR products were custom-sequenced. Following confirmation, the isolates were stored at -70ºC.

The test cultures were propagated in PPLO broth, supplemented with 0.5% (w/v) sodium pyruvate and 0.09% (w/v) yeast extract, while 0.004% (w/v) phenol red, 1% (v/v) 10× minimum essential medium (MEM), 20% (v/v) donor equine serum and 80,000 U of penicillin G/100 mL of medium after autoclavation, acquiring a final pH of 7.3. The measurement of color changing unit (ccu) was executed through viable count method for liquid MIC assay (Hannan, 2000). Before the conduction of susceptibility test for any drug, stock culture aliquots from each isolate were sub-cultured twice in PPLO broth (10% v/v) to log phase (24 h incubation, acquiring 10^9-10^{10} ccu/mL), whereas by third term log phase, susceptibility test was performed. The end-points were read as red to yellow/orange color changes in test wells.

Testing of the PG45 had allowed a direct comparison between the MIC values obtained in this study and from previous reports (Hannan *et al.,* 1997). To ensure the reproducibility and validity, PG45 was incorporated in the first row of each tray. MIC value of PG45 had already been reported for one of the presently used agent's chemical analogue (Hannan, 2000).

Antimicrobial agents and dilution ranges: The agents were nalidixic acid, ciprofloxacin, levofloxacin, lomefloxacin and norfloxacin. To have the comparable results, the un-sterilized stock solutions of 1 mg/mL were prepared on the antimicrobial base basis using correction factors (Hannan, 2000) and according to CLSI guidelines (Anonymous, 2010). The stock solutions were stored at -70ºC, while the working solutions were stored at 4ºC. The two fold serial dilutions ranging from 128 to 0.25 µg/mL for nalidixic acid, 64 to 0.125 µg/mL for norfloxacin and 16 to 0.03 µg/mL for ciprofloxacin, levofloxacin and lomefloxacin were used.

Broth micro-dilution susceptibility method: The adhoc working group of international research program on comparative mycoplasmology (IRPCM) had proposed serial broth dilution method as the most useful and reproducible assay (Ter Laak *et al.,* 1993). The broth microtiter trays' layout was like so; columns 1 to 9 contained 100 µL/well of broth with antimicrobial agent (Test wells), column 10 contained 100 µL/well of broth without antimicrobial agent (Growth control), columns 11 and 12 contained 200 µL/well pH adjusted broth (pH 6.8, pH control) and standard broth medium (Sterility control), respectively. The columns from 1 to 9 were inoculated with 100 µL/well of ccu adjusted mycoplasma inocula (10^4-10^5 ccu/mL). The column 10 was inoculated with 100 µL/well of 24 h incubated log phase broth culture (10^9-10^{10} ccu/mL) of each clinical isolate from which ccu dilutions were prepared. The trays were sealed with adhesive tape and incubated aerobically at 37ºC.

Interpretation of results: Since there were no specific test guidelines regarding susceptibility analysis for mycoplasmas from CLSI, MICs break-points of the tested quinolones were based on their CLSI break-points for other common bacteria (Anonymous, 2010; Soehnlen *et al.,* 2011). The trays were examined periodically until the broth color in the column No. 10 first/just matched or exceeded to that of the column No. 11 and MICs values at this change were recorded as initial MICs, which were on day 2 of incubation assuming the lowest concentration of antimicrobial agent that showed no color change when the color change in growth control well was equal to pH control well and to ensure any trailing effect of antimicrobial agents, final MICs, when there was no further color change for upto at least two days, were recorded on day 7 of incubation (Hannan, 2000).

Tests were performed in duplicate pattern and parallely to evaluate the effect of solvents and diluent, separate broth samples were prepared without antimicrobial agent but containing the same volumes of solvents and diluent those had already been used. These taped trays were also incubated under the same conditions of temperature and air.

Amplification of QRDRs, detection of point mutation and phylogeny: Genomic DNA was extracted from 1 mL log phase broth cultures by boiling at 100ºC for 10 min, followed by 1 min thawing and finally centrifugation for 10 sec to get it in the supernatant. The sequencing was performed at two different DNA sequencing units, Sangon® Biotech (Shanghai) Co Ltd and Beijing Genomics Institute® using ABI-PRISM3730 sequencing reagent for Big Dye TerminatorV3.1. The translations of the sequence results were performed using NCBI's DNA & RNA tool available at (http://www.ncbi.nlm.nih.gov /gorf/gorf.html). Multiple Sequence alignments of the isolates' proteins were done to find out mutation evidence by ClustalX2.0.12 (http://www.clustal.org/). Phylogenetic neighbor joining (N-J) trees were also constructed by the same software.

The susceptibility status of isolates for different drugs was statistically analyzed by T-test analysis. The isolates were classified as susceptible (sensitive) and non-susceptible (intermediate and resistant) by comparison of the MIC to CLSI breakpoints. Differences were considered statistically significant when $P<0.05$.

RESULTS

Confirmation of the isolates: The isolates as *M. bovis* were confirmed by PCR through *uvrC*'s 238 bp

amplicons. For 16S rRNA, sequence alignment for these PCR products revealed 99-100% similarity.

Calibration of the drug susceptibility test: These susceptibility results were exclusively of the quinolones because the trays inoculated with quinolone-free broth but containing the solvents and diluent as same as had previously been used, showed color change in all twofold serially diluted test wells. The validity of the currently executed method was assessed by the PG45, as its MIC value for one of the presently used fluoroquinolone, ciprofloxacin was 1 µg/mL that was comparable with the earlier report (1-2 µg/mL) using the same method (Hannan, 2000).

Susceptibility of *M. bovis* isolates: *In vitro* susceptibility analysis is presented in Table 1. All isolates and PG45 grew against the highest used concentration of nalidixic acid, so, isolates and PG45 were measured resistant to it. The isolate with Ser. No. 4 from Huanggang, rendered complete resistance to all the tested quinolones and was excluded from susceptibility result interpretation.

For ciprofloxacin, 96% isolates remained "Sensitive" while 4% were measured "Intermediate" (moderately sensitive). MIC value of 1 µg/mL for PG45 was also in the sensitive range. For levofloxacin all the isolates were measured "Sensitive" alongwith PG45 because according to author's best of knowledge, its MIC break-points have yet not been standardized for this agent. For lomefloxacin, isolates and PG45 were measured "Sensitive". There were also no MIC break-points reported for PG45 against lomefloxacin. For norfloxacin, 76 and 24% isolates were remained "Intermediate" and "Resistant" respectively. The PG45 was considered "Intermediate" to norfloxacin.

Evidence for point mutations in QRDRs and phylogeny: The PCRs performed using regions specific primers confirmed the amplification of QRDRs by producing the amplicons of 531, 555, 488 and 502 bp for *gyrA, gyrB, parC* and *parE* genes, respectively. The nucleotide sequences of *gyrA* were same in all the isolates except one (Huanggang). It had a single substitution of codon's second nucleotide "C" at position 2381 {*E. coli* numbering, reverse complementary (RC)}, 449 (PG45 numbering) and 2312 (*M. bovis* Hubei-1 and *M. bovis* HB0801 numbering, RC) by "T" resulting in amino acid change in GyrA protein from "S-F" at position 83 (*E. coli* numbering) and 150 (PG45, *M. bovis* Hubei-1 and *M. bovis* HB0801 numbering). About *gyrB,* no change was observed in QRDRs of isolates except for a non-QRDR transition in one isolate (Ser. No. 23); Where "G" had been substituted by "A" nucleotide that resulted in "G-D" amino acid shift at position 475 (*E. coli* numbering) and 496 (PG45, *M. bovis* Hubei-1 and *M. bovis* HB0801 numbering) in GyrB protein. This change did not affect the MICs of this isolate.

Comparison of the ParC QRDRs revealed a substitution of an amino acid "S-I" at position 80 (*E. coli* numbering), which corresponds to position 91 (PG45, *M. bovis* Hubei-1 and *M. bovis* HB0801 numbering), resulting from the point mutation of "G-T" nucleotide at position 2021 (*E. coli* numbering), 2315 (PG45 numbering) and 272 (*M. bovis* Hubei-1 and *M. bovis* HB0801 numbering) of *parC*. No difference was found in QRDRs of *parE* and its coordinate protein. In both QRDR mutations, a polar amino acid lying between acidic and polar amino acids, has been replaced by a non-polar amino acid (Table 2).

To find out the phylogenetic evidence for susceptibility criteria of isolates, phylogenetic N-J trees

Table 1: Initial (day 2) and final (day 7) MICs for used quinolones

Isolates	MICs (µg/mL)								
	NAL	CIP		LVX		LOM		NOR	
	F	I[a]	F[b]	I	F	I	F	I	F
Yanling	>128	1	1	1	1	2	2	8	16
Yuliang 2086	>128	0.5	1	0.5	0.5	2	2	4	8
Jingshan 1015	>128	1	1	0.5	1	2	2	8	8
Huanggang	>128	>16	>16	>16	> 16	>16	>16	>64	>64
NNH	>128	1	1	1	1	2	2	8	16
Yanling 0724	>128	1	1	1	1	2	2	8	8
Yanjin 0719	>128	0.5	1	1	1	2	2	8	16
Fangxian	>128	1	1	1	2	2	2	8	16
Jiang xixinyu	>128	0.5	0.5	0.5	0.5	1	2	4	8
Kaifeng	>128	0.5	0.5	1	1	1	2	8	8
Lianjiang 1225	>128	0.5	1	1	1	1	2	8	16
Xiamen	>128	1	1	0.5	1	1	2	4	8
Xiantao	>128	2	2	1	2	2	2	8	8
Suizhou	>128	1	1	1	1	1	2	4	8
Xinzhou-2	>128	0.5	1	1	1	2	2	8	8
Xinzhou-1	>128	1	1	0.5	1	2	2	8	16
Yingcheng HB0801	>128	1	1	1	1	2	2	8	8
Zhongxiang	>128	0.25	0.5	0.5	1	1	2	4	8
Ezhou-8	>128	0.25	0.5	0.5	1	1	2	8	8
Daye	>128	1	1	1	1	1	2	4	8
Ezhou-3	>128	0.5	1	0.5	1	1	2	4	8
Bozhou	>128	0.5	1	1	1	1	2	8	8
Ezhou-2	>128	1	1	1	1	2	2	4	8
Zhuma dian	>128	1	1	1	1	1	2	4	8
LLLQ	>128	1	1	1	1	1	2	4	8
Ezhou	>128	0.5	1	1	1	2	2	8	8
PG45	>128	1	1	0.5	1	1	2	8	8

Notes: [a]Initial; [b]Final; Nalidixic Acid (NAL); Ciprofloxacin (CIP); Levofloxacin (LVX); Lomefloxacin (LOM) and Norfloxacin (NOR); According to T-test statistical analysis, the isolates from all the provinces were sensitive to all the drugs except nalidixic acid.

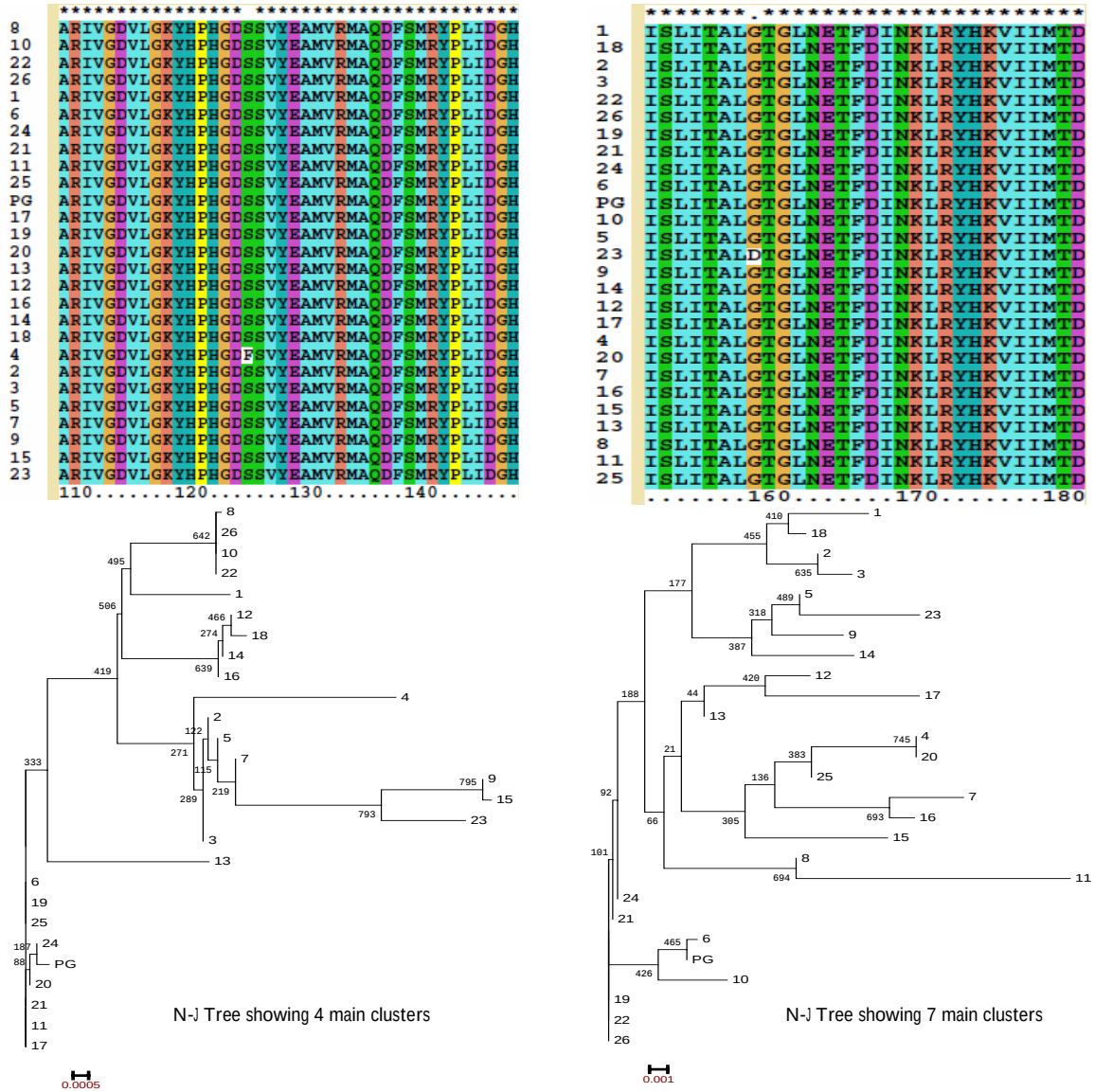

Fig. 1: MSA and Phylogeny for GyrA and GyrB of *M. bovis* isolates and PG45. *numbers on the left side are isolates Ser. No.

were constructed for proteins (Fig. 1 and 2). From results, it was obvious that resistant isolate did not fall in any of the four main clusters of GyrA. The analysis of phylogenetic tree for ParC pointed out that all the isolates those gave intermediate susceptibilities to norfloxacin fall in cluster E, except the isolates with Ser. No. 5 and 1. There was no information from GyrB and ParE phylogeny, harboring no change in QRDRs of isolates. Statistically no influence of geographic origin of isolates on susceptibility status was observed but there was significant difference among susceptible and non-susceptible isolates for all the used drugs from all the provinces.

DISCUSSION

Our study has obviously oriented the inevitability for periodic quinolones susceptibility testing as the source for coherent chemotherapy against bovine mycoplasmosis that is responsible for huge economic losses due to the non-provision of effective vaccine. This research work has also produced the first in vitro susceptibility report for Chinese *M. bovis* strains against quinolones. Two of the China's isolated *M. bovis* strains (Hubei-1 and HB0801) from same province have been completely sequenced (Li *et al.*, 2011; Qi *et al.*, 2012); the later has been used in our study (Table 1, Ser. No. 17). The replicate's results were accepted when they were identical or differ by a single two fold serial dilution between initial and final MICs (Qaiyumi, 2007).

Table 2: Most common position of QRDRs proteins transition in mycoplasmas

Reference	Species	GyrA	ParC
		83*	80
Current study	*M. bovis* Huanggang isolate	S-F	S-I
Lysnyansky *et al.* (2009)	*M. bovis*	S-F	-
Gruson *et al.* (2005)	*M. hominis*	S-L	S-I
Bébéar *et al.* (1998)	*M. hominis* PG21	S-L/W	S-I
Li *et al.* (2012)	*M. gallisepticum*	S-I	S-L
Reinhardt *et al.* (2002)	*M. gallisepticum* MG ATCC15302	S-R	S-L
Shimada *et al.* (2010)	*M. ganitalium*	-	S-N
Vicca *et al.* (2007)	*M. hyopneumoniae*	A-V	S-Y
Hirose *et al.* (2004)	*M. bovirhinis*	-	S-L

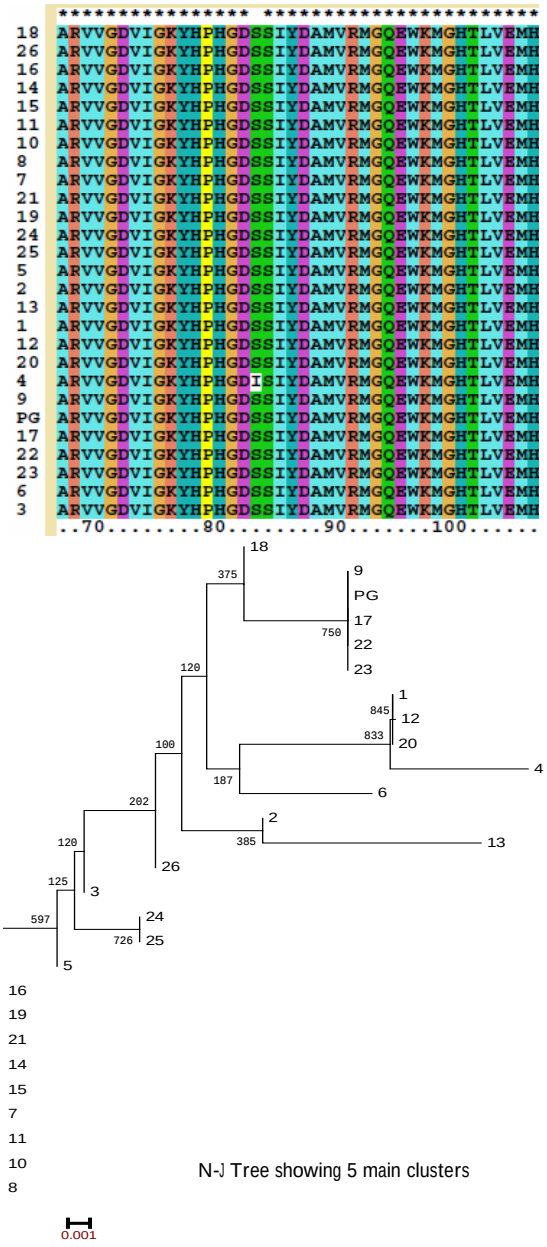

Fig. 2: MSA and Phylogeny for ParC of *M. bovis* isolates and PG45

We had read the final MICs after 7 days to seek out any trailing effect of these bactericidal agents by re-incubating the trays after taking initial MICs on day 2. But the other authors read these after 2-3, 3, 4, 5, 6 or 7 days, except Hannan (2000) who had taken the final MIC values after day 7 and also yielded the MIC value for PG45, none of others had reported the MICs values of PG45 against any of the presently used quinolones.

The antimicrobial susceptibilities were obtained at physiological pH of 7.3 in this study to reflect physiological conditions rather than usually used 7.6-7.8. Thus, the *in vitro* results were obtained at *in vivo* condition of pH, which might be clinically more applicable and accurate.

The practice of using a frozen culture of a previously determined titer as an inoculum was having pitfalls like loss of viability during storage or thawing. Even if this does not occur, the lag phase of thawed cultures is likely to be prolonged over that of a fresh culture and MICs could be falsely elevated. To eliminate such factors along with the effect of cryoprotectant, the isolates were sub-cultured to log phase twice and by third sub-culturing, susceptibility test was performed. The sub-culturing with 10% v/v was done as larger sampling volume provides a greater number of colonies, enhancing the test precision (Donna *et al.,* 1999).

The MIC_{50}s and MIC_{90}s values of lomefloxacin for Chinese *M. bovis* differ from corresponding values of *M. pneumoniae* and *M. hominis*. For Chinese *M. bovis*, they were same i.e. 2 µg/mL but for *M. pneumoniae* and *M. hominis,* they were within accepted difference of single two fold dilution (Renaudin and Bébéar, 1990).

The Huanggang isolate mutation in GyrA at position 83 (S-F) was the most common coordinate for amino acid transition in mycoplasma. Above all, the ParC mutation in Huanggang isolate at position 80 (S-I) is reported for first time for *M. bovis* strain in this study, although this mutation had been reported in other species of genus mycoplasma. In *M. hyopneumoniae*, mutation at the same coordinate gave low-level resistance, while ParC and GyrA mutation combination resulted in high-level resistance. For *M. bovis*, mutation in GyrA was considered to be responsible for intermediate resistance (Lysnyansky *et al.,* 2009). But the N-J tree for GyrA protein of Chinese isolates did not provide any phylogenetic evidence for this consideration, rather it showed that the intermediate resistant isolates were falling in different cluster and none of them was accompanying resistant Huanggang isolate. On the other hand, if we analyze the N-J tree for ParC, it was quite realistic and also followed the target specificity like other mycoplasmas, that all the intermediate resistant isolates were in one cluster except 2 isolates (Ser. No. 1 and 5). Isolate No. 5 was the closest neighbor to this cluster, while isolate No. 1 was lying in the same cluster that for Huanggang isolate. Except for a silent mutation in non-QRDR, no transition was observed in GyrB, which was in apposition to *M. hominis*, *M. hyopneumoniae, U. urealyticum and A. laidlawii* but in opposition to *M. gallisepticum*. No mutation was seen in ParE as for *M. hyopneumoniae* and *M. hominis*, whereas *M. gallisepticum* has mutation in this protein (Carrou *et al.,* 2006).

In China, use of quinolone drugs is very common which is even indicated from very high rate of their resistance, reaching even upto 100% in some areas (Wang *et al.,* 2006). This is also positively supported from our study, where Huanggang isolate has shown resistance to all of the tested quinolones. Furthermore, this is an alarming indication that the number of resistant isolates is increasing as evident from *M. bovis* isolates' and PG45's resistances to nalidixic acid, norfloxacin and ciprofloxacin in different amplitudes.

Conclusion: The broth micro-dilution susceptibility method has been successfully executed for the determination of quinolones MICs against *M. bovis* field isolates and PG45. Huanggang isolate remained resistant/ mutant through broth micro-dilution method, sequencing of QRDRs of *gyrA* and *parC* genes and phylogeny. These

facts also provide the authenticity to our designed susceptibility methodology. Levofloxacin, lomefloxacin and ciprofloxacin remained the most effective *in vitro* fluoroquinolones for mycoplasmosis. The development of resistance to quinolones was related to point mutations in QRDRs of target genes following the mutation trend from ParC to GyrA.

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REFERENCES

Anonymous, 2010. Performance Standards for Antimicrobial Susceptibility Testing; Twentieth Informational Supplement. CLSI document M100-S20. Clinical and Laboratory Standards Institute; Wayne, PA, USA.

Arcangioli MA, H Aslan, F Tardy, F Poumarat and D Le Grand, 2012. The use of pulsed-field gel electrophoresis to investigate the epidemiology of *Mycoplasma bovis* in French calf feedlots. Vet J, 192: 96-100.

Bébéar CM, H Renaudin, A Boudjadja and C Bébéar, 1998. In vitro activity of BAY 12-8039, a new fluoroquinolone, against mycoplasmas. Antimicrob Agents Chemother, 42: 703-704.

Carrou JL, KA Reinhardt, I Kempf and AV Gautier-Bouchardon, 2006. Persistence of *Mycoplasma synoviae* in hens after two enrofloxacin treatments and detection of mutations in the *parC* gene. Vet Res, 37: 145-154.

Donna MH, CD Dana and RP Lance, 1999. Highly reproducible bactericidal activity test results by using a modified national committee for clinical laboratory standards broth macrodilution technique. J Clin Microbiol, 37: 1881-1884.

Gruson D, S Pereyre, H Renaudin, A Charron, C Bébéar and CM Bébéar, 2005. In vitro development of resistance to six and four fluoroquinolones in *Mycoplasma pneumoniae* and *Mycoplasma hominis*, respectively. Antimicrob Agents Chemother, 49: 1190-1193.

Hannan PC, GD Windsor, Ade Jong, N Schmeer and M Stegemann, 1997. Comparative susceptibilities of various animal-pathogenic mycoplasmas to fluoroquinolones. Antimicrob Agents Chemother, 41: 2037-2040.

Hannan PCT, 2000. Guidelines and recommendations for antimicrobial minimum inhibition concentration (MIC) testing against veterinary mycoplasma species. Vet Res, 31: 373-395.

Hirose K, Y Kawasaki, K Kotani, K Abiko and H Sato, 2004. Characterization of a point mutation in the *parC* gene of *Mycoplasma bovirhinis* associated with fluoroquinolone resistance. J Vet Med, 51: 169-175.

Kornspan JD, M Tarshis and S Rottem, 2011. Adhesion and biofilm formation of *Mycoplasma pneumonia* on an abiotic surface. Arch Microbiol, 193: 833-836.

Lei S, G Rui, Y Zheng-yan, Z Yong, P Jie, H Zhi-bin, W Li-xia, H Chang-min, L Tao, C Ying-yu, L Juan-hong, Z Jun-long, C Huan-chun and G Ai-zhen, 2008. Diagnosis of cattle infectious *Mycoplasma bovis* pneumonia. J Huazhong Agric Univ, 27: 629-633.

Li J, DH Lu, ZJ Liu, XH Zhang, FL Wei, YH Liu and HX Jiang, 2012. Role of mutation in DNA gyrase and topoisomerase iv in fluoroquinolones-resistance of *Mycoplasma gallisepticum* obtained in vitro and in vivo. J Anim Vet Adv, 11: 2327-2332.

Li Y, H Zheng, Y Liu, Y Jiang, J Xin, W Chen and Z Song, 2011. The complete genome sequence of *Mycoplasma bovis* strain Hubei-1. PLoS ONE 6: e20999. doi:10.1371/journal.pone.0020999.

Lysnyansky I, I Mikula, I Gerchman and S Levisohn, 2009. Rapid detection of a point mutation in the *parC* gene associated with decreased susceptibility to fluoroquinolones in *Mycoplasma bovis*. Antimicrob Agents Chemother, 53: 4911-4914.

Nicholas R, R Ayling and L McAuliffe, 2008. Bovine Respiratory Disease. In: Mycoplasma Diseases of Ruminants. (Nicholas R, R Ayling and L McAuliffe, eds): Veterinary Laboratory Agency, CABI, UK; pp: 135, 139, 153 & 154.

Nicholas RAJ and RD Ayling, 2003. *Mycoplasma bovis*: disease, diagnosis and control. Res Vet Sci, 74: 105-12.

Pfützner H, 1990. Epizootiology of the *Mycoplasma bovis* infection of cattle. Zentralblatt fur Bakteriologie Supplement, 20: 394-399.

Qaiyumi S, 2007. Macro- and Micro-dilution Methods of Antimicrobial Susceptibility Testing. In: Antimicrobial Susceptibility Testing Protocols. (Schwalbe R, Steele-Moore L, Goodwin AC, eds): CRC Press: Taylor & Francis Group, pp: 76.

Qi J, A Guo, C Peng, Y Chen, R Mustafa, X Ba, C Hu, Z Bai, X Chen, L Shi and H Chen, 2012. Comparative Geno-Plasticity Analysis of Mycoplasma bovis HB0801 (Chinese Isolate). PLoS ONE 7: e38239. doi:10.1371/journal.pone.0038239.

Reinhardt AK, CM Bébéar, M Kobisch, I Kempf and AV Gautier-Bouchardon, 2002. Characterization of mutations in DNA gyrase and topoisomerase IV involved in quinolone resistance of *Mycoplasma gallisepticum* mutants obtained in vitro. Antimicrob Agents Chemother, 46: 590-593.

Renaudin H and C Bébéar, 1990. Comparative *in Vitro* Activity of Azithromycin, Clarithromycin, Erythromycin and Lomefloxacin against *Mycoplasma pneumoniae*, *Mycoplasma hominis* and *Ureaplasma urealyticum*. New Antimicrobial Agents. Eur J Clin Microbiol Infect Dis, 9: 838-841.

Shimada Y, T Deguchi, K Nakane, T Masue, M Yasuda, S Yokoi, S Ito, M Nakano, S Ito and H Ishiko, 2010. Emergence of clinical strains of *Mycoplasma genitalium* harbouring alterations in ParC associated with fluoroquinolone resistance. Int J Antimicrob Agents, 36: 255-258.

Soehnlen MK, ME Kunze, KE Karunathilake, BM Henwood, S Kariyawasam, DR Wolfgang and BM Jayarao, 2011. In vitro antimicrobial inhibition of *Mycoplasma bovis* isolates submitted to the Pennsylvania Animal Diagnostic Laboratory using flow cytometry and a broth microdilution method. J Vet Diagn Invest, 23: 547-551.

Ter Laak EA, JH Noordergraaf and MH Verschure, 1993. Susceptibilities of *Mycoplasma bovis*, *Mycoplasma dispar* and *Ureaplasma diversum* strains to antimicrobial agents in vitro. Antimicrob Agents Chemother, 37: 317-321.

Vicca J, D Maes, T Stakenborg, P Butaye, F Minion, J Peeters, ADe Kruif, A Decostere and F Haesebrouck, 2007. Resistance mechanism against fluoroquinolones in *Mycoplasma hyopneumoniae* field isolates. Microb Drug Resist, 13: 166-170.

Wang B, JS Xu, CX Wang, ZH Mi, YP Pu, M Hui, TK Ling and CY Chan, 2006. Antimicrobial susceptibility of *Neisseria gonorrhoeae* isolated in Jiang su Province, China, with a focus on fluoroquinolone resistance. J Med Microbiol, 55: 1251-1255.