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ORIGINAL RESEARCH

Drug Susceptibility Test and Analysis of Quinolone Resistance Genes in *Mycoplasma hyopneumoniae* Vaccine Strains and Field Isolates from China

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Background: Enrofloxacin is a commonly used animal-specific drug in veterinary clinics. However, this drug has no epidemiological cutoff values (ECVs/ECOFFs) for *Mycoplasma hyopneumoniae* in CLSI and EUCAST. Defining the epidemiological cutoff values (ECOFFs) of enrofloxacine to *Mycoplasma hyopneumoniae (M. hyo)* can inform an early detection of bacterial resistance to better manage the resistance prevention and also help in establishing drug resistance breakpoints;.

Methods: We determined the susceptibility breakpoint of *M. hyo* to enrofloxacin by the American Clinical and Laboratory Standards Institute (CLSI) standard method based on the PCR of vaccine strains and wild strains drug resistance genes;.

Results: Eighty strains of *M.hyo* isolated in Tibet were moderately sensitive (S) to tetracycline, florfenicol, spiramycin, erythromycin thiocyanate, tilmicosin, tiamulin, lincomycin, clindamycin, ofloxacin, enrofloxacin, gentamicin, amikacin, with MICs below 0.5 μg/mL. For vaccine 168L, RM48, and J strains, the susceptibility to the same antibacterial drugs was lower compared to the Tibetan isolates. The resistance of J strain to erythromycin thiocyanate was confirmed. Gene point mutation was confirmed in Quinolone Resistance Determining Regions (QRDR) of HNSH strain Topoisomerase IV subunit A, this finding is compared with the sequencing results of 168L strain reference sequence (Accession number: CP003131). Arg-Lys amino acid mutation (G921A and G1179A) was confirmed for the increase of MIC value involved in *M.hyo* to enrofloxacine;.

Conclusion: The cut-off value of *M.hyo* to enrofloxacin was set as 1 μg/mLby ECOFFinder XL 2010 V2.1.

Keywords: *Mycoplasma hyopneumoniae*, drug resistance, Tibetan pigs, MIC, enrofloxacin

Introduction

Although imported and domestic vaccines prevented *M. hyo* infection in P. R. China, there is no clinical vaccine that can 100% prevent and control *M. hyo* infection in Tibetan pigs, possibly because of the gene variation in this species. According to reports from China and abroad, antimicrobial drugs have always played an irreplaceable role in preventing and treating swine enzootic pneumonia.[1–5] High MIC values of fluoroquinolones (enrofloxacin 2.5 μg/mL; marbofloxacin 5 μg/mL) were observed against one M.hyo strain (MycSu17) by Felde, O. et al.[6] Due to the long-term low-dose prophylaxis and high-dose medications,[7] the resistance of Mycoplasmas (including *M. hyo*) to antimicrobials is becoming more widespread,[8] bringing severe challenges to the prevention and control of *M.hyo* in P. R.China.

Epidemiological survey results have demonstrated the prevalence of Mycoplasma pneumonia of swine (MPS) in Tibetan pig herds to be 20.48% to 50%[9] in the Qinghai-Tibet Plateau. MPS reduced the growth rate of Tibetan pigs, causing considerable losses to the Tibetan pig-breeding industry. However, there are relatively few basic data on drug susceptibility of *M.hyo* in Tibetan pigs by searching public databases.

The resistance of *M.hyo* to fluoroquinolones is becoming more widespread in China, with fluoroquinolones treatment failures occurring more frequently in veterinary clinics. Quinolones target DNA gyrase, an essential enzyme in bacterial replication that generates negative and positive supercoils in DNA by transiently introducing double-stranded breaks in an ATP-dependent reaction. DNA gyrase is a heterotetramer consisting of two A and two B subunits, respectively.[10,11]

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The drug resistance mechanism includes a quinolone resistance-determining region (QRDR) target gene mutation,[12–16] plasmid-mediated quinolone resistance (PMQR) gene,[17] the efflux pump,[18] transferable multidrug resistance element, and other not bacteria to be discovered.[19] China is strengthening the monitoring of the drug resistance of animal-derived microorganisms, especially bacteria; the monitoring needs to formulate relevant drug resistance determination standards.

The International Research Programme on Comparative Mycoplasmology (IRPCM) proposed as early as 2000 the antibacterial drug susceptibility testing on animal mycoplasmas.[20] However, only a few animal mycoplasmas have been tested for antimicrobial susceptibility due to various factors including the different media and the protracted cultivation process of animal mycoplasmas. A unified susceptibility breakpoint standard has not yet been established.[21] At present, many countries have established systematic drug resistance detection and judgment standards. The American Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Drug Susceptibility Testing (EUCAST) standards are the most widely used. Criteria for determining drug resistance are divided into three types: microbial, pharmacodynamic, and clinical breakpoints.[22,23] EUCAST has replaced the term microbiological breakpoints with epidemiological cut-off values (ECOFFs) or wild-type cut-off values (COWT) to prevent confusion about the different meanings of breakpoints. The academic and clinical breakpoints are standardized as pharmacodynamic cut-off and clinical cut-off values. Wild-type strains are those that do not carry any mechanisms of acquired resistance. ECOFFs or COWT represent the minimum inhibitory concentration (MIC value) used to distinguish wild-type strains from acquired or selective drug-resistant strains. The epidemiological monitoring of bacteria can play a prompting role in the early development of drug resistance and is of great significance for optimizing clinical drug selection and slowing down the emergence of drug-resistant bacteria.

There are relatively few studies in China and abroad on determining criteria of drug resistance for animal-specific drugs. Enrofloxacin is a commonly used animal-specific drug in veterinary clinics, but there are no epidemiological cut-off values (ECVs/ECOFFs) for *M.hyo* in CLSI and EUCAST. Establishing ECOFFs of this drug can serve as a warning for the early development of antimicrobial resistance,[24] and provide reference to initiating the formulation of drug resistance prevention and control, and establishing the final drug resistance breakpoints.[25–27]

It is urgently necessary to test the drug susceptibility of *M.hyo* and understand the occurrence and mechanism of resistance to reduce the resulting losses and improve the targeted and clinical effects of the prevention and treatment of MPS of Tibetan pigs. However, not even the reference guide of CLSI that sets standards for antimicrobial susceptibility testing provides cut-off information on Mycoplasma species of animals. This makes it difficult to evaluate results and obtain a consensus among researchers. Therefore, the current test is based on preliminary exploratory trials research methods (Reference CLSI guides), and discoloration is the primary basis for judgment.

Drugs used in Tibetan pigs have been selected for in vitro drug susceptibility testing. These drugs include macrolides, lincosamides, quinolones, tetracyclines, florfenicol, and traditional Chinese medicines.

Materials and Methods

Materials

Test Strains

All methods were performed in accordance with the relevant guidelines and regulations. Each pig was euthanized by intramuscular injection of xylazine hydrochloride (Changsha Better Biotechnology Research Institute Co., Ltd., 100 mg/mL) and Pentobarbital sodium (Shanghai Chemical Reagent Purchasing Station of Chinese pharmaceutical company, Each pig was injected 3% Pentobarbital sodium normal saline solution at a dose of 0.2 mL/kg). Then, each animal was intravenously injected with 50 mL of potassium chloride (Tianjin Zhiyuan Chemical Reagent Co., Ltd. 40%). This experiment was performed according to the AVMA (the American Veterinary Medical Association) Guidelines for the Euthanasia of Animals (2020 Edition) to minimize pain in the animals. All animal experiments have been approved by Animal Ethics and Welfare Committee (AEWC) of Xinyang Agriculture and Forestry University with the approval number of AEWC–2021011803.

Lung typical lesions were taken from the Tibetan pigs in Nyingchi, Tibet, P.R.China. Eighty *M.hyo* strains from Tibetan pigs, labeled TB1 (a-j), TB2 (a-j), TB3 (a-j), TB4 (a-j), TB5 (a-j), TB6 (a-j), TB7 (a-j) and TB8

(a-j) were isolated, purified, identified and expanded in the Tibetan Plateau Animal Disease Epidemiology Laboratory and Veterinary Pharmacology Laboratory. The isolation, purification, identification method of *M. hyo* is consistent with that of Qiu G (2019).[28] Eight of the isolates were isolated in 2018 and the rest were isolated from March to May 2022. The strains were stored at −80 °C in the Laboratory of Veterinary Pharmacology, Tibet Agricultural and Animal Husbandry College, and Huazhong Agricultural University Laboratory. Other forty strains were obtained as indicated: RM48 (CVCC4049) (a-j) from China Veterinary Microbiological Culture Collection Management Center; J strain (CVCC359) (a-j) from China Veterinary Microbiological Culture Collection Management Center; and 168L strains (batch number: 20181101) (a-j) from Chuan Haojia, Qianyuanhao Biological Co., Ltd., DJ-166 strains (batch number: 20210312) (a-j) from China Animal Husbandry Industry Co., Ltd. TB1 (a-j), TB2 (a-j), TB3 (a-j), TB4 (a-j), TB5 (a-j), TB6 (a-j), TB7 (a-j), TB8 (a-j), HNSH, HNPQ, HNHC, HNNY, AHFY, and HNZZ strains are wild strains. The vaccine strains were stored at −20°C and −80°C, recently used samples are kept at −20 °C and the rest at −80 °C, within the effective period. The storage time is less than six months. The preservation conditions of wild strain and vaccine strain are consistent. The strains source and date of isolation is shown in Table 1.

Test Drugs

The current drugs used in the treatment of porcine respiratory diseases syndrome in Nyingchi, Tibet, are as follows:

tetracycline, oxytetracycline, chlortetracycline, doxycycline, florfenicol, tylosin tartrate, spiramycin, kitasamycin, erythromycin thiocyanate, tilmicosin, spectinomycin, tiamulin, lincomycin, clindamycin, ofloxacin, ciprofloxacin, enrofloxacin, ampicillin, streptomycin, gentamicin, amikacin, berberine reference material, nanosilver, mequindox, quinoquinone, cyadox, patchouli oil, tea tree oil, garlic oil, oregano oil, and cinnamic aldehyde. The above 31 drugs are commercially available as raw powders oil, drugs, or standard reference materials. Other drugs and reagents used in the tests include bacitracin and polymyxin b. All drugs, reagents, and standard reference materials are within the validity period. Detailed information was provided in Supplementary Material 1.

Table 1 The Strains Source and Date of Isolation

The Strains Name	Date of Isolation	Enrofloxacin Treatment Failed or Not	Source
TB_1	January 2018	□	Field isolates
TB_2	February 2018	□	Field isolates
TB_3	March 2018	□	Field isolates
TB_4	April 2018	□	Field isolates
TB_5	May 2018	□	Field isolates
TB_6	May 2018	□	Field isolates
TB_7	June 2018	□	Field isolates
TB_8	July 2018	□	Field isolates
HNPQ	March 2022	□	Field isolates
HNSH	March 2022	■	Field isolates
HNHC	April 2022	□	Field isolates
HNNY	April 2022	□	Field isolates
HNZZ	May 2022	□	Field isolates
AHFY	May 2022	□	Field isolates
J strain	CVCC359, May 2021	–	Vaccine
168L strains	Batch number:20181101	–	Vaccine
RM48	CVCC4049, May 2021	–	Vaccine
DJ-166 strains	Batch number:20210312	-	Vaccine

Notes: ■Enrofloxacin treatment failed. □Treatment with enrofloxacin is effective. - Commercially available.

Methods

Preparation of Test Bacteria Suspension

One hundred and forty wild strains of *M. hyo* (TB1 (a-j), TB2 (a-j), TB3 (a-j), TB4 (a-j), TB5 (a-j), TB6 (a-j), TB7 (a-j), TB8 (a-j), HNPQ (a-j), HNSH (a-j), HNHC (a-j), HNNY (a-j), HNZZ (a-j), AHFY (a-j)), isolated from fourteen different pig farms (Ten strains of *M.hyo* from ten different pigs per farm) in China and stored in the Central Laboratory of Xinyang Agriculture and Forestry University, were inoculated in a modified KM_2 liquid medium (the modified Friis medium) at a ratio of 1:10. Resuscitation was performed and then aseptically diluted to 10^6 CCU/mL as a bacterial test suspension. Isolation and culture method of *M.hyo* refer to Chinese Patent CN201710782410.X.

Farm pigs in Henan Province of P.R.China that failed enrofloxacin treatment were necropsied, the lungs were collected aseptically (Supplementary Figure 1), and *M.hyo* was isolated, purified, identified, and tested for drug susceptibility and drug resistance. The HNSH strain is field strain isolated from the lungs of pigs that failed enrofloxacin treatment (Figure 1); the presence of *M.hyo* was confirmed by throat swabs PCR testing.

Preparation of Drug Solution

Thirty-one drugs commonly used for the treatment of *M.hyo* were selected, doses were calculated according to the drug content and drug weight, and each was formulated using a modified KM_2 liquid medium (the modified Friis medium) at a concentration of 1 mg/mL (2x the highest concentration of the drug) or 0.01 μL/mL (for plant essential oils).

Test Plan

Measurement Method

The specific method of MIC determination is consistent with CLSI.[29] The susceptibility test was performed in a 96-well cell culture plate using a micro broth dilution method. Table 2 gives details of the sample addition method. The first column is the negative control (200 μL of KM_2 medium (modified Friis medium)); the second column is the drug control (100 μL of modified KM_2 medium + 100 μL of modified KM_2 medium containing twice the highest concentration of drug), and the third to eleventh columns are drug gradients. In culture, each drug was tested in parallel; the 12th column shows the positive control (100 μL of modified KM_2 medium + 100 μL of bacterial suspension). The detailed information is provided in Supplementary Table 1.

Operating Procedure

0.9 mL of culture medium was placed into each tube, and 0.1 mL of the corresponding positive bacteria solution was added to each tube, and mixed thoroughly; 0.1 mL was drawn into the second tube. In this order, the solution was diluted 10^{-1} to 10^{-12} and incubated at 37 °C. Changes in the culture fluid color were used to determine the results; the highest dilution at which color changes determines the CCU. For example, When the solution was diluted to 10^{-1}~10^{-6}, the color

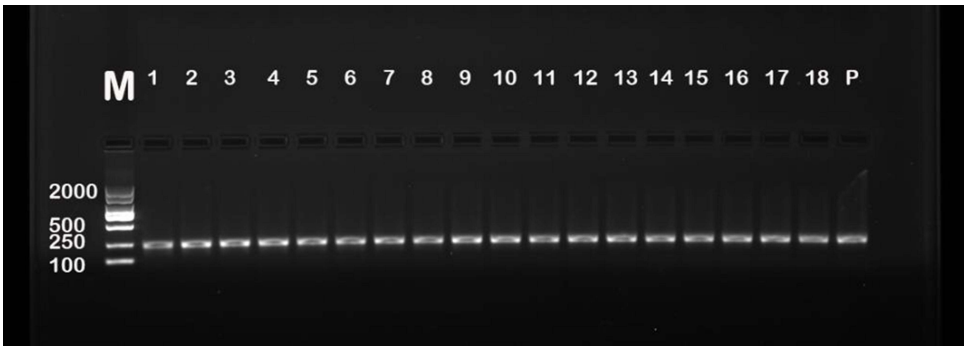

Figure 1 PCR Results of *16 sRNA* gene from *M. hyo*.
Notes: 1 = TB1 (field strain from Tibetan pigs), 2 = TB2 (field strain from Tibetan pigs), 3 = TB3 (field strain from Tibetan pigs), 4 = TB4 (field strain from Tibetan pigs), 5 = TB5 (field strain from Tibetan pigs), 6 = TB6 (field strain from Tibetan pigs), 7 = TB7 (field strain from Tibetan pigs), 8 = TB8 (field strain from Tibetan pigs), 9 = 168L (vaccine strain), 10 = RM48 (vaccine strain), 11 = 5722 (vaccine strain), 12 = HNPQ (field strain), 13 = HNSH (field strain), 14 = HNHC (field strain), 15 = HNNY (field strain), 16 = AHFY (field strain), 17 = HNZZ (field strain), 18=DJ-166 (vaccine strain), P = Positive Control J strain (CVCC359).

Table 2 Adding Samples Method of Drug Sensitivity Test of *M. Hyo*

Drug	The First Column	The Second Column	The 3th-11th Columns	The 12th Column
1 two parallel samples 2 Two parallel samples 3 Two parallel samples 4 Two parallel samples	Negative control	Drug control	Drug concentration gradient	Positive control

changed, the solution was diluted to $10,^{-7}$ the color did not change, then the test result is CCU = 10^6, that is, Mh still grows when the measured bacterial liquid is diluted to 10^{-6}.

The modified KM_2 medium (200 μL) was added to the first column of the plate; 100 μL of modified KM_2 medium was added to each well of columns 2–12, and 100 μL of modified KM_2 base medium containing 2-times the highest concentration of the drug was added to the second and third columns. The third well was mixed thoroughly, and 100 μL was drawn into the fourth well. Then, the fourth well was mixed thoroughly, 100 μL was drawn into the fifth well, etc. to dilute wells serially to the 11th well. Then, 100 μL of the bacterial suspension was aspirated after mixing and added to columns 3–12, gently tapped the plate to mix them, making two rows of each treatment (parallel); plate was covered and cultured in a constant temperature incubator with 5% CO_2 at 37 °C for 7–10 days. Then, the color changing values (CCUs) of *M.hyo* were determined.

When the color of the positive control wells turned yellow, and the color of the negative control wells and the drug control wells did not change, and the result of two consecutive naked-eyes observations was same, the lowest concentration of drug in the wells that did not change color was the lowest concentration of drug (MIC) that completely inhibited the growth of *M. hyo*. According to the result of the preliminary experiment, the observation results of the 7th day were used to judge, as shown in Figure 2.

According to the data and preliminary experimental results, the following primers were used to detect *M.hyo* specific genes and quinolone-related resistance genes. The primer sequence is shown in Table 3.

For amplification of the 16S rDNA, the PCR conditions included 34 cycles of initial denaturation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 60°C for 0.5 min, and extension at 72°C for 7 min. After amplification, 5μL amplicon was electrophoreted in 1% agarose gel (Goldview Nucleic Acid Gel Stain 2μL) at 0.5×TAE at 120 V for 60 min. For amplification of the P36 gene, the PCR conditions included 34 cycles of initial denaturation at 95°C for 5 min, denaturation at 95°C for 0.5 min, annealing at 42°C for 0.5 min, and extension at 72°C for 8 min. After amplification, 5μL amplicon was electrophoreted in 1% agarose gel (Goldview Nucleic Acid Gel Stain 2μL) at 0.5×TAE at 120 V for 60 min. The PCR products

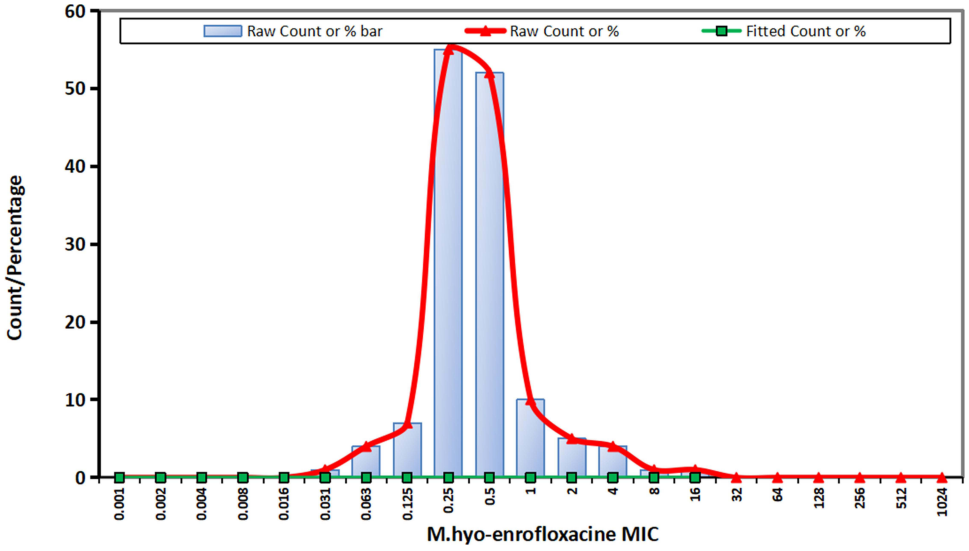

Figure 2 MIC distribution of *M.hyo* strains to enrofloxacin.

Table 3 Primers Used to Detect *M.hyo* and Quinolone-Related Resistance Genes

Gene Name	Primers (5'-3')
Topoisomerase 4 subunit A seqence 1-F	CTAAAACCGGTTCAGAGGCG
Topoisomerase 4 subunit A seqence1-R	TCATTCCTGGGCAAGTCTGA
Topoisomerase 4 subunit A seqence2-F	GCTCAACCGCCCTTATATCG
Topoisomerase 4 subunit A seqence2-R	GGTCCATTGTCGTTTGCCAA
Topoisomerase 4 subunit A seqence3-F	GAACTCGAACAGGACGGAAA
Topoisomerase 4 subunit A seqence3-R	CGGCAACTTTGAGAAACCCA
Topoisomerase 4 subunit A seqence4-F	TTCAATGTGACAGTTGGGGC
Topoisomerase 4 subunit A seqence4-R	GCAGGTGGATCGTCATCAAT
Topoisomerase 4 subunit A seqence5-F	GCATATGCGGCCTTTAATCGA
Topoisomerase 4 subunit A seqence5-R	CCCCAAGTCCTTTATAGCGC
Gyr A-F	TTGATGACGATCCACCTGCC
Gyr A-R	GTTGCATCGATCACTTCGCC
Gyr B-F	CGAGCTTCATTCAGGAGCGA
Gyr B-R	AAACAAGTTTCGCGTAGCCG
ParC-F	TTGATGACGATCCACCTGCC
ParC-R	GTTGCATCGATCACTTCGCC
ParE-F	TGATTCTGCTGGTGGTAGTGC
ParE-R	TTTTCCCACACCAGCTCCAAT
16S-F	CTGGCTCAGGATAAACGCTAG
16S-R	GCTGTGTCGCTCCATCAAG
P36-F	AGTATCGCCTAATTCGGTTCAG
P36-R	CCGTGAAATCCGTATTCTCCTC

Notes: 168L strain of *M.hyo* in Genbank was set as the reference strain, the Primer designed by Primer blast in NCBI, and the specific primers were determined through preliminary experiments and Reference 9.

were sequenced by the shotgun method. The Method of Amplification of gyrA, gyrB, parE and parC gene and follow-up experiment steps are consistent with Le Carrou J et al.[30]

Minimum Inhibitory Concentration Data Collection

Web of Science, China National Knowledge Infrastructure, and Wanfang databases were searched manually to obtain the minimum inhibitory concentration (MIC) data for different entities, and drug susceptibility tests and resistance assays were conducted on *M.hyo* strains.

MIC Data Correction

The MIC distribution data obtained in this study originated from different sources and needed to be processed with the same standard. After merging the data of the same drug, by collecting relevant literature on the quinolone-related drug resistance mechanism and epidemiological cut-off values of *M. hyo*, the data were revised with reference to the distribution range of wild-type *M.hyo* MIC; MICs of non-wild-type *M.hyo* strains were excluded.

ECOFFinder Fitting MIC Distribution Map and Determining Epidemiological Cut-off Values

The data were fitted using the integrated statistical software ECOFFinder XL 2010 V2.1 produced by J. Turnidge et al.

Linear regression analysis was used to fit the cumulative distribution of MIC to obtain the MIC distribution map and to determine the epidemiological cut-off value at different confidence intervals within the best fitting range.

Determination of Drug Resistance Rate and Collection of Related Literature

The epidemiological cut-off value with a confidence interval of 95% was used for the drug resistance breakpoint.

The MIC distribution of the strains was observed, and the drug resistance rate was calculated. All strains were divided into wild strains and clinical isolates based on the obtained epidemiological cut-off values and the proportion of non-wild strains to all strains calculated, giving the resistance ratio of *M.hyo* to the drug.

The pure culture and PCR product of *M.hyo* HNSH strain were sent to Shanghai Sangon Bioengineering Co., Ltd. for sequencing and used for retrieval-specific genes and quinolone-related resistance genes.

Criteria for Color Change of Drug Susceptibility Test

If *M. hyo* strain could grow and discolor the medium (red to yellow), the medium eventually turns yellow, indicating resistance to drug. If *M. hyo* strain could not grow and discolor the medium, the medium eventually appears red, indicating sensitive to enrofloxacin. Same with other drugs.

CCU=10^{-6}, CCU is the highest dilution when the medium changes from red to yellow, then the bacterial solution concentration is calculated as CCU/mL.

The colors used to determine whether *M. hyo* is growing, with yellow indicating growth (resistance to drugs) and red indicating no growth (susceptibility to drugs). During the culture period, the color change was recorded every 12 hours. The color of the modified KM_2 medium changed from red to yellow, indicating the growth of *M. hyo*.

Results

M. h field strains and vaccine strains were identified by PCR of 16 sRNA gene as shown in Figure 1.

Observation of Test results

On the 3rd day of the experiment, the MIC values of the 31 antibacterial drugs and 80 strains of *M.hyo* from Tibetan pigs were found not to have changed. After the 7th day of culture, the color of the 96-well culture plate used to determine the drug susceptibility test results were as follows:

Negative drug control wells did not discolor (red), the positive control wells discolored (yellow), and wells with the gradient of drug concentration from high to low gradually changed from red to yellow.

Drug Susceptibility Test Result of *M. hyo* Isolated from Swine

Eighty isolates from Tibet were highly sensitive to diterpenes, macrolides, tetracyclines, and quinolones (S), with MICs lower than 0.5 μg/mL; sensitivity to lincosamides was moderate, with MIC of 0.13 to 0.3 μg/mL, low sensitivity to quinocetone and ampicillin, MIC ≥ 16 μg/mL, high sensitivity to enrofloxacine, MIC50 = 0.25 μg/mL, and higher MIC for berberine than common chemical drugs MIC ≥ 32 μg/mL. The MIC of nano-silver was 0.5 ~ 1 μg/mL, considered sensitive. For the vaccine strains 168L strains, RM48 strains, and avirulent strain J strains, the susceptibility to the same antibacterial agents was lower than that of the Tibet isolates; J strains were low sensitivity to erythromycin thiocyanate more frequently. The MIC_{50} of HNSH strain to enrofloxacin was 16 μg/mL. To enrofloxacine, the MIC_{50} of HNPQ strain was 0.25μg/mL, MIC_{90}=0.5μg/mL, MIC range was between 0.008μg/mL and 8μg/mL. The MIC_{50} of HNHC strain was 0.25μg/mL, MIC_{90}=0.5μg/mL, MIC range was between 0.008μg/mL and 4μg/mL. The MIC_{50} of HNNY strain was 0.25μg/mL, MIC_{90}=0.5μg/mL, MIC range was between 0.008μg/mL and 4μg/mL. The MIC_{50} of AHFY, strain was 0.25μg/mL, MIC_{90}=0.5μg/mL, MIC range was between 0.008μg/mL and 4μg/mL. The MIC_{50} of HNZZ strain was 0.25μg/mL, MIC_{90}=0.5μg/mL, MIC range was between 0.008μg/mL and 8μg/mL.

Drug susceptibility test resultof *M.hyo* isolated from swine is shown in Table 4.

MIC distribution of *M.hyo* strains to enrofloxacin is shown in Figure 2.

Identification of DNA topoisomerase 4 subunit A from *M.hyo* HNSH strain is shown inFigure 3.

PCR results of genes (*16sRNA gene, P36 gene, parC, parE, gyrA, gyrB*) from *M.hyo* strains are shown in Figure 4.

The following gene mutations in the quinolone resistance-determining region were found by sequencing of Topoisomerase IV subunit A in the HNSH strain of *M.hyo* as shown in Table 5.

When compared the QRDRs region nucleotide sequence of HNSH strain with the reference strain CP002274.1, the results showed that the amplified sequence had multiple-mutation sites. Subsequently, we compared amino acid sequence of the gyrA, gyrB, parE and parC in HNSH strain QRDRs with the standard strain CP002274.1, specific gene

Table 4 MICs of Antimicrobial Agents Used Against Field Strains from Tibetan Pigs and Vaccine Strain 168L Strain, RM48 Strain and Type Strain J Strain of M. Hyo, Determined by a Serial Broth Dilution Method

Drugs (Unit: Solid, μg/mL, Liquid, μL/mL)	M. hyo						HNSH (Field Strain)
	TB (Field Strain from Tibetan Pigs) (n=80)			168L (Vaccine Strain)	RM48 (Vaccine Strain)	J (CVCC359)	
	MIC50	MIC90	RANGE				
Berberine (pH7.6–7.8)	32	64	32–64	32	32	64	64
Patchouli oil (μL/mL)	0.01	0.02	0.01–0.02	0.01	0.01	0.02	0.08
Tea tree oil (μL/mL)	0.02	0.04	0.02–0.04	0.02	0.02	0.04	0.08
Garlic oil (μL/mL)	0.01	0.02	0.01–0.02	0.01	0.01	0.02	0.04
Oregano oil (μL/mL)	0.01	0.02	0.01–0.02	0.01	0.01	0.02	0.04
Cinnamic aldehyde (μL/mL)	0.03	0.06	0.03–0.06	0.03	0.03	0.06	0.13
Nanosilver (0.1%)	0.5	1	0.5–1	0.5	0.5	1	1
Mequindox	16	32	16–64	64	32	32	64
Quinocetone	16	32	16–64	64	64	16	64
Cyadox	16	32	16–64	64	64	32	64
Tetracycline	0.03	0.06	0.03–0.06	1	1	2	8
Oxytetracycline	0.03	0.06	0.008–1	2	2	2	2
Chlortetracycline	0.5	1	0.13–1	1	1	2	2
Doxycycline	0.03	0.06	0.008–2	2	2	2	2
Florfenicol	0.25	0.5	0.016–0.5	0.13	0.13	0.13	0.13
Tylosin tartrate	0.03	0.5	0.008–2	2	2	2	16
Spiramycin	0.25	0.25	0.06–0.5	0.25	0.25	0.13	0.13
Kitasamycin	0.5	1	0.12–1	1	1	0.25	0.25
Erythromycin thiocyanate	0.13	0.5	0.13–0.5	2	2	2	2
Tilmicosin	0.06	0.5	0.008–0.5	0.25	0.25	0.13	0.25
Spectinomycin	0.5	1	0.5–1	1	1	1	1
Tiamulin	0.016	0.062	0.002–0.13	0.125	0.25	0.25	0.25
Lincomycin	0.3	0.3	0.06–0.25	0.3	0.3	0.13	0.13
Clindamycin	0.13	0.25	0.13–0.25	0.25	0.25	0.13	0.13
Ofloxacin	0.01	0.25	0.01–0.5	0.25	0.25	0.25	0.25
Ciprofloxacin	0.03	0.5	0.008–1	0.5	0.5	0.5	0.5
Enrofloxacin	0.25	0.5	0.008–0.5	0.5	1	0.5	16
Ampicillin	32	64	16–64	32	64	64	64
Streptomycin	0.5	1	0.5–1	1	1	2	2
Gentamicin	0.25	0.5	0.25–0.5	0.5	0.5	1	1
Amikacin	0.13	0.25	0.13–0.25	0.13	0.13	0.25	0.5

Notes: MICs were read when color changes had stopped for 1 days (M.h grows steadily), ie, around day 7 after inoculation (final MICs).

polymorphisms were observed. After excluding the interference of species-specific or serotype-specific polymorphisms, we summarized the corresponding amino acid changes in QRDRs of the tested strains, as shown in Table 5. The MIC value of enrofloxacine corresponding to the above-mentioned QRDRs mutant strains was significantly higher than other strains without mutation in the QRDRs.

The MIC90 data of *M. hyo* strains and 31 drugs resistance rates were collected after conducting drug susceptibility tests according to CLSI standards. The cut-off values for enrofloxacin–*M.hyo* combinations are shown in Table 6.

Discussion

Although individual resistance strain to macrolides was found, on the whole, different from the resistance of *M. hyo* to Beta lactam drugs, the Tibet isolate strain showed sensitivity to enrofloxacin and macrolides in this test. HNSH field strain showed insensitivity to enrofloxacin. It suggests regional differences in drug use history and drug resistance phenotype. When compared to Mycoplasma gallisepticum,[15] Mycoplasma bovis,[3] Mycoplasma hominis,[11] the drug resistance rate of *M. hyo* obtained are somewhat different. For *M.hyo* differences in the drug resistance rate were

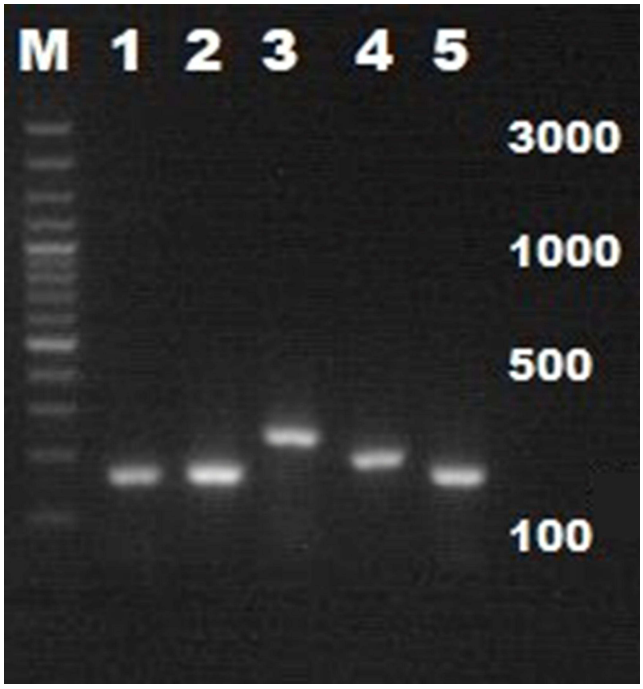

Figure 3 PCR results of DNA topoisomerase 4 subunit A from *M.hyo* HNSH strain. 1= *parC*. 2= *parE*. 3=*16sRNA gene*. 4= *gyrA*. 5= *gyrB*.

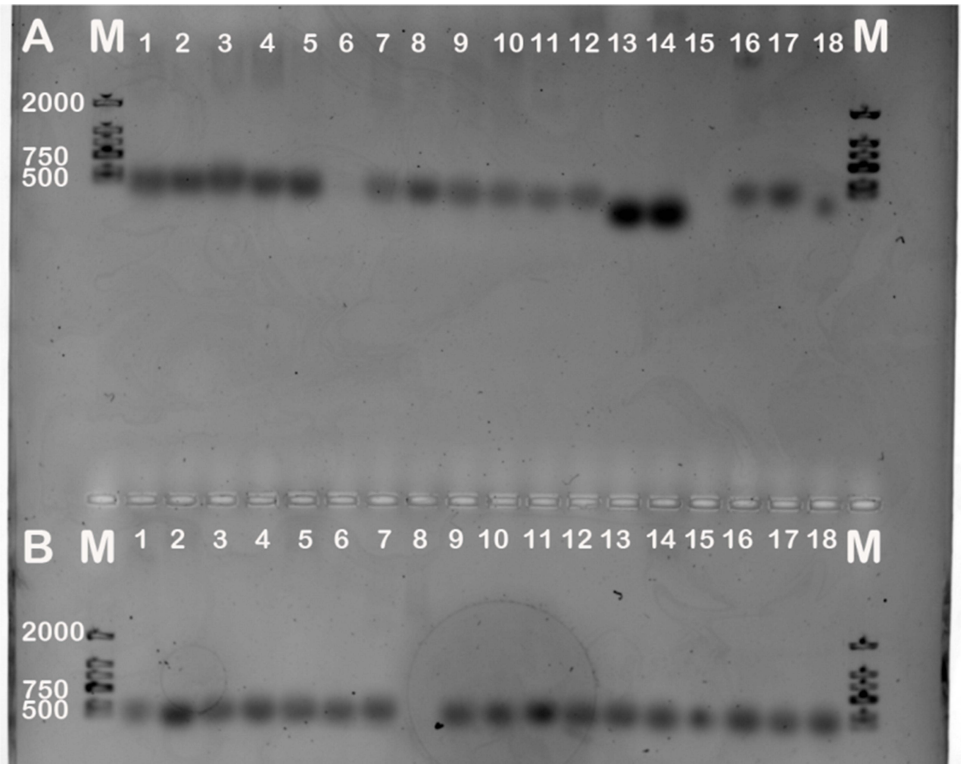

Figure 4 PCR results of genes (*16sRNA gene, P36 gene, parC, parE, gyrA, gyrB*) from *M.hyo* strains. Vaccine strains: RM48 strain, J strain, 168L strain, DJ-166 strain. Field strains: HNPQ strain, HNSH strain, AHFY strain. Conserved genes for identification of *M.hyo:16sRNA gene, P36 gene*. QRDR genes: *gyrA, gyrB, parE and parC*.
Notes: A line: M = DL 2000 DAN Marker, A 1 = HNPQ strain *16sRNA gene*, A 2 = J strain *16sRNA gene*, A 3 = HNPQ strain *P36 gene*, A 4 = HNPQ strain *16sRNA gene*, A 5=DJ-166 strain *16sRNA gene*, A 6 = HNPQ strain *gyrA gene*, A 7 = AHFY strain *P36 gene*, A 8 = J strain *parE gene*, A 9 = HNSH strain *parC gene*, A 10=DJ-166 strain *parC gene*, A 11 = AHFY strain *16sRNA gene*, A 12 = RM48 strain *P36 gene*, A 13 = RM48 strain *gyrB gene*, A 14 = 168L strain *gyrB gene*, A 15 = Negative control sample (KM2 culture medium), A 16 = 168L strain *p36 gene*, A 17 = J strain *gyrB gene*, A 18 = RM48 strain *parC gene*. B line: M = DL 2000 DAN Marker, B 1 = 168L strain *parC gene*, B 2 = RM48 strain *16sRNA gene*, B 3 = AHFY strain *P36 gene*, B 4 = J strain *P36 gene*, B 5 = 168L strain *P36 gene*, B 6 = RM48 strain *parC gene*, B 7 = HNSH strain *P36 gene*, B 8 = AHFY strain *parC gene*, B 9 = HNSH strain *16sRNA gene*, B 10 = 168L strain *16sRNA gene*, B 11 = J strain *p36 gene*, B 12 = AHFY strain *p36 gene*, B 13 = AHFY strain *gyrA gene*, B 14 = 168L strain *16sRNA gene*, B 15 = 168L strain *16sRNA gene*, B 16 = 168L strain *parC gene*, B 17 = J train *parC gene*, B 18 = HNSH strain *parC gene*.

Table 5 Resistance-Associated Nucleotides Mutations and Amino Acid Changes in *M.hyo* HNSH Strain Topoisomerase IV Subunit a

Sample Serial Number	Location	Reference Strain (168L)	HNSH Strain	Amino Acid Changes
41,334,316	No.418	A	G	(GIT-Gly) +
41,334,316	No.437	G	A	(Cys-Tyr) +
41,334,316	No.580	G	A	(Ser-Asn) +
41,334,316	No.696	T	C	(LeT-Pro) +
41,334,316	No.845	T	C	(Val-Ala) +
41,334,316	No.915	C	A	(Ser-Ter) +
41,334,316	No.921	G	A	(Arg-Lys) +
41,334,316	No.1101	A	G	(Asn-Ser) +
41,334,316	No.1140	G	T	(Gly-Val) +
41,334,316	No.1179	G	A	(Arg-Lys) +
41,334,316	No.1220	A	G	(Asn-Ser) +
41,334,316	No.1351	A	G	(Tyr-Cys) +
41,334,316	No.1355	A	G	(Ter-Ter) -
41,334,316	No.1359	C	T	(Gln-Ter) +
41,334,316	No.1446	A	G	(Arg-Arg) -
41,334,316	No.1454	G	A	(Ter-Ter) -
41,334,316	No.1539	G	A	(Ser-Ser) -
41,334,316	No.1908	C	T	(Ile-Ile) -
41,334,316	No.2339	A	C	(Ser-Ser) -

Notes: +Indicates amino acid changed, - Indicates the amino acid has not changed. Reference Sequence: CP002274.1.

Table 6 Cut-off Values for Enrofloxacin–*M. hyo* Combinations

Species	Antibiotic	No. of Distributions in Pool	ECOFFinder XL 2010 v2.1 cut-off (μg/mL)
M.hyo	Enrofloxacine	140	1

observed in different farms in the same year. The drug resistance rate generally shows an upward trend year by year in Central China.

Drugs with high antibacterial activity for *M. hyo* are mainly bisterpenes, tetracyclines, macrolides, quinolones, lincosamides, amido alcohols and some aminoglycosides. In this experiment, the 80 wild *M.hyo* strains (TB1 (a-j), TB2 (a-j), TB3 (a-j), TB4 (a-j), TB5 (a-j), TB6 (a-j), TB57 (a-j), TB8 (a-j)) were highly sensitive to macrolides, including tilmicosin and diterpenoid tiamulin. The results are consistent with the literature reports.[31–35] The vaccine strains are moderately sensitive to lincomycin, consistent with relevant literature reports,[36] possibly because lincomycin has been used as a feed additive for many years throughout the P.R.China, resulting in increased drug resistance.[37] Vaccine strains are resistant to oxytetracycline attributed to long-term use of oxytetracycline in pig industry. *M.hyo* isolates in Tibet are more sensitive to chemotherapeutic drugs that can inhibit protein synthesis commonly used in veterinary clinics in P.R.China, while vaccine strains 168L strain, RM48 strain, and avirulent J strain are more sensitive to erythromycin and oxytetracycline. Strains 168L, RM48, and avirulent J strain are presumed to have different levels of resistance and resistance transfer due to the widespread use of oxytetracycline in different provinces and regions of the country.

Tylosin powder, tilmicosin, tiamulin, ciprofloxacin, doxycycline, and florfenicol are all highly effective with *M.hyo* in pigs and are recommended as the first choice for the prevention and treatment of Tibetan pig mycoplasma pneumonia. This result is consistent with the test results of Qiu H.L.[38] and Zhang C.P.[39] The more effective drugs, including lincomycin, spectinomycin, can be used as the second-choice drugs for the treatment of Tibetan pig mycoplasma pneumonia. The Tulathromycin is effective for eradicating *M. hyo* and is used abroad on pig farms in farm. However,

due to the higher price of Tulathromycin and taking into account the depressed market conditions in the pig industry now, it is not recommended for large-scale eradication of *M. hyo*. Tylosin, tilmicosin, tiamulin, ciprofloxacin, enrofloxacin, oxytetracycline, tetracycline, doxycycline, and florfenicol are reasonable choices that can achieve adequate therapeutic effects in the treatment of Tibetan pig mycoplasma pneumonia. For industrially farmed Tibetan pigs, we can refer to the aforementioned Swiss reduction group method or partial reduction group method or unreduced population for the eradication of *M. hyo*, to select the appropriate program. Gene point mutation was confirmed in Quinolone Resistance Determining Regions (QRDR) of *M.hyo* HNSH strain Topoisomerase IV subunit A, this finding is compared with the sequencing results of Topoisomerase IV subunit A in *M. hyo* 168L strain reference sequence (Accession number: CP003131).[40] The resistance phenotype of *M.hyo* to enrofloxacin is consistent with that of Enterococcus faecalis from healthy chickens and pigs in Taiwan.[41]

Arg-Lys amino acid mutation (G921A and G1179A) is the main reason for the increase of MIC value involved in *M. hyo* to enrofloxacin.[30,42–48]

M. Hyo-enrofloxacine cut-off value is calculated by the CLSI Official website statistical software ECOFFinder XL 2010 V2.1 (J. Turnidge et al). This method is the standard method worldwide.

Conclusions

Eighty wild *M.hyo* strains (TB1 (a-j), TB2 (a-j), TB3 (a-j), TB4 (a-j), TB5 (a-j), TB6 (a-j), TB7 (a-j), TB8 (a-j)) were sensitive to Tylosin, Tilmicosin, Tiamulin, Ciprofloxacin, enrofloxacin, Oxytetracycline, Tetracycline, Doxycycline, Florfenicol, and resistant to Ampicillin. Therefore, drug selection is more reasonable and efficient when considering the advantages and disadvantages of various types of drugs to prevent and treat swine mycoplasma pneumonia. Multiple genetic mutations associated with elevated MIC of *M.hyo* to enrofloxacin were identified. The cut-off value of *M.hyo* to enrofloxacin was set as 1 μg/mLby the CLSI Official website statistical software ECOFFinder XL 2010 V2.1 (J. Turnidge et al).

Data Sharing Statement

The datasets generated and/or analysed during the current study are available in the GenBank repository,(https://www.ncbi.nlm.nih.gov/nuccore/CP003131.1/), (https://www.ncbi.nlm.nih.gov/nuccore/CP002274.1/).

Institutional Review Board Statement

The animal study protocol was approved by the Animal Ethics and Welfare Committee (AEWC) of Xinyang Agriculture and Forestry University (AEWC −2021011803 and January 17, 2021).

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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