Antibiotic resistance of *Mycoplasma Synoviae* strains isolated in China from 2016 to 2019

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### Abstract

**Background:** In the past decade, *Mycoplasma synoviae* (*M. synoviae*) infection has become widely prevalent in China, has caused serious economic losses and has become one of the most important diseases in the chicken industry. Medication is a general approach for the control of *M. synoviae* infection, but antibiotics are sometimes ineffective in clinical practice. To investigate the sensitivity of *M. synoviae* to antimicrobials commonly used in the treatment of *M. synoviae* infection, the antibiotic susceptibility of 32 *M. synoviae* strains isolated from China from 2016 to 2019 were determined using the minimum inhibitory concentration (MIC) method.

**Results:** All isolates had low MIC values for the combination of lincomycin and spectinomycin, pleuromutilin, and macrolides. However, the *M. synoviae* isolates displayed variance in MICs for doxycycline hydrochloride with a range of 0.25 to 8 μg/mL, and oxytetracycline hydrochloride with a range of 0.5 to 8 μg/mL. Three and one *M. synoviae* isolates showed intermediate MIC values to doxycycline hydrochloride and oxytetracycline hydrochloride, respectively. High MIC values for enrofloxacin were detected in all isolates with MICs ranging from 4 to 32 μg/mL. Furthermore, comparison of the parC QRDR identified a mutation at nucleotide position 254 (C254T) resulting in a Thr 85 Ile amino acid change in all *M. synoviae* isolates and the reference strain ATCC 25204 being resistant to enrofloxacin. Moreover, mutations at Glu 804 Gly and Thr 686 Ala of gyrA QRDR were identified in all *M. synoviae* isolates and ATCC 25204. The mutation in the QRDR of the parE gene resulted in amino acid changes at positions 197 (Pro to Ser) in 27/32 *M. synoviae* isolates.

**Conclusion:** Three nonsynonymous mutations in gyrA and parE were first identified to be related to enrofloxacin resistance. Our results showed that *M. synoviae* resistance to enrofloxacin is widespread.

**Keywords:** *Mycoplasma synoviae*, Antimicrobial susceptibility, Minimum inhibitory concentrations, Resistance genes, Enrofloxacin resistance
Other studies reported a prevalence of 15% in South America [6] and 27% in Middle East [7]. In China, the incidence rate of chickens varies greatly in different provinces from 5.10 to 100%, which has caused serious economic losses to the chicken industry [8]. Therefore, there has been a growing emphasis on the prevalence of *M. synoviae*, especially its subclinical infection.

The three general approaches for the control of *M. synoviae* infection are eradication strategies (maintaining flocks free of infection), vaccination and medication. Although eradication strategies and vaccination provide long-term solutions for the control of *M. synoviae* infection, medication can be very useful in preventing vertical transmission and clinical symptoms as well as economic losses [9]. However, antibiotic susceptibility should first be determined to maximize treatment efficacy. In most countries, excessive use of a range of antimicrobials in feed, on the one hand, prevents and treats disease and, on the other hand, improves growth performance. Antibiotic resistance is a global health threat, and the abuse of antimicrobials in animal production is the main contributing factor [10].

*Mycoplasmas* are readily resistant to β-lactam antibiotics and sulfones, but susceptible to other classes of antibiotics, including tetracyclines, macrolides, fluoroquinolones, and pleuromutilins [11–13]. Previous studies also demonstrated the efficacy of tiamulin fumarate (TIF) and the combination of lincomycin and spectinomycin (LS) against *M. synoviae* [14, 15]. However, increasing resistance of *Mycoplasma* against tetracyclines and quinolones has been reported [16, 17]. Quinolone resistance is related to point mutations in the quinolone resistance-determining regions (QRDRs) of the A subunits of DNA gyrase and topoisomerase IV (*parC* and *gyrA* gene) or B subunits of DNA gyrase and topoisomerase IV (*gyrB* and *parE* gene) in *M. synoviae* [18].

As mentioned above, in recent years, *M. synoviae* infection has caused serious economic losses to the chicken industry. In addition to medication, vaccines also play an important role in controlling *M. synoviae* infection in China. Even when the oil emulsion vaccine is inoculated, it will be mixed with some antimicrobials. The liquid MIC test was carried out in 96-well microdilution plates as described in a previous study [20]. The *M. synoviae* culture was diluted in *Mycoplasma* broth medium in the range of $10^3$ ccu/mL and $10^5$ ccu/mL. The concentration of antibiotic dilution was usually obtained by doubling dilutions with 100 μL *Mycoplasma* broth medium (pH = 7.8). After the dilution of antibiotic was completed, the 100 μL dilution *M. synoviae* culture was inoculated into each well. The liquid MIC test was carried out in 96-well microdilution plates as described in a previous study [20]. The *M. synoviae* culture was diluted in *Mycoplasma* broth medium in the range of $10^3$ ccu/mL and $10^5$ ccu/mL. The concentration of antibiotic dilution was usually obtained by doubling dilutions with 100 μL *Mycoplasma* broth medium (pH = 7.8). After the dilution of antibiotic was completed, the 100 μL dilution *M. synoviae* culture was inoculated into each well. *M. synoviae* culture and antibiotics were included in all tests as negative controls and antibiotic controls, respectively. Plates were incubated at 36 ± 1 °C. The lowest concentration of antibiotic to show a color change denoted MIC. The MIC was read when the phenol red indicator in the negative control had just turned orange–yellow. There is a lack of standardized methods and official breakpoints of susceptibility testing criteria for animal mycoplasmas in vitro. Thus, MIC values measured in this study were compared to previous studies.

To screen the most effective antimicrobials, it is urgent to investigate the sensitivity of *M. synoviae*.

### Materials and methods

#### Strains

The *M. synoviae* strains used in this study were isolated from China from 2016 to 2019 [19]. The isolates were cultured aerobically at 37 °C in *Mycoplasma* broth (pH = 7.8) with an MB base (OXOID Ltd., Hampshire, UK) containing 15% porcine serum, 3.3 g/L glucose, 100 mg/L L-cysteine, 400 mg/L L-arginine, 100 mg/L β-nicotinamide adenine dinucleotide trihydrate (β-NAD), and 0.02% phenol red until the color of the culture medium changed from red to orange–yellow. These cultures were subsequently titrated in *Mycoplasma* broth medium to determine the number of color changing units (ccu). Then, sterile glycerol (5%) was added to the cultures and stored at −70 °C.

#### Antimicrobials

All strains were tested by the MIC method using the antimicrobials commonly used for *M. synoviae* treatment in farms as follows. LS, doxycycline hydrochloride (DO), valnemulin hydrochloride (VA), tylosin (TY), TIF, oxytetracycline hydrochloride (OT), enrofloxacin (ENR) and tilmicosin (TIL) were purchased from Solarbio Life Sciences (Beijing, China). The concentration of antibiotic solution was diluted to 128 μg/mL. Then the solutions were sterilized with a 0.22-μm membrane filter. The concentration range of antibiotics is shown in Table 3.

#### Antimicrobial sensitivity test in vitro

The liquid MIC test was carried out in 96-well microdilution plates as described in a previous study [20]. The *M. synoviae* culture was diluted in *Mycoplasma* broth medium in the range of $10^3$ ccu/mL and $10^5$ ccu/mL. The concentration of antibiotic dilution was usually obtained by doubling dilutions with 100 μL *Mycoplasma* broth medium (pH = 7.8). After the dilution of antibiotic was completed, the 100 μL dilution *M. synoviae* culture was inoculated into each well. *M. synoviae* culture and antibiotics were included in all tests as negative controls and antibiotic controls, respectively. Plates were incubated at 36 ± 1 °C. The lowest concentration of antibiotic to show a color change denoted MIC. The MIC was read when the phenol red indicator in the negative control had just turned orange–yellow. There is a lack of standardized methods and official breakpoints of susceptibility testing criteria for animal mycoplasmas in vitro. Thus, MIC values measured in this study were compared to previous studies.
Quinolone resistance-determining regions and nucleotide sequence analysis
To elucidate the mechanism of acquired ENR resistance in *M. synoviae* isolates, the sequences gyrA, gyrB, parC, and parE of QRDRs were further analyzed. To amplify the QRDRs of *M. synoviae* isolates, the primers of gyrA, gyrB, parC, and parE (Table 1) were based on a previous study [17]. *M. synoviae* isolate cultures were harvested for DNA extraction using the TIANamp Bacteria DNA Kit (Transgen Biotech Co., Ltd., Beijing, China) according to the manufacturer’s instructions. The sequences were aligned using Lasergene 7.1 software and blasted with those of reference *M. synoviae* strains MS-H (GenBank accession number: NZ_KP704286) and ATCC 25204 (GenBank accession number: NZ_CP011096). The nucleotide and amino acid positions were located based on the reference *M. synoviae* strain MS53 (GenBank accession number: AE017245).

Results

MIC determinations
In this study, 32 *M. synoviae* isolates were tested for antibiotic susceptibility in vitro with 9 commonly used antimicrobials. The MICs of 32 *M. synoviae* isolates was shown in Table 2. All isolates had low MIC values for LS, with MICs ranging from 0.063 to 2 μg/mL. An unimodal distribution of LS MIC value was observed. Thirty-one *M. synoviae* isolates showed MIC values for LS equal or lower to 1 μg/mL (MIC50 = 0.5 μg/mL), while the rest were inhibited by a concentration of 2 μg/mL. The MIC50 and MIC90 values for DO were same as OT, being 2 and 4 μg/mL respectively (Table 3).

**Table 1** Primer sequences used in this study

| Primer name | Sequences (5′ → 3′) | Position (bp) |
|-------------|---------------------|---------------|
| gyrAF       | GAAGATCAGCCTGAATTATTT | 58–78        |
| gyrAR       | GCCATCTCTGCTGGTTAA   | 531–551      |
| gyrBF       | CAAGGTGAGAATTTCAGAGA | 964–984      |
| gyrBR       | TGTGCTCCTGTTAAGCG    | 1677–1694    |
| parCF       | CCAACCGTGCAATTTGTGAT | 95–114       |
| parCR       | TTATGCGGCGGATTCGCG   | 546–563      |
| parEF       | GGCATATCGTGCGAGAAATGG | 1034–1055   |
| parER       | AGTGGTTTCACAAAGTTG   | 1741–1758    |

However, the *M. synoviae* isolates displayed variance in MICs for tetracyclines, and intermediate MIC results were detected. MIC values for DO and OT, 3/32 and 1/32 *M. synoviae* strains had the highest value at 8 μg/mL. The MIC50 and MIC90 values for DO were same as OT, being 2 and 4 μg/mL respectively (Table 3). Importantly, high MIC values for ENR were detected in all *M. synoviae* isolates with MICs ranging from 4 to 32 μg/mL. ENR MIC values were distributed unimodally and revealed that most of the *M. synoviae* (28/32) were inhibited by concentrations of ≥8 μg/mL, with 12 isolates and 3 isolates showing MIC values of 16 μg/mL and 32 μg/mL respectively. MIC50 and MIC90 values of ENR were the highest, which were 8 μg/mL and 16 μg/mL respectively (Table 3).

Molecular characterization of quinolone resistance-determining regions in *M. synoviae* isolates
To further investigate the mechanism of ENR resistance in *M. synoviae*, four resistance genes in the QRDRs of 32 *M. synoviae* isolates were amplified by PCR and sequenced. The reference strain ATCC 25204 is resistant to ENR and MS-H is susceptible to ENR. Four QRDR genes in the reference genome of *M. synoviae* strain MS53 were aligned with the sequences of ATCC 25204, MS-H and 32 *M. synoviae* isolates. Several non-synonymous mutations were found to be potentially resistance-related.

Sequence analysis of the gyrA QRDR showed that three SNPs in all *M. synoviae* isolates and ATCC 25204 resistant to ENR were identified. Mutations at nucleotide positions 2410 (A2410G) and 2411 (A2411G) resulted in Glu 804 Gly amino acid changes. A mutation at nucleotide position 2058 (A2058G) resulted in a Thr 686 Ala amino acid change. In the gyrB gene, a SNP mutation was found at position 1250 (G1250A) and resulted in a Ser 417 Asn amino acid change in 11/32 *M. synoviae* isolates with high MIC values for ENR. Comparison of the parC QRDR identified a mutation at nucleotide position 254 (C254T) resulting in a Thr 85 Ile amino acid change. In the parE gene, a Pro 197 Ser amino acid change was also found in 27/32 *M. synoviae* isolates (Table 4).

Discussion

Antimicrobial resistance is a growing global concern for animals and humans. During the past decade, few studies have investigated the MICs of *M. synoviae* isolates in vitro. At present, the commonly used clinical antimicrobial medications for the treatment of mycoplasma disease are macrolides (e.g., TIL and TIL), pleuromutilins (e.g., valnemulin and TIL), tetracyclines (e.g., DO and
Table 2  MICs of *M. synoviae* isolates

| Isolates            | MIC (μg/mL) |
|---------------------|-------------|
|                     | LS          | VA | TIF | TY | TIL | DO | OT | ENR |
| Ningxia/2019–1      | 0.5         | <0.016 | <0.016 | 0.063 | <0.016 | 4  | 1  | 4   |
| Ningxia/2019–2      | 0.5         | <0.016 | <0.016 | 0.031 | <0.016 | 4  | 2  | 4   |
| Hebei/2016–1        | 0.5         | <0.016 | 0.031 | 0.25  | 0.063  | 4  | 4  | 16  |
| Hebei/2016–2        | 0.5         | <0.016 | <0.016 | 0.063 | 0.031  | 1  | 2  | 8   |
| Hebei/2016–3        | 0.5         | <0.016 | <0.016 | 0.063 | 0.031  | 4  | 2  | 8   |
| Shandong/2016–1     | 1           | <0.016 | 0.063 | 0.25  | 0.063  | 8  | 4  | 16  |
| Shandong/2017–1     | 0.5         | <0.016 | <0.016 | 0.031 | 0.031  | 1  | 1  | 8   |
| Shandong/2017–2     | 0.25        | <0.016 | 0.031 | 0.063 | 0.063  | 2  | 4  | 8   |
| Shandong/2018–1     | 0.5         | <0.016 | <0.016 | 0.031 | 0.031  | 1  | 1  | 8   |
| Hebei/2016–1        | 2           | <0.016 | <0.016 | 0.063 | 0.031  | 2  | 4  | 8   |
| Jiangsu/2018–1      | 0.5         | <0.016 | 0.031 | 1     | 1     | 1  | 1  | 16  |
| Jiangsu/2018–2      | 0.5         | <0.016 | 0.031 | 1     | 0.5   | 0.5 | 0.5 | 16  |
| Jiangsu/2018–3      | 1           | <0.016 | 0.063 | 0.5   | 1     | 1  | 1  | 32  |
| Jiangsu/2018–4      | 0.25        | <0.016 | <0.016 | 0.25  | 0.5   | 1  | 0.5 | 8   |
| Jiangsu/2018–5      | 0.5         | <0.016 | <0.016 | 0.031 | 0.125 | 0.5 | 0.5 | 4   |
| Jiangsu/2018–6      | 1           | <0.016 | 0.031 | 0.5   | 1     | 1  | 0.5 | 16  |
| Anhui/2019–1        | 1           | <0.016 | 0.125 | 0.031 | 0.125 | 4  | 4  | 16  |
| Anhui/2019–2        | 1           | <0.016 | 0.063 | 0.5   | 0.125 | 1  | 1  | 32  |
| Jiangsu/2019–1      | 0.063       | <0.016 | <0.016 | <0.016 | 0.063 | 0.25 | 0.5 | 16  |
| Shandong/2019–2     | 0.5         | <0.016 | <0.016 | 0.063 | 0.063 | 1   | 0.5 | 16  |
| Shandong/2019–3     | 1           | <0.016 | 0.063 | 0.125 | 0.5   | 2  | 2  | 32  |
| Shandong/2019–4     | 0.125       | <0.016 | <0.016 | 0.031 | 0.125 | 0.063 | 1   | 0.5 | 16  |
| Ningxia/2019–3      | 0.125       | <0.016 | <0.016 | 0.125 | <0.016 | 2  | 4  | 8   |
| Henan/2019–1        | 1           | <0.016 | 0.063 | 0.125 | <0.016 | 2  | 2  | 8   |
| Jiangsu/2019–2      | 0.25        | <0.016 | <0.016 | 0.125 | <0.016 | 2  | 2  | 16  |
| Jiangsu/2019–3      | 0.5         | <0.016 | 0.031 | 0.5   | 0.5   | 1  | 0.5 | 16  |
| Ningxia/2019–4      | 0.5         | <0.016 | 0.031 | 0.25  | 0.063 | 8   | 2  | 8   |
| Ningxia/2019–5      | 0.5         | <0.016 | 0.031 | 0.25  | 0.063 | 4   | 4  | 16  |
| Heilongjiang/2019–1  | 0.5         | 0.031 | 0.031 | 0.125 | 0.063 | 4   | 2  | 8   |
| Hebei/2018–1        | 0.125       | <0.016 | <0.016 | 0.031 | 0.031 | 2   | 2  | 8   |
| Hebei/2018–2        | 1           | <0.016 | 0.063 | 0.125 | 0.031 | 8   | 8  | 16  |
| Shandong/2019–5     | 0.125       | <0.016 | <0.016 | 0.063 | <0.016 | 2   | 1  | 4   |

Table 3  Distribution of MIC values of the tested antimicrobials against the 32 *M. synoviae* isolates

| Antimicrobials | MIC Values (μg/mL) |
|---------------|--------------------|
|               | 0.016   | 0.031   | 0.063   | 0.125   | 0.25   | 0.5     | 1     | 2     | 4     | 8     | 16    | 32    | 64    |
| LS            | 1       | 4       | 3       | 10<sup>50</sup> | 8<sup>50</sup> | 1       |
| VA            | 1<sup>10</sup>–<sup>90</sup> | 1       |
| TIF           | 16<sup>10</sup> | 10      | 6<sup>30</sup> |
| TY            | 4       | 3       | 8       | 5<sup>30</sup> | 6       | 4<sup>20</sup> | 2       |
| TIL           | 7       | 7       | 7<sup>20</sup> | 3       | 5<sup>30</sup> | 3       |
| DO            | 1       | 3       | 10      | 7<sup>30</sup> | 8<sup>30</sup> | 3       |
| OT            | 7       | 6       | 10<sup>30</sup> | 8<sup>30</sup> | 1       |
| ENR           | 4       | 13<sup>30</sup> | 12<sup>30</sup> | 3       |

The MIC values are expressed in μg/mL. Superscript numbers indicate the MIC<sub>50</sub> and MIC<sub>90</sub> values.
OT), fluoroquinolones (e.g., ENR), as well as LS. In this study, we investigated the antimicrobial susceptibility of 32 *M. synoviae* strains isolated from China from 2016 to 2019. Our results showed that the MIC values of *M. synoviae* isolates were generally low for LS, pleuromutilin, and macrolides. Similar results were observed in a recent study in Asia, except for TIL [21]. When spectinomycin was applied in combination with lincomycin, it improved the efficacy of two antimicrobials against most *M. synoviae* [22]. In this study, 31/32 isolates were sensitive to low concentrations of LS (MICs 0.063 ~ 1 μg/mL). A previous study investigated the antibiotic susceptibility of 41 *M. synoviae* strains originating from Central and Eastern Europe between 2002 and 2016, including Hungary, Austria, the Czech Republic, Slovenia, Ukraine, Russia, and Serbia. Overall, similar low MIC values (0.25 ~ 2 μg/mL) were detected for LS [13]. The macrolides showed good activity against *M. synoviae* strains worldwide, but higher MIC values (> 2 μg/mL) were also identified in Europe [13, 23, 24]. In the current study, all isolates were sensitive to low concentrations of TY and TIL with MICs

| Strains          | gyrA SNP | AA     | gyrB SNP | AA     | parC SNP | AA     | parE SNP | AA     |
|------------------|----------|--------|----------|--------|----------|--------|----------|--------|
| MS-H             | 2442     | 814    | 2410–2411| 804    | 1250     | 417    | 254      | 85     |
| ATCC25204        | C        | Gln    | A        | Thr    | G        | Ser    | C        | Thr    | C      | Pro    |
| Hebei/2016–1     | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Hebei/2016–2     | C        | Gln    | AA       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Hebei/2016–3     | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Hebei/2018–1     | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Hebei/2018–2     | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Jiangsu/2018–1   | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Jiangsu/2018–2   | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Jiangsu/2018–3   | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Jiangsu/2018–4   | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Jiangsu/2018–5   | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Jiangsu/2018–6   | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Jiangsu/2019–1   | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Jiangsu/2019–2   | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Jiangsu/2019–3   | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Jiangsu/2019–4   | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Jiangsu/2019–5   | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Shandong/2016–1  | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Shandong/2017–1  | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Shandong/2017–2  | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | C      | Pro    |
| Shandong/2018–1  | A        | Lys    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Shandong/2019–2  | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Shandong/2019–3  | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | C      | Pro    |
| Shandong/2019–4  | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
| Shandong/2019–5  | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Anhui/2019–1     | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | C      | Pro    |
| Anhui/2019–2     | C        | Gln    | GG       | Gly    | G        | Ala    | A        | Asn    | T      | Ile    | T      | Ser    |
| Henan/2019–1     | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | C      | Pro    |
| Hubei/2016–1     | C        | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | C      | Pro    |
| Heilongjiang/2019–1 | C    | Gln    | GG       | Gly    | G        | Ala    | G        | Ser    | T      | Ile    | T      | Ser    |
of only 2/32 and 3/32 isolates = 1 μg/mL. In contrast, TIL MICs clearly showed a time-dependent gradual transition to high concentrations in 154 M. synoviae isolates from Italy collected from 2012 to 2017. Seven M. synoviae isolates showed an MIC > 32 μg/mL for TIL between 2013 and 2016 [25]. High MIC values were also detected in another study, which showed 25/87 M. synoviae strains with high MIC values (> 8 μg/ml for TIL and/or > 1 μg/ml for TY and/or > 0.5 μg/ml for tylovadosin) from 18 different countries from 1982 to 2019 [26]. As mentioned above, increased TIL MICs (≥ 64 μg/ml MIC<sub>90</sub> values) were also detected in M. synoviae isolates collected from China, India, Indonesia, Malaysia, the Philippines, the Republic of Korea, and Thailand [21]. Our results confirmed the high efficiency of TY and TIL against M. synoviae in China. Previous research has shown that pleuromutilins display high efficacy against avian mycoplasmas [27]. To date, the MIC values for TIF in the Europe mentioned above are relatively low (0.004 ~ 2.5 μg/mL). All M. synoviae isolates remained sensitive to TIF with MICs ranging from 0.12 to 2.5 μg/mL in South Africa between 2003 and 2015 [16]. The MIC values of VA were ≤ 0.039 μg/mL in Central and Eastern Europe [26]. The M. synoviae isolates examined in this study showed high susceptibility to VA (MIC < 0.016 ~ 0.031 μg/mL) and TIF (MIC < 0.016 ~ 0.063 μg/mL). Especially, 31 M. synoviae isolates had MIC values equal or lower than the lowest concentration of VA (0.016 μg/mL). Therefore, pleuromutilins are supposed to be preferable in the treatment of M. synoviae infection.

For tetracyclines, 3/32 of M. synoviae isolates showed intermediate MIC values for DO, and only one strain showed an intermediate MIC value for OT. The MIC<sub>50</sub> and MIC<sub>90</sub> values for DO were same as OT, being 2 and 4 μg/mL respectively. The finding of tetracycline resistance was not unexpected because of the long-term widespread use of tetracyclines in feed in China. Aureomycin, DO and OT are the most widely used antimicrobials. It can not only prevent bacterial infection but also improve the growth performance of animals. Our results indicated that long-term use of tetracycline antimicrobials can reduce the sensitivity of M. synoviae. Interestingly, even though the M. synoviae isolates did not have high MIC values of tetracyclines, our results do not align with previous studies in Europe, which showed that the MIC values of OT were higher than those of DO. For example, MIC values of OT and DO to M. synoviae were 0.031 ~ 32 μg/mL and 0.062 ~ 2 μg/mL, respectively, from six European countries from 2014 to 2016 [24]. In another study, 84 M. synoviae field strains were collected from 18 different countries from 2010 to 2019, and the majority of strains were from Hungary, Italy, the Netherlands, Israel, and Spain. The MIC values of OT and DO were ≤ 0.25 ~ 8 μg/mL and ≤ 0.039 ~ 1.25 μg/mL, respectively [26]. The difference in MIC value may be due to the geographic area, density of poultry flocks, and different quantitative uses among countries. Since 2020, all forms of growth-promoting antimicrobials, except for traditional Chinese medicines have been forbidden to be used as feed additives in China. With the increase in the number of laws and regulations concerning the use of antimicrobials, we speculate that the resistance of M. synoviae to tetracyclines may be decreased in the future.

Previous studies showed that resistance to ENR increased rapidly [17, 28, 29]. In this study, high MIC values (4 ~ 32 μg/mL) for ENR were present in all M. synoviae isolates. The MIC<sub>50</sub> and MIC<sub>90</sub> values of ENR were the highest, with 12 isolates showing MIC values of 16 μg/mL, 3 isolates showing MIC values of 32 μg/mL. In Israel and Europe, decreased susceptibility to ENR was detected in 59% of M. synoviae field strains, with MICs ranging from 1 to > 16 μg/mL [17]. There is no standardized method for MIC testing in animal mycoplasma, but genetic mutations can determine the presence of antimicrobial resistance. To further investigate the mechanism of ENR resistance, gyrA, gyrB, parC, and parE genes in the QRDRs of 32 MS isolates were sequenced and analyzed. Topoisomerase IV (parC) is considered to be the primary target of ENR in M. synoviae, based on decreased susceptibility after experimental infection in vivo [18]. Amino acid positions 85 ~ 89 (80 ~ 84 according to Escherichia coli strain K-12 substrain MG1655) of parC were identified as hot spot regions that seem to have a principal role [26]. Amino acid substitutions at positions 80 and 84 of parC are known as important spots for ENR resistance in many bacteria, including mycoplasmas and may be alone or together with a mutation of gyrA [30 ~ 33]. In addition, amino acid substitutions at positions 79 and 81 of parC were also identified in mycoplasma [17, 34]. In the current study, comparison of the parC QRDR identified a mutation at nucleotide position 254 (C254T) resulting in a Thr 85 lle amino acid change in all M. synoviae isolates and ATCC 25204, which was similar to a previous study [26]. In the gyrB gene, a SNP mutation has been found at position 1250 (G1250A) and resulted in a Ser 417 Asn amino acid change in 11/32 M. synoviae isolates with high MIC values to ENR, which has also been reported [17].

Comparison of the gyrA QRDR found the presence of different amino acid substitutions at positions 686, 804 and 814. Mutations at Glu 804 Gly and Thr 868 Ala were identified in all M. synoviae isolates and ATCC 25204. To our knowledge, these nonsynonymous mutations have not been reported in previous research. Our results revealed that there is a correlation between MIC values and amino acid mutations at positions 804 and 686 of
the gyrA QRDR. Indeed, *M. synoviae* isolates containing these two amino acid substitutions had MICs ranging from 4 to 32 μg/mL. These two amino acid mutations may together affect *M. synoviae* with decreased susceptibility to ENR. More strains with a broader spectrum of MICs should be identified to prove this conclusion, and the relevance of the mutations that occurred in gyrA QRDR should be further investigated. The mutation in the QRDR of parE at positions 197 (Pro to Ser) in 27/32 *M. synoviae* isolates has also not been reported. Amino acid substitution at position 420 of parE corresponds to residue 426 Asp of gyrB in *E. coli*, which is a multiple possible marker for quinolone resistance in many bacteria [35, 36]. The role of position 197 of parE in quinolone resistance needs to be further established.

In conclusion, 32 *M. synoviae* isolates had low MIC values for the combination of lincomycin and spectinomycin, pleuromutilin and macrolides. However, 3/32 and 1/32 *M. synoviae* isolates showed intermediate MIC values for DO and OT. High MIC values for ENR were detected in all isolates, with MICs ranging from 4 to 32 μg/mL. Furthermore, mutations at Glu 804 Gly and Thr 866 Ala of gyrA QRDR were identified in all *M. synoviae* isolates and ATCC 25204. The mutation in the QRDR of the parE gene resulted in amino acid changes at positions 197 (Pro to Ser) in 27/32 *M. synoviae* isolates. These nonsynonymous mutations were first identified to be related to ENR resistance.

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Authors’ contributions
XRZ and MJG designed the study, performed most of the experiments. DX performed the molecular biology experiments. YC and YZC participated in the molecular biology experiments. CCZ and YTW discussed the results and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analysed during the current study available from the first author (E-mail: zxr@yzu.edu.cn) on reasonable request.

Declarations
Ethics approval and consent to participate
No animals or animal samples were used in the study. The ethics approval and consent to participate was not required.

Consent for publication
Not applicable.

Competing interests
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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