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Nebulization for *Mycoplasma* Control in Goats - Enhancing the Therapeutic Efficacy of Tiamulin Fumarate

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ABSTRACT

***Background*:** Contagious caprine pleuropneumonia (CCPP) presents a significant peril to the well-being of goats, manifesting in clinical indications such as coughing, elevated body temperature, and the presence of fibrinous and serous inflammation in the pleura and lungs. Furthermore, it can give rise to secondary complications, including arthritis, urinary and reproductive tract infections, and mastitis. Tiamulin fumarate is a frequently employed pharmaceutical agent for the management of CCPP, exhibiting good therapeutic efficacy against *Mycoplasma* infection in goats. Presently, the predominant route of administration for tiamulin fumarate is through injection. Nevertheless, this approach possesses inherent constraints and fails to adequately address the exigencies of prompt treatment for goats harboring latent infections or displaying subclinical symptoms. Consequently, the optimization of drug administration methods has emerged as a crucial imperative in the realm of agricultural production.

***Materials, Methods & Results*:** A total of 15 goats naturally infected with *Mycoplasma* were selected and randomly divided into 3 groups. Group A received nebulization, Group B received intramuscular injection (IM) as the control, and Group C served as the blank control. Nasal swab samples were collected from the 3 groups of goats at 0, 7, and 15 days after the start of treatment. Quantitative real-time PCR was used to detect the intensity of *Mycoplasma* infection. The weight and clinical symptom changes of the goats in the 3 groups were recorded before and after treatment. The results showed that in Group A, the reduction rates of *Mycoplasma* infection intensity were recorded as 78.27% and 85.29% at both 7 and 15 days following the initiation of treatment compared to day 0. In Group B, the reduction rates of *Mycoplasma* infection intensity were calculated to be 76.39% and 80.60% at 7 and 15 days at both 7 and 15 days following the treatment compared to day 0. In Group C, the average intensity of *Mycoplasma* infection exhibited an increase at both 7 and 15 days in comparison to day 0. In Group A, the average weight gain rates of goats at 7, 15, and 30 days after the start of treatment were 5.64%, 14.11%, and 31.87% in comparison to day 0. These rates were better than those of Group B (5.20%, 11.20%, and 25.44%). The weight gain rates were also superior to those of Group C (5%, 9.54%, and 21.86%). All goats in Group A had their clinical symptoms disappear within 7 days of treatment, while in Group B, all goats had their clinical symptoms disappear within 15 days of treatment. However, in Group C, the clinical symptoms of goats showed no improvement within 30 days.

***Discussion*:** The study investigates the efficacy of nebulized tiamulin fumarate in treating 15 naturally infected goats with CCPP by employing quantitative methods in order to investigate changes in infection intensity, weight, and clinical symptoms following medication through nebulization and IM injection. The findings revealed that nebulization exhibits marginally superior therapeutic effects compared to intramuscular injection. Moreover, nebulized tiamulin fumarate has shown a more pronounced impact on promoting goat growth. Additionally, nebulization serves a preventive function for other goats within the same group while concurrently treating symptomatic goats.

Keywords: tiamulin fumarate, therapeutic efficacy, nebulization, *Mycoplasma* sp., caprine, goats, CCPP.

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INTRODUCTION

Animal husbandry is crucial for global food security and economic development, but the persistent threat of diverse infectious diseases poses a multitude of challenges to the field of animal husbandry[6,8-10]. Contagious caprine pleuropneumonia (CCPP), a highly transmissible respiratory ailment in goats[3,13], threatens the global goat farming industry [17]. CCPP manifests with clinical indications like coughing, elevated body temperature, and inflammation in the pleura and lungs, leading to secondary complications and increased mortality in goats[5,13,16,19,22,23].

In recent times, there has been a growing prevalence of CCPP in goats on a global scale [4,19], with its widespread occurrence observed in numerous provinces engaged in goat farming within China [24].

Tiamulin fumarate, a highly effective antibiotic [7,12,21], exhibits efficient antibacterial properties, rapid assimilation, and widespread dissemination [14,15,21]. Nevertheless, conventional administration techniques exhibit certain constraints in their implementation.

This study focuses on investigating the efficacy of nebulized tiamulin fumarate for CCPP treatment in goats. Through analysis of data about Mycoplasma infection intensity, weight changes, and clinical symptoms, this research aims to provide a theoretical framework for rapid mass drug administration. The goal is to improve treatment options for *Mycoplasma* infection in goat farming, enhancing industry well-being and sustainable growth.

MATERIALS AND METHODS

Bacteria, Chemicals and Animals

- Bacteria: The reference strains used in this study, including MO strain Y98, MCCP strain F38, and MMC strain PG3, were generously provided by Professor Yuefeng Chu from the Veterinary Institute, Chinese Academy of Agricultural Sciences. *Escherichia coli* (C83600), *Salmonella* (ATCC25923), *Staphylococcus* (ATCC26003), and *Mycoplasma arginini* were preserved strains in the College of Veterinary Medicine, Yangzhou University.

- Chemicals: Tiamulin Fumarate Premix[1], TB Green® Premix Ex Taq™ II (Tli RNaseH Plus)[2], Premix Taq[2], DH-5α[2], TIANquick Midi Purification Kit3, pGM-T3, TIANprep Mini Plasmid Kit[3], DNA Dilution Buffer (for Real Time PCR)[4], *Mycoplasma* Broth Medium[5].

- Animals: A total of 15 goats displaying intermittent cough, nasal discharge, and other clinical signs indicative of contagious caprine pleuropneumonia (CCPP), which were subsequently confirmed as having clinical *Mycoplasma* infection through routine PCR analysis, were sourced from a farm located in Nantong City, Jiangsu Province. The goats were then allocated into 3 distinct groups, namely, A, B, and C, each consisting of 5 goats. Group A received nebulization, Group B received IM injection as the control, and Group C served as the blank control. Group A was administered nebulization of tiamulin fumarate premix [5 mg/kg once daily for 3 consecutive days], in accordance with the recommended dosage provided by the company. Group B received IM injection of tiamulin fumarate premix [5 mg/kg once daily for 3 consecutive days], following the recommended dosage by the company. Group C did not receive any medication treatment. Nasal swab samples were collected from the affected goats in Groups A, B, and C at 0, 7, and 15 days after the initiation of medication in Groups A and B.

Quantitative real-time PCR detection of Mycoplasma

A highly conserved segment of the 16S rRNA gene sequences of MA and MO was identified by aligning the published data in GenBank. This segment was selected for the design of universal primers for the detection of both species (Table 1). The expected size of the amplification fragment was 278bp. Reference strains were cultured after retrieval from the ultralow temperature freezer, and DNA templates were extracted using the boiling method. Subsequently, gel electrophoresis was performed following PCR amplification. PCR products obtained from positive samples were isolated through agarose gel electrophoresis and subsequently ligated into the T vector employing Taq enzyme. The ligated products were then introduced into DH-5α competent cells, cultured, and sequenced, and plasmids were extracted. A standard curve was constructed utilizing qPCR, which was followed by experiments to assess the repeatability, sensitivity, and specificity.

Table 1. Primer sequences for qPCR.

Primers	Sequence	Amplified product
M-F	GCTAACTATGTGCCAGCAGCC	278 bp
M-R	TGTTTGCTCCCCACGCTTTC	

Mycoplasma *detection and clinical observation in goats after nebulization*

Extract DNA templates by boiling 0.5 mL nasal swab samples. The qPCR methodology was utilized to assess the magnitude of *Mycoplasma* infection, conducting 3 tests per sample and subsequently determining the average value. The relative reduction rate of *Mycoplasma* infection in Groups A and B after 7 and 15 days of nebulization was calculated in comparison to the initial day. Ascertain the weight measurements of Groups A and B at days 0, 7, 15, and 30 and subsequently compute the average weight and growth rate. Document the clinical symptom observations of Groups A, B, and C at days 0, 7, 15, and 30 following nebulization.

Statistical analysis

Statistical *t* tests were conducted using GraphPad Prism to assess the mean pathogen infection intensity within each of the 3 groups (A, B, and C) at various time intervals, as well as the average weight gain rates of the goats between each pair of groups during the same time intervals. $P > 0.05$ indicates no statistically significant difference; $0.01 < P < 0.05$ suggests the presence of a statistically significant difference; $0.001 < P < 0.01$ implies a highly significant difference and $P < 0.001$ indicates an extremely significant difference.

RESULTS

Establishment of the qPCR method

The specificity of PCR primers was verified through amplification using MA and MO, showing specific bands at 278bp for both strains, while the negative control exhibited no bands. Subsequent targeted fragment sequencing and comparison with the NCBI database confirmed 100% homology with the MA 16S rRNA gene sequence (accession number: MN401232.1) and 99% to 100% homology with other MA 16S rRNA gene sequences. The successful construction of the recombinant plasmid standard was confirmed by PCR amplification and subsequent quantification to 9.2×10^{10} copies/μL. A robust standard curve was established through fluorescence quantitative PCR, displaying a desirable amplification dynamic curve with an "S"- shaped pattern, a distinct melting peak at 84.16°C, and a linear correlation from 10^3 to 10^{10} copies/μL, with an equation of Y=-3.423X+39.50, R^2 value of 0.9972, and an amplification efficiency (E) of 96%. The method demonstrated strong reproducibility, with coefficients of variation not exceeding 2% for within-batch and between-batch repeatability experiments. Moreover, the sensitivity of the method was determined to be 2.5×10^2 copies/μL, surpassing that of conventional PCR by 100-fold. Last, the specificity experiment confirmed the method's remarkable specificity in detecting *Mycoplasma* strains GY1 and Y98 while yielding negative results for other strains, such as PG3, F38, *Escherichia coli*, *Salmonella*, and *Staphylococcus*.

Detection of Mycoplasma *in nasal swab samples from goats*

In Group A, the average intensity of *Mycoplasma* infection exhibited a significant decrease at both 7 and 15 days following the initiation of treatment compared to day 0, indicating highly significant statistical disparities ($P < 0.001$). Furthermore, a decrease was observed at 15 days in comparison to 7 days, with significant differences ($0.01 < P < 0.05$). The reduction rates of *Mycoplasma* infection intensity were recorded as 78.27% and 85.29% at 7 and 15 days post-administration, respectively (Figure 1).

In Group B, the average intensity of *Mycoplasma* infection exhibited a significant decrease at both 7 and 15 days following the treatment compared to day 0 ($P < 0.001$). Additionally, there was a decrease in intensity observed at 15 days compared to 7 days, although this difference did not reach statistical significance ($P > 0.05$). The reduction rates of *Mycoplasma* infection intensity were calculated to be 76.39% and 80.60% at 7 and 15 days postadministration, respectively (Figure 2).

In Group C, the average intensity of *Mycoplasma* infection exhibited an increase at both 7 and 15 days in comparison to day 0; however, these changes were not statistically significant ($P > 0.05$). Furthermore, a further increase was observed at 15 days in comparison to 7 days, but again, no significant differences were observed ($P > 0.05$) [Figure 3].

Statistics on weight gain in goats after nebulization

The average weight gain rates of Group A goats at 7 days, 15 days, and 30 days were 5.93%, 15.92%, and 31.45%. The average weight gain rates of Group B goats at 7 days, 15 days, and 30 days were 5.79%, 15.10%, and 30.61%. The average weight gain rates of Group C goats at 7 days, 15 days, and 30 days were

4.71%, 13.44%, and 28.89%. Through a comparison of the weight changes and average weight gain rates among 3 distinct groups of goats at 7 days, 15 days, and 30 days post administration, it was observed that both Groups A and B exhibited higher average weight gain rates than Group C at 7 days, 15 days, and 30 days. The weight gain rates of goats in Group A at 15 days and 30 days were found to be significantly different from those of goats in Group C ($0.01 < P < 0.05$). Conversely, the weight gain rates of goats in Group B at 7 days, 15 days, and 30 days did not exhibit any statistically significant differences when compared to those of goats in Group C ($P > 0.05$). Furthermore, although the weight gain rates of goats in Group A were higher than those of goats in Group B at 7 days, 15 days, and 30 days, these differences were not statistically significant ($P > 0.05$) [Figure 4].

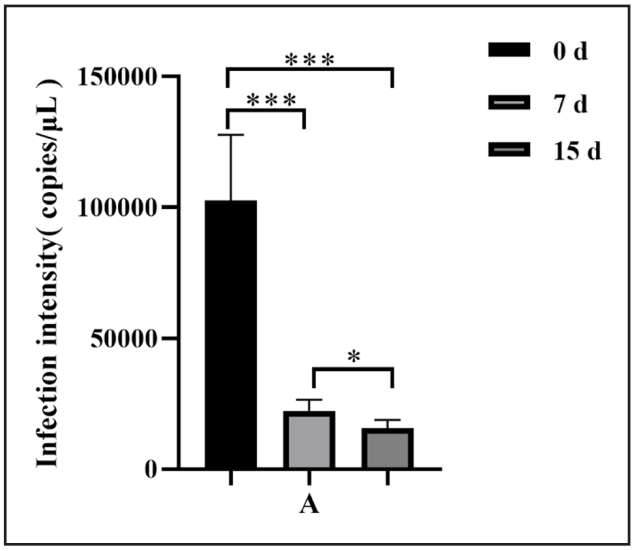

Figure 1. qPCR results of goat nose swab samples in group A. [*$0.01 < P < 0.05$ (statistically significant difference); ***$P < 0.001$ (extremely significant difference)].

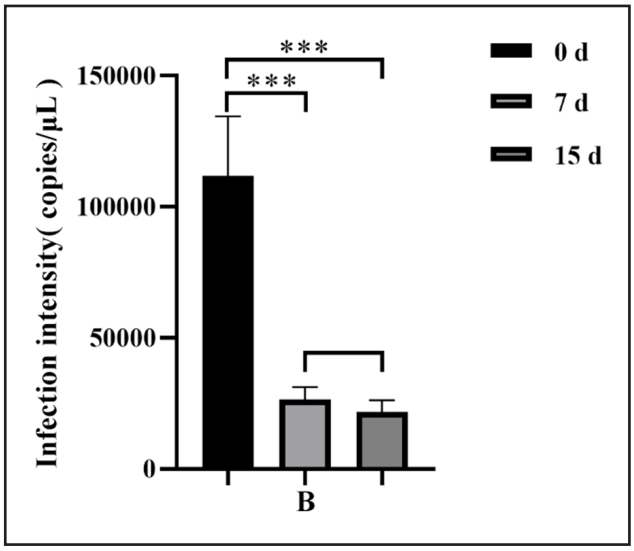

Figure 2. qPCR results of goat nose swab samples in group B. [***$P < 0.001$ (extremely significant difference); Unmarked represents $P > 0.05$].

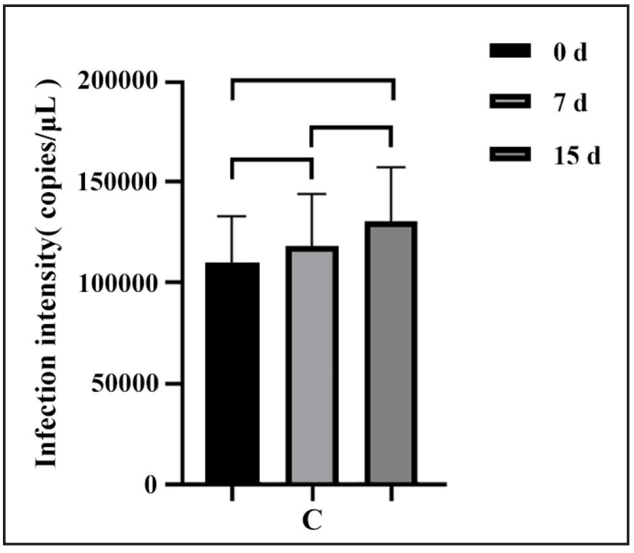

Figure 3. qPCR results of goat nose swab samples in group C. [Unmarked represents $P > 0.05$].

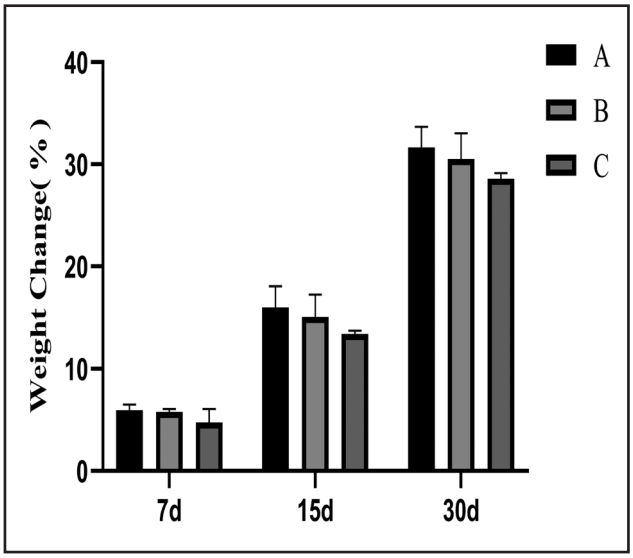

Figure 4. Results of weight change of goats in the 3 groups.

Clinical symptoms in goats after nebulization

Clinical symptoms were observed in 3 distinct groups of goats at 4 different time points (0 day, 7 days, 15 days, and 30 days) following the commencement of medication. Specifically, at the initial time point (0 day), all 3 groups of goats displayed varying degrees of intermittent coughing, nasal discharge, and other clinical symptoms. In Group A, all 5 goats demonstrated the resolution of coughing, nasal discharge, and other clinical symptoms after 7 days. No recurrence of clinical symptoms was observed at 15 and 30 days. In Group B, 60% goats exhibited the resolution of clinical symptoms at 7 days. The remaining 40% goats showed significant improvement in intermittent coughing, nasal discharge, and other symptoms. All 5 goats in Group B showed the resolution of clinical symptoms at 15 days, and there was no recurrence of clinical symptoms within 30 days. However, all 5 goats Group C did not exhibit any improvement in intermittent coughing, nasal discharge, or other clinical symptoms at 7, 15, and 30 days when compared to 0 day. These findings indicate that the clinical symptoms of the affected goats will experience a significant improvement following treatment with tiamulin fumarate, with nebulization resulting in a better therapeutic effect compared to IM injection.

DISCUSSION

Quantitative real-time PCR not only inherits various advantages of conventional PCR, such as high sensitivity and rapid detection[2] but also enables the quantitative analysis of *Mycoplasma* [1]. It is currently extensively applied in the detection and quantitative analysis of *Mycoplasma* infections [11,18,20]. In this work, a pair of universal primers was designed based on the conserved 16S rRNA gene sequences of MO and MA to establish a fluorescent quantitative TB GREEN II staining method. The R2 value of the fluorescent qPCR detection method was 0.9972, and the amplification efficiency E was 96%. The standard curve of the recombinant plasmid shows a strong linear relationship between 103 and 1010 copies/μL. The detection limit was 2.5x102 copies/μL, whereas the conventional PCR detection limit of the primer was 2.5x104 copies/μL. The sensitivity of the qPCR detection method is 100 times higher than that of the conventional PCR detection method. Both the intra- and intergroup variation coefficients of the qPCR detection method were within 2%, indicating good reproducibility. The sensitivity and reproducibility of the detection method meet the requirements of this experiment and can be used to measure the intensity of *Mycoplasma* infection in diseased goats after medication.

Analysis of the *Mycoplasma* infection intensity of 3 groups of diseased goats at 0 day, 7 days, and 15 days. The average *Mycoplasma* infection intensity of diseased goats in the A group and B group decreased significantly at 7 days and 15 days compared to 0 day ($P < 0.001$). Furthermore, there was a decrease at 15 days compared to 7 days. Conversely, the average *Mycoplasma* infection intensity in the C group increased at 7 days and 15 days compared to 0 day. This suggests that tiamulin fumarate has a discernible therapeutic effect on CCPP infection. Meanwhile, the average *Mycoplasma* infection intensity in the A group decreased by 78.27% and 85.29% at 7 days and 15 days after the commencement of administration compared to 0 d, slightly outperforming the B group's 76.39% and 80.60%, respectively. This suggests that the eradication effect of tiamulin fumarate on *Mycoplasma* within diseased goats via nebulization is marginally superior to that via IM injection. Meanwhile, based on the observed changes in body weight, the average weight gain rates of Group A goats at 7 days, 15 days, and 30 days were 5.93%, 15.92%, and 31.45%, respectively. Similarly, Group B goats exhibited weight gain rates of 5.79%, 15.10%, and 30.61% at the corresponding time points. Notably, both Group A and Group B demonstrated superior average weight gain rates compared to Group C, which recorded rates of 4.71%, 13.44%, and 28.89%, respectively. These findings suggest that the administration of tiamulin fumarate contributes to the promotion of goat growth to a certain extent and that nebulization demonstrates a more substantial effect in enhancing goat growth. Observations of clinical symptoms in the A group and the B group of diseased goats revealed that the improvement in clinical symptoms at 7 days was more pronounced in the A group than in the B group. Moreover, during this period, no diseased goat was observed among the initially clinically healthy goats in the same group. This suggests that tiamulin fumarate via nebulization can serve as a preventive measure for other goats within the same group while treating clinically symptomatic goats.

CONCLUSION

Both the nebulization and IM injection of tiamulin fumarate demonstrate promising therapeutic effects in treating contagious caprine pleuropneumonia (CCPP) in goats. Notably, nebulization exhibits marginally superior therapeutic effects compared to IM injection. Moreover, nebulized tiamulin fumarate has shown a more pronounced impact on promoting goat growth. Additionally, nebulization serves a preventive function for other goats within the same group while concurrently treating symptomatic goats.

MANUFACTURERS

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***Ethical approval*.** All animals were handled in strict accordance with good animal practice as defined by the Animal Ethics Procedures and Guidelines of the People's Republic of China. The study protocol was approved by the Animal Care and Use Committee of the College of Veterinary Medicine, Yangzhou University (Approval ID: SCXK [Su] 2021-0013).

***Declaration of interest*.** 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. The authors alone are responsible for the content and writing of paper.

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