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Antimicrobial Susceptibility Testing of *Mycoplasma bovis* Isolated from Dairy Cows in Ningxia Hui Autonomous Region, Province of China

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Abstract: The susceptibilities of 13 recent field isolates of *Mycoplasma bovis* from the Ningxia province of China to 13 antimicrobial agents were detected using a microbroth dilution method. The rates of resistance to gentamicin, tilmicosin, erythromycin, lincomycin were 8/13, 7/13, 12/13 and 8/13, respectively according to the CLSI resistance breakpoint. Less than 50% of strains were resistance to ciprofloxacin, doxycycline and florfenicol with the resistance rates of 4/13 and 3/13, respectively. The lower resistance rates were observed for tiamulin (0/13), spectinomycin (1/13), enrofloxacin (2/13) and ofloxacin (2/13) suggesting the higher susceptibility of tiamulin, spectinomycin, enrofloxacin and ofloxacin to the *M. bovis*.

Key words: *Mycoplasma bovis*, antimicrobial susceptibility, minimum inhibitory concentration, mycoplasma culture, China

INTRODUCTION

Among the pathogenic mycoplasma that have been isolated from cattle, *Mycoplasma bovis* (*M. bovis*) is the most pathogenic agent which causes mastitis in dairy cattle, respiratory tract diseases in calves and arthritis in all age groups of cattle except for the *Mycoplasma mycoides* sub sp. *mycoides* small-colony type (Arcangioli *et al*., 2008). Since, *M. bovis* was first isolated in USA cattle during a severe case of mastitis, its infection has spread worldwide inducing considerable economic losses in milk and meat production. In natural, *M. bovis* usually coexists with other bacteria and virus, even as a primary disease to be responsible for high infection rate in Europe or the United States (Nicholas and Ayling, 2003).

In china, *M. bovis* was firstly isolated from a case of pneumonia in beef cattle and reported by Xin *et al*. (2008). Since, then it has spread to >11 Chinese provinces. For all this, little is reported in dairy cows. By 2010, more strains were isolated from the cases of dairy mastitis in many provinces of China in particular a latest surveillance of seroprevalence of *Mycoplasma bovis* infection in dairy cows demonstrating the infection rate is up to 20% (Xu *et al*., 2012). Because there have no effective vaccine applying in China, antimicrobial agents are still considered one of the optimal choices to early control mycoplasmal infectious mastitis in cattle. However, the use of antimicrobials is suggested to create a positive selective pressure on the development of resistance in species of mastitis pathogens (Barlow, 2011).

Although, some documents have already reported the *in vitro* antimicrobial activity and inhibition profiles of certain antimicrobial agents against *M. bovis* isolates in Japan, Europe and the United States using different methods (Thomas *et al*., 2003; Soehnlen *et al*., 2011; Francoz *et al*., 2005; Gerchman *et al*., 2009; Uemura *et al*., 2010), few were conducted to Chinese *M. bovis* clinical isolates. In the present study, 13 *M. bovis* isolated strains were cultured and identified from the Ningxia Hui Autonomous Region of China and then subjected to evaluate the *in vitro* susceptibility by using a microbroth dilution method to several antibiotics which are commonly used in treatment and control of mycoplasmas.

MATERIALS AND METHODS

Samples collection: A total of 186 milk samples were collected aseptically from dairy cows with cases of mastitis from several scale farms and the individual farms during the 2010 to 2011 period. The farms are all located in the four different prefectures in Ningxia Hui Autonomous Region, China because most of the large-scale dairy farms and individual farms are concentrated in the four districts. Only one isolate per cow was included. Each milk sample was then subjected to detected *M. bovis* specific antibody

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using the *Mycoplasma bovis* Test kit (ELISA) (Insert/Product No. B021, Canada) following the manufacturer's instructions before submitting to the laboratory.

Mycoplasma culture: After filtered by 0.45 μm filtration, each antibody positive milk sample was diluted 10 fold and then incubated in modified PPLO broth (Difco) medium containing 20% heat inactivated horse serum, 10% fresh yeast extract, 0.5% glucose and supplemented with sodium pyruvate with the final pH adjusted to 7.6. The broth medium was incubated at 37°C in 5% CO_2 until the color changed. Three passages were performed and were then purified on agar plates. Colonies with typical *M. bovis* morphology were plugged into broth medium. Species identification was simultaneously confirmed by standard mycoplasma identification techniques (Poveda, 1998) and by PCR (Subramaniam *et al*., 1998). Finally, aliquots of pure culture were prepared and held at -70°C until required.

Antimicrobial agents: The thirteen antimicrobial agents used in this study is as following: Tiamulin hydrogen fumarate (K0340808), Erythromycin (K0120906), Lincomycin hydrochloride (K0101003), Enrofloxacin (H0080904), Ciprofloxacin Lactate (H0141009), Gentamicin (K0070806), Neomycin sulfate (K0090911), Spectinomycin hydrochloride (K0260907), Tylosin tartrate (K0160911), Tilmicosin (K0310711), Florfenicol (K0301004), Doxycycline Hyclate (K0131001) and Ofloxacin (H0090912). Stock solutions of each of the antimicrobials were prepared with appropriate solvents according to the manufacturers' recommendations and were sterilized by membrane filtration (0.22 mm pore size) and then were stored in onetime-use aliquots at -70°C.

The antimicrobial susceptibility tests: The *in vitro* Minimum Inhibition Concentration (MICs) of thirteen antimicrobial agents for the thirteen *M. bovis* isolates were performed using a microbroth dilution method and essentially following the guidelines of Hannan (2000). In briefly, the drug susceptibility assay was carried out in the 96 well microtiter plate format. Each antimicrobial agent was serially diluted two fold in culture broth in 15 wells of a microtiter plate; each well contained 100 μL then the thawed cultures diluted containing 10^4/0.2 mL Color-Changing Units (CCU) 100 μL was added to each well. The tested concentration range of each drug was 0.03-128 μg mL^{-1}. A control with no antimicrobial agent was included. The plates were sealed and incubated at 37°C. The MIC was defined as the lowest concentration in which there was no bacterial growth as evidenced by a lack of pH color change at the time the drug-free growth control showed a color change. Each strain was tested in duplicate at least three times for each antimicrobial on different days.

RESULTS AND DISCUSSION

Mycoplasma bovis is a complex pathogen in cattle, its infection is responsible for a high proportion of the chronic and debilitating disease. Early intervention with antibiotic therapy is critical for successful treatment and control *M. bovis* spread (Caswell and Archambault, 2007). It is demonstrated that *Mycoplasma bovis* infection exists currently in most of the local cattle farms. And *M. bovis* related epidemiological information is not complete in China. Herein, researchers isolated this species from milk samples in Ningxia Hui Autonomous region, China, for the first time. For the 186 milk samples, antibodies to *M. bovis* were found in 42 isolates yielding the relatively higher isolation rate (22.5%). This result further indicates that the position of *M. bovis* in the cow mastitis pathogenic process is of vital important. Of this 42 milk samples, a total of 13 isolates were obtained by culture.

At present, no established breakpoints available for *Mycoplasma bovis*, the Clinical and Laboratory Standards Institute (CLSI) criteria for veterinary pathogenic bacteria in cattle were used to interpret the MIC results (NCCLS, 2002). Especially when the tested MIC values are significantly higher than those established by CLSI, the antibiotics are regarded as resistance (Soehnlen *et al*., 2011). As observed in Table 1, among the thirteen *Mycoplasma bovis* isolates, the rates of resistance to

Table 1: MIC50s, MIC90s and MIC ranges of the antimicrobial agents (μg mL^{-1}) and the rates of resistance of 13 *M. bovis* isolates

Antimicrobial agents	MIC50	MIC90	MIC range	I	R
Fluoroquinolones					
Enrofloxacin	2	4	0.25-16	2/13	>2
Ofloxacin	4	8	0.5-32	2/13	>8
Ciprofloxacin	4	16	0.5-32	4/13	>4
Aminoglycosides					
Spectinomycin	16	128	4 to >128	1/13	>128
Neomycin	128	>128	32 to >128		*
Gentamicin	128	>128	2 to >128	8/13	>16
Macrolides					
Tylosin	8	16	0.5-64		*
Tilmicosin	128	>128	2 to >128	7/13	>32
Erythromycin	32	>128	8 to >128	12/13	>8
Lincosamines					
Lincomycin	32	128	0.0625-128	8/13	>4
Chloramphenicol					
Florfenicol	8	64	1-64	3/13	>32
Tetracyclines					
Doxycycline	16	32	0.25-64	4/13	>16
Pleuromutilin					
Tiamulin	2	8	0.06-16	0/13	>32

*No defined breakpoints; R: MIC breakpoints (resistance) used for susceptibility determination according to NCCLS recommendations for other bovine pathogens; I: The ratio of 13 isolates which MIC is above the NCCLS recommendations MIC breakpoints (resistance)

gentamicin, tilmicosin, erythromycin, lincomycin were 8/13, 7/13, 12/13 and 8/13, respectively. Less than 50% of strains were resistance to ciprofloxacin, doxycycline and florfenicol with the resistance rates of 4/13 and 3/13. The lower resistance rates were observed for tiamulin (0/13), spectinomycin (1/13), enrofloxacin (2/13) and ofloxacin (2/13).

Macrolides is a class of antimicrobials which bind to the ribosomal subunit to inhibit the bacterial protein synthesis commonly used for the treatment of Mycoplasma infections. The acquired resistance has been reported everywhere for human or avian mycoplasmal pathogens as observed in the study. The MIC values (>128 μg mL^{-1}) for erythromycin is far more than those required breakpoint resistance (32 μg mL^{-1}), showing higher resistance consistent with Francoz report using the E test method Francoz *et al.* (2005). Meanwhile, the significantly high MIC90 (16 μg mL^{-1}) of tylosin shows less susceptibility to the currently isolated *M. bovis* strains. In addition, a bimodal distribution of MICs values for tylosin and lincomycin (lincosamine class have the same modes of antimicrobial action with macrolides) as well as the higher MICs for tilmicosin than for tylosin was seen consistent with those described in the Israel and the United State reports. It is noted that the MICs values for macrolides in current study is slightly higher than those earlier reported by others (Thomas *et al*., 2003; Soehnlen *et al*., 2011; Francoz *et al*., 2005; Gerchman *et al*., 2009) and slightly lower or equal to those recently detected in Japan (Uemura *et al*., 2010).

Among the tested atibiotics, the MICs of aminoglycosides are relatively higher in comparison with the macrotides, fluoroquinolones as well as Pleuromutilin antibiotics suggesting acquired resistance because of continued and ineffective use of this class of antibiotics to control bovine related diseases in Chinese veterinary. Resistance to spectinomycin against *M. bovis* was previously observed in Belgium (Thomas *et al*., 2003) and also was found recently in Israel and the United States (Gerchman *et al*., 2009; Soehnlen *et al*., 2011) differing from that reported in Japan where spectinomycin was effective to most of the tested isolates. In this study, the MIC value of 4-128 μg mL^{-1} for spectinomycin is within the required susceptible range (32-128 μg mL^{-1}) showing susceptibility to *M. bovis* isolates. But most of strains show more high MIC values (MIC50 = 128 μg mL^{-1}) for gentamycin and neomycin presenting obvious resistance.

Due mainly to inhibition of DNA replication, fluoroquinolones are frequently used to treat *M. bovis* infections in many countries. But the decreased antibacterial activity is detected against *M. bovis* and other mycoplasmas (Ben Shabat *et al*., 2010). In the study, enrofloxacin, ofloxacin and ciprofloxacin have relatively lower MICs against majority of the isolates than other tested antimicrobial agents. And the lower resistance rate (2/13) especially for enrofloxacin and ofloxacin is expected that both of these drugs would be effective against *M. bovis* isolated in China in agreement with the findings by Thomas *et al.* (2003) in Belgium and by Marty *et al.* (2011) in Pennsylvania.

Pleuromutilin antibiotics such as tiamulin and valnemulin have been exclusively used in veterinary medicine to control bacterial infection exhibiting excellent antibacterial activity against *Mycoplasma* sp. Among the tested antibiotics tiamulin was the most effective agent with MIC ranges of 0.06-16 μg mL^{-1} although, it is not licensed to treat *M. bovis* infections by CLSI. The alternative valnemulin would be expected to become the best treatment choice. Its highly activity against *M. bovis* has been confirmed in controlled studies under experimental infections and field conditions (Stipkovits *et al*., 2001, 2005).

Tetracycline resistance has been described for other mycoplasmas species due to contain streptococcal tetM sequence (Roberts *et al*., 1985), the same acquired resistance to *M. bovis* was also described by Francoz *et al.* (2005) and Thomas *et al.* (2003). Herein, the doxycycline MICs of 4/13 *M. bovis* isolates were over the resistance breakpoint. So, the doxycycline should be considered as active to the current isolates consistent with the Israel and the Pennsylvania isolates reported by Gerchman *et al.* (2009) and Soehnlen *et al.* (2011). The similar results were also observed for florfenicol.

CONCLUSION

Compared all tested antibiotics in present study, tiamulin, spectinomycin, enrofloxacin and ofloxacin are superior to ciprofloxacin, doxycycline and florfenicol to control *M. bovis* infection. In particular when the cost is the first consideration, spectinomycin, enrofloxacin and ofloxacin have better activity against *M. bovis* in the areas. Other clinical experiments need to be performed to validate the *in vivo* validity. In addition, owing to the currently isolated *M. bovis* strains are limited in only one province, the regional heterogeneity (Gerchman *et al*., 2009) in antibiotic MIC patterns for *M. bovis* isolates from different farms was not observed but the cows tested in Ningxia were mainly introduced from domestic market, the MIC dates obtained in this study reflect to some extent the general situation of the antibiotic use in China. This also provides baseline information for rational therapeutic management in time as well as for future monitoring susceptibility patterns especially at local and national levels.

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REFERENCES

Arcangioli, M.A., A. Duet, G. Meyer, A. Dernburg, P. Bezille, F. Poumarat and D. le Grand, 2008. The role of Mycoplasma bovis in bovine respiratory disease outbreaks in veal calf feedlots. Vet. J., 177: 89-93.

Barlow, J., 2011. Mastitis therapy and antimicrobial susceptibility: A multispecies review with a focus on antibiotic treatment of mastitis in dairy cattle. J. Mammary Gland Biol. Neoplasia, 16: 383-407.

Ben Shabat, M., I. Mikula, I. Gerchman and I. Lysnyansky, 2010. Development and evaluation of a novel single-nucleotide-polymorphism real-time PCR assay for rapid detection of fluoroquinolone-resistant *Mycoplasma bovis*. J. Clin. Microbiol., 48: 2909-2915.

Caswell, J.L. and M. Archambault, 2007. *Mycoplasma bovis* pneumonia in cattle. Anim. Health Res., 8: 161-186.

Francoz, D., M. Fortin, G. Fecteau and S. Messier, 2005. Determination of Mycoplasma bovis susceptibilities against six antimicrobial agents using the E test method. Vet. Microbiol., 105: 57-64.

Gerchman, I., S. Levisohn, I. Mikula and I. Lysnyansky, 2009. *In vitro* antimicrobial susceptibility of *Mycoplasma bovis* isolated in Israel from local and imported cattle. Vet. Microbiol., 137: 268-275.

Hannan, P.C., 2000. Guidelines and recommendations for antimicrobial Minimum Inhibitory Concentration (MIC) testing against veterinary mycoplasma species. International research programme on comparative mycoplasmology. Vet. Res., 31: 373-395.

NCCLS, 2002. Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated from Animals: Approved Standard. 2nd Edn., National Committee for Clinical Laboratory Standards, Wayne, PA., USA., ISBN: 1-56238-461-9.

Nicholas, R.A. and R.D. Ayling, 2003. *Mycoplasma bovis*: Disease, diagnosis and control. Res. Vet. Sci., 74: 105-112.

Poveda, J.B., 1998. Biochemical characteristics in mycoplasma identification. Methods Mol. Biol., 104: 69-78.

Roberts, M.C., L.A. Koutsky, K.K. Holmes, D.J. LeBlanc and G.E. Kenny, 1985. Tetracycline-resistant *Mycoplasma hominis* strains contain streptococcal *tetM* sequences. Antimicrob. Agents Chemother., 28: 141-143.

Soehnlen, M.K., M.E. Kunze, K.E. Karunathilake, B.M. Henwood, S. Kariyawasam, D.R. Wolfgang and B.M. 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.

Stipkovits, L., P.H. Ripley, J. Varga and V. Palfi, 2001. Use of valnemulin in the control of *Mycoplasma bovis* infection under field conditions. Vet. Rec., 148: 399-402.

Stipkovits, L., P.H. Ripley, M. Tenk, R. Glavits, T. Molnar and L. Fodor, 2005. The efficacy of valnemulin (Econor) in the control of disease caused by experimental infection of calves with *Mycoplasma bovis*. Res. Vet. Sci., 78: 207-215.

Subramaniam, S., D. Bergonier, F. Poumarat, S. Capaul, Y. Schlatter, J. Nicolet and J. Frey, 1998. Species identification of Mycoplasma bovis and Mycoplasma agalactiae based on the uvrC genes by PCR. Mol. Cell. Probes, 12: 161-169.

Thomas, A., C. Nicolas, I. Dizier, J. Mainil and A. Linden, 2003. Antibiotic susceptibilities of recent isolates of *Mycoplasma bovis* in Belgium. Vet. Rec., 153: 428-431.

Uemura, R., M. Sueyoshi and H. Nagatomo, 2010. Antimicrobial susceptibilities of four species of *Mycoplasma* Isolated in 2008 and 2009 from Cattle in Japan. J. Vet. Med. Sci., 72: 1661-1663.

Xin, J.Q., Y. Li and D. Guo, 2008. First isolation of Mycoplasma bovis from calf lung with pneumoniae in China. Chin. J. Prev. Vet. Med., 30: 661-664.

Xu, M.J., Q.Y. Liu, J.H. Fu, A.J. Nisbet and D.S. Shi *et al.*, 2012. Seroprevalence of *Mycoplasma bovis* infection in dairy cows in subtropical southern China. Parasitology, 2: 1-4.