Original Article

Effects of in vitro-induced drug resistance on the virulence of Streptococcus

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Abstract

This study aimed to evaluate the effects of in vitro-induced drug resistance on the virulence of Streptococcus. Micro-dilution method was used to determine the minimal inhibitory concentration (MIC). In vitro-induced drug resistance was conducted for S. agalactiae (CVCC1886) and S. dysgalactiae (CVCC3701) by gradually increasing the antimicrobial concentration (strains were from IVDC, China). PCR was used to detect the resistance and virulence genes of the strains before and after resistance induction. Colony morphology was observed to compare the physiological and biochemical properties of the strains. A total of 88 clean-grade Kunming mice (obtained from Inner Mongolia University, Hohhot, China) were used in half of the lethal dose (LD50) test for detecting the changes in virulence of strains. The results showed that S. agalactiae (CVCC1886) and S. dysgalactiae (CVCC3701) developed resistance against seven kinds of antibiotics, respectively. Resistance and virulence genes of CVCC3701 were changed when treated by the Penicillin-inducing. The growth of the CVCC3701-PEN was decreased compared to the CVCC3701. Virulence test in mice indicated that the LD50 of CVCC3701 before induction and CVCC3701-PEN after induction were 5.45 × 10^6 and 5.82 × 10^8 CFU/ml, respectively. Compared with the untreated bacteria, the bacterial virulence was reduced 1.1 × 10^2 times after resistance induction. In conclusion, S. dysgalactiae (CVCC3701) is a susceptible strain of drug resistance to antibiotics, in vitro-induced drug resistance reduced the virulence of CVCC3701, but the virulence is still existing and also could result in the death of mice. For public health safety, it must be alert to the emergence of drug resistance of Streptococcus in animal production.

Keywords
drug resistance, Streptococcus, virulence
1 | INTRODUCTION

Streptococcus agalactiae (S. agalactiae) are the main pathogens that causing mastitis and invasive disease in cows (Jain et al., 2012), whereas Streptococcus dysgalactiae (S. dysgalactiae) is environmental pathogens (Guérin-Faublée et al., 2002). Various species of Streptococcus are known to be associated with bacterial infections in cattle, pigs, horses, sheep, birds, aquatic mammals, and fishes (Ding et al., 2016). Zoonotic transmission of S. dysgalactiae subsp. Equisimilis has been previously suggested by Silva et al. (2015), and S. agalactiae is also a human pathogen that mainly cause neonatal infections (Poyart et al., 2003). In recent decades, animal production has tended to a high-density and intensive production model (Guo et al., 2020a; Liu et al., 2020), leading to frequent occurrence of animal diseases, and antibiotics are therefore widely used to prevent and treat animal infectious diseases (Guo et al., 2020b; Liu et al., 2019). The antibiotic environment in livestock production already existed that was below the antimicrobial concentration and could induce bacterial resistance (Stolker et al., 2013). On the other hand, the sub-inhibitory concentration of antibiotics will also affect the virulence of bacteria by toxin production, adhesion, movement (Dal Sasso et al., 2002, 2003). In the process of adapting to antibiotics of sub-inhibitory concentration, ecological characteristics of bacteria will change adaptively, thus showing polymorphism (Friman et al., 2015). In particular, the reproductive performance of cows has been found to decrease as the antimicrobial resistance increases (Guérin-Faublée et al., 2002). The issue of resistance of Streptococcus is not only a great concern in animal health and production, but also exert serious effects on both the environment and public health safety (Tian et al., 2019).

Streptococcus possesses a variety of virulence factors that contribute to pathogenicity, such as surface proteins and adhesion factors are involved in adhesion, invasion of host cells, and escape of the immune system (Brooks & Mias, 2018). It has been demonstrated that the surface components of bacteria, including capsulated polysaccharides, protein components (Ca, Cβ, ribose), laminin-binding proteins (Lmb), and C5a peptidase, which were related to virulence (Maisey et al., 2008). The α-antigen and β-antigen were found to be encoded by the bac and bca genes (Beigverdi et al., 2014; Lindahl et al., 2005). The cly and cfb genes encode haemolysin and the cyclic adenosine mono-phosphate (CAMP) factor, respectively (Dmitriev et al., 2002). However, to the best of our knowledge, the effect of Streptococcal resistance on virulence has been poorly described. Therefore, the purpose of this study was to induce drug resistance of Streptococcus, and analyse the resistance changes and virulence-related genes, with the expectation to explore the relationship between drug resistance and virulence, so as to lay a scientific basis for effective therapy of animal diseases caused by Streptococcus.

2 | MATERIALS AND METHODS

2.1 | Antimicrobial susceptibility test

The S. agalactiae (CVCC1886) and S. dysgalactiae (CVCC3701) were obtained from the China Institute of Veterinary Drug Control (IVDC, China), and they were revived by subculture on Mueller-Hinton agar plates supplemented with 5% defibrinated sheep blood and incubated at 37°C for 18–24 hr aerobically in 5% CO2 for antimicrobial susceptibility test. Minimal inhibitory concentrations (MICs) were determined using the micro-dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2018). This method used Muller Hinton (MH) broth and consisted of manually prepared 96-well microtitre plates containing the following seven antibiotics from the IVDC: penicillin, amoxicillin, cefalotin, norfloxacino, ofloxacin, ciprofloxacin, erythromycin.

Each antibiotic tested was diluted using a two-fold dilution pattern and wells containing different concentrations (0.125, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, 32.0, 64.0 and 128 μg/ml) were prepared. The inoculum was prepared by suspending several colonies of Streptococcus in MH broth and adjusting the value of optical density at 625 nm (OD625) to 0.1 (about 1 × 10^8 CFU/ml). The final bacterial concentration was diluted to 1 × 10^6 CFU/ml of 50 ml per well. The plates were covered and incubated at 37°C for 18–20 hr. At the same time, S. pneumoniae ATCC 49,619 was used as the control strain. All susceptibility results were complied with the quality control ranges.

2.2 | In vitro-induced drug resistance assay

The in vitro-induced drug resistance study was based on a previous report (Yao et al., 2018). Briefly, the preserved standard strains were inoculated into the Brain Heart Infusion (BHI) broth with serum, and incubated at 37°C for 6 hr. A small amount of bacterial solution was picked to streak MH agar plates and incubated at 37°C for 16–20 hr. BHI broth containing the sub-inhibitory concentration of antibiotic was prepared and incubated at 37°C. Meanwhile, the negative control of broth was made and transferred every 3 days. The concentration of the induced antibiotic was gradually increased by two times until the MIC of the test strain rose to the resistance range.

2.3 | Detection of antimicrobial resistance genes and virulence factors

The resistant strains that displayed phenotypic resistance to antibiotics were further tested for the presence of antimicrobial resistance genes. PCR was used to amplify GyrA and GyrB genes contributing to quinolone resistance. Pbp1α, Pbp1b and Pbp2x conferring resistance to β-lactam. ermA, ermB and mefA genes encoding resistance
to erythromycin. Primers, amplicon size and annealing temperatures are listed in Table 1. PCR method was also performed to detect virulence genes (\textit{bca}, \textit{cfb}, \textit{cyl}, \textit{glnA}, \textit{hyIB}, \textit{lmb}, \textit{scpB}) in strains before and after induction.

### 2.4 Detection of physiological and biochemical properties in strains before and after induction

The conventional microbiological method was used to detect the physiological and biochemical properties, and the growth of bacteria (Cui et al., 2003). The test strains were selected with large differences before and after induction according to the detection of genes, which were streaked on MH agar plates supplemented with 5% defibrinated sheep blood and incubated at 37°C for 18–24 hr to observe colony morphology and hemolysis. Single colony was selected and placed in MH broth that adjusting the bacterial concentration to $1 \times 10^8$ CFU/ml. One ml of the tested bacteria solution was added to 99 ml of MH broth with 5% serum for oscillating culture in 37°C. The absorbance value of the solution was measured every 1–14 hr at OD625 nm, and the growth curve was plotted.

### 2.5 Detection of LD50 on tested strains

Half of the lethal dose (LD50) was determined by Bliss method as previous reported (Bliss, 1936). The experimental design was divided into a blank control and two experimental groups, they are shown in Table 2. Briefly, a total of 88 clean-grade Kunming mice (7 weeks, the body weight is between 18–22 g, purchased from Inner Mongolia
University, Hohhot, China) were used in the animal LD50 detection experiment. Firstly, after one week of adaptive feeding, they are divided into equal groups, and half of the male and female in each group. Both drinking water and litter were autoclaved. Each group of five mice, adjust the concentration of bacterial solution, determine LD0 and LD100. Based on the results, expand the number of experimental animals and verify the results. According to the values of LD0 and LD100, three concentrations were set in equal proportions in the middle to form five concentration gradients, with eight mice in each group. After the challenge, observe for 14 days, record the number of deaths, and calculate LD50 according to the Bliss method.

3 | RESULTS

3.1 | Antimicrobial resistance in Streptococcus and detection of antimicrobial resistance genes

Streptococcus were tested for their susceptibility to seven antimicrobial agents before and after induction, and the results are shown in Table 3. All induced strains were resistant to the antibiotics tested. The induced strains that demonstrated resistance to quinolones, the results are shown in Table 4. S. agalactiae (CVCC1886) had contained resistance genes before induction. The resistance phenotypes of S. dysgalactiae (CVCC3701) all reached the level of resistance, whereas no quinolone resistance genes were detected after induction. For the strains demonstrating resistance to β-lactams, the results are shown in Table 5. Pbp1a and Pbp2b were identified from CVCC1886 before and after induction. CVCC3701 did not carry three resistance genes before induction, whereas Pbp1a and Pbp2b genes were detected by penicillin induction. In the erythromycin-induced resistant strains, ermB, ermA and mefA genes were not identified.

3.2 | Detection of virulence genes

The strains before and after resistance induction were tested by PCR for the presence of seven genes potentially involved in virulence, and the results are presented in Table 6. Seven virulence genes were present in CVCC1886 and induced strains. In contrast, cyl gene was only identified in CVCC3701, all virulence genes were discovered in CVCC3701-PEN and other induced strains did not change against CVCC3701.

3.3 | Detection of physiological and biochemical properties in strains before and after induction

According to Tables 5 and 6, CVCC3701 increased resistance genes Pbp1a and Pbp2b, virulence genes bca, scpB, lmb, glnA, cfb, scaA and hydB had been also found after being induced by penicillin. So CVCC3701 and CVCC3701-PEN were selected as tested strains. Their appearance presented smooth, raised and round small colonies on the blood nutrient agar plate with typical rings of hemolysis. CVCC3701 and CVCC3701-PEN were stained by the gram, and the bacteria were observed as round, blue-purple, double or short chain arrangement under an oil microscope. The growth curve of tested strains was plotted by OD625 measurement, the growth and propagation rate of CVCC3701-PEN was slower than that before induction.

3.4 | Detection of LD50 on tested strains

The LD0 and LD100 of the tested strains were obtained by pretest. The LD0 and LD100 of CVCC3701 were 4.0 × 10⁵ and 1.0 × 10⁸ CFU/ml, whereas CVCC3701-PEN were 1.4 × 10⁸ and 1.4 × 10¹⁰ CFU/ml, respectively. Bliss statistical software was used to analyse the data. The LD50 of tested strains are shown in Table 7. The results suggested that the LD50 of the induced strain was 1.1 × 10² times higher than before, indicating that the virulence of the induced strain was lower than that before induction.

4 | DISCUSSION

With the wide application of antimicrobial agents in clinical therapy and prevention, the antimicrobial resistance has been increasing, especially the unreasonable use of antibiotics. The emergence of resistance brings great obstacles to the clinical treatment of

| Strain                  | MIC (μg/ml) | NOR | OFLX | CIP | PEN | AMX | CEF | ERY |
|-------------------------|-------------|-----|------|-----|-----|-----|-----|-----|
| CVCC1886 (S. agalactiae)|             |     |      |     |     |     |     |     |
| Before                  | 4           | 2   | 0.5  | 4   | 4   | <0.25| <0.25|
| After                   | 64          | 256 | 128  | 64  | 64  | 8   | 64  |
| CVCC3701 (S. dysgalactiae)|            |     |      |     |     |     |     |     |
| Before                  | 2           | 1   | <0.25| 1   | 1   | <0.25| <0.25|
| After                   | 64          | 256 | 128  | 64  | 64  | 8   | 64  |

Abbreviations: AMX, amoxicillin; CEF, cefalotin; CIP, ciprofloxacin; ERY, erythromycin; MIC, minimum inhibitory concentration; NOR, norfloxacin; OFLX, ofloxacin; PEN, penicillin.
**TABLE 4**  Resistance genes of quinolones

| Resistance genes | **S. agalactiae** | **S. dysgalactiae** |
|------------------|------------------|-------------------|
|                  | CVCC1886 | NOR | OFLX | CIP | CVCC3701 | NOR | OFLX | CIP |
| GyrA             | +        | +   | +    | +   | −        | −   | −    | −   |
| GyrB             | +        | +   | +    | +   | −        | −   | −    | −   |

Abbreviations: −, negative; +, positive; CIP, ciprofloxacin; NOR, norfloxacin; OFLX, ofloxacin.

**TABLE 5**  Resistance genes of β-lactam antibiotics

| Resistance genes | **S. agalactiae** | **S. dysgalactiae** |
|------------------|------------------|-------------------|
|                  | CVCC1886 | PEN | AMX | CEF | CVCC3701 | PEN | AMX | CEF |
| Pbp1a            | +        | +   | +   | +   | −        | +   | −    | −   |
| Pbp2b            | +        | +   | +   | +   | −        | +   | −    | −   |
| Pbp2x            | −        | −   | −   | −   | −        | −   | −    | −   |

PEN: Penicillin, AMX: Amoxicillin, CEF: Cefalotin, +: positive, −: negative.

**TABLE 6**  Virulence genes of strains before and after induced resistance

| Tested strains | Virulence genes |
|----------------|-----------------|
|                | **bca** | **scpB** | **lmh** | **cyl** | **glnA** | **cfb** | **hylB** |
| **S. agalactiae** (CVCC1886) | +  | +  | +  | +  | +  | +  | +  |
| NOR             | +  | +  | +  | +  | +  | +  | +  |
| OFLX            | +  | +  | +  | +  | +  | +  | +  |
| CIP             | +  | +  | +  | +  | +  | +  | +  |
| PEN             | +  | +  | +  | +  | +  | +  | +  |
| AMX             | +  | +  | +  | +  | +  | +  | +  |
| CEF             | +  | +  | +  | +  | +  | +  | +  |
| ERY             | +  | +  | +  | +  | +  | +  | +  |

| **S. dysgalactiae** (CVCC3701) | −  | −  | −  | +  | −  | −  | −  |
| NOR             | −  | −  | −  | +  | −  | −  | −  |
| OFLX            | −  | −  | −  | +  | −  | −  | −  |
| CIP             | −  | −  | −  | +  | −  | −  | −  |
| PEN             | +  | +  | +  | +  | +  | +  | +  |
| AMX             | −  | −  | −  | +  | −  | −  | −  |
| CEF             | −  | −  | −  | +  | −  | −  | −  |
| ERY             | −  | −  | −  | +  | −  | −  | −  |

NOR: Norfloxacine, OFLX: Ofloxacin, CIP: Ciprofloxacin, PEN: Penicillin, AMX: Amoxicillin, CEF: Cefalotin, ERY: Erythromycin, +: positive, −: negative.

Abbreviations: −, negative; +, positive; AMX, amoxicillin; CEF, cefalotin; CIP, ciprofloxacin; ERY, erythromycin; NOR, norfloxacin; OFLX, ofloxacin; PEN, penicillin.
production has led to an increase in the resistance of Streptococcus et al., 2012). In recent years, the abuse of antibiotics in livestock has brought difficulties to prevention and control the infectious diseases, thereby reducing the production performance (Jain et al., 2001) found that the virulence of Streptococcus, which has brought difficulties to prevention and control the Streptococcus caused diseases (Ding et al., 2016). Previous studies have demonstrated that the acquisition of antimicrobial resistance has an important effect on the change in their virulence (Alejandro et al., 2013). It has also been reported that the adsorption, colonization, invasion and tissue damage of pathogen are always related to virulence factors (Brooks & Mias, 2018). In regards to the Streptococcus, invasion and tissue damage of pathogen are always related to virulence factors (Brooks & Mias, 2018). In regards to the Streptococcus, Rieux et al. (2001) found that the virulence of Streptococcus pneumoniae was significantly reduced due to the changes of PbpX and Pbp2b after the action of penicillin. Padilla et al. (2010) suggested that the efflux pump caused resistance while virulence would be increased. Meanwhile, Ismaeel et al. (2005) found that the ability of cytotoxicity of drug resistance and virulence in S. dysgalactiae and S. dysgalactiae. In this study, seven virulence genes were found in S. agalactiae CVCC1886, only cyl gene was detected in S. dysgalactiae CVCC3701 before and after induction, which indicated S. agalactiae might be more virulent. The resistance genes (pbl1a and pbl2b) were detected in the CVCC3701-PEN, and the virulence genes were increased compared with those before induction. However, the virulence test showed that the virulence of the strain was reduced after induction, which was about 100 times. The virulence of S. dysgalactiae CVCC3701 was greatly weakened when resistance was induced in vitro. Similar to our study, it has been previously indicated that tetracycline and clindamycin significantly increased the expression of the agr virulence regulator at sub-inhibitory concentrations, and increased the production of phenol-soluble modulin cytolysins of MRSA (Joo et al., 2010). However, Ohlsen et al. (1998) revealed that β-lactam up-regulated alpha toxin (hla) gene expression.

| Strains                   | Groups | Concentration (CFU/ml) | Number of mice | Inoculation method | Inoculation dose | Deaths number | LD50 (CFU/ml) |
|---------------------------|--------|------------------------|----------------|--------------------|----------------|---------------|---------------|
| S. dysgalactiae CVCC3701  | 4-LD100| 1.0 × 10^9             | 8              | Intraperitoneal injection | 0.5 ml         | 8             | 5.45 × 10^8   |
|                           | 4-A    | 1.4 × 10^6             | 8              |                     |                | 7             |               |
|                           | 4-B    | 1.98 × 10^7            | 8              |                     |                | 3             |               |
|                           | 4-C    | 2.8 × 10^6             | 8              |                     |                | 1             |               |
|                           | 4-LD0  | 4.0 × 10^5             | 8              |                     |                | 0             |               |
| S. dysgalactiae CVCC3701-PEN (penicillin) | 5-LD100| 1.4 × 10^10            | 8              | Intraperitoneal injection | 0.5 ml         | 8             | 5.82 × 10^6   |
|                           | 5-A    | 4.4 × 10^9             | 8              |                     |                | 7             |               |
|                           | 5-B    | 1.1 × 10^9             | 8              |                     |                | 6             |               |
|                           | 5-C    | 4.4 × 10^9             | 8              |                     |                | 3             |               |
|                           | 5-LD0  | 1.4 × 10^8             | 8              |                     |                | 0             |               |

TABLE 7 Detection of LD50 in mice
of Staphylococcus aureus, while aminoglycoside down-regulated hla, lacZ gene expression. Also, it was reported that low concentrations of antibiotics could also act as intraspecific or interspecific signalling molecule in bacteria (Rutherford & Bassler, 2012; Yim et al., 2007). These findings implied that the influence of sub-inhibitory concentration of antibiotics on bacterial virulence could enhance or weaken, both of which are possible.

There might be several considerations: the expression of virulence factor decreased after induced resistance. Mutations in the genes of bacteria during induction might lead to abnormalities in the genes encoding the pathogenic factors or their regulators. For instance, Sakoulas et al. (2002) found that the decreased expression of virulence factor of vancomycin intermediate Staphylococcus aureus (VISA) was related to the abnormal function of the regulatory gene in S. aureus. Under the pressure of long-term drug selection, bacteria are less virulent in order to adapt to the environment. In Salmonella typhimurium, the mutation of rpsL gene conferred resistance to streptomycin. The resulting amino acid substitution (K42N) in ribosomal protein S12 caused an increased rate of ribosomal proofreading, which slowed down protein synthesis, bacterial growth rate and virulence (Mainsnier-Patin et al., 2002). Some structures of bacteria (such as ribosomes, RNA polymerases, DNA rotating enzymes, cell walls, etc.) were changed when it develop resistance, resulting in reduced growth rate, colonization ability and pathogenicity of bacteria. In other words, the acquisition of resistance of bacteria requires the fitness cost (Enne et al., 2004). The decreased virulence of bacteria might be associated with the slower growth and reproduction rate. The VanA gene, which mediates vancomycin resistance in S. aureus, it can be divided into induced resistance and intrinsic resistance due to differences in operand level expression (Arthur et al., 1992). There were different types of VanA in MRSA, their adaptability is different, and the growth capacity of the bacterial strains decreased by 20%–38% after in vitro-resistance induction. In contrast, the growth capacity decreased by only 0.04%–0.3% (Foucault et al., 2009). The growth and reproduction rate of VISA was significantly slower than that of the standard strain (Cui et al., 2003). Because of the slowing down of growth and reproduction, the amount of toxin released by bacteria is reduced, which is beneficial for the body to produce an immune response. Therefore, the relationship between bacterial resistance and virulence needs to be further studied.

5 | CONCLUSIONS

To summarize, S. dysgalactiae (CVCC3701) is a susceptible strain of drug resistance to antibiotics, in vitro-induced drug resistance reduced the virulence of CVCC3701, but the virulence is still exists and also could result in the death of mice. For the public health safety, it must be alert to the emergence of drug resistance of Streptococcus in animal production.

CONFLICT OF INTEREST

The authors report that they have no conflicts of interest.

AUTHOR CONTRIBUTION

Yue-Xia Ding: Conceptualization; Data curation; Formal analysis; Methodology; Writing-original draft; Writing-review & editing. Qun Wu: Data curation; Methodology. Yan Guo: Writing-review & editing. Man Li: Formal analysis. Pei-Feng Li: Formal analysis. Yi Ma: Conceptualization; Funding acquisition; Supervision; Writing-review & editing. Wen-Chao Liu: Writing-original draft; Writing-review & editing.

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DATA AVAILABILITY STATEMENT

All public data generated or analysed during this study are included in this article. Data sharing is not applicable to this article as no new data were created or analysed in this study.

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