



The formation and drug resistance mechanism of biofilm for *Streptococcus pneumoniae* infection in severe respiratory patients

Haiyan Sun^{1*}, Xinlou Chai², Guangxun Xu³, Shuangying Wei¹

¹Department of Respiratory Fever, Dongfang Hospital Beijing University of Chinese Medicine, Beijing, 100078, China

²College of Basic Medicine, Beijing University of Traditional Chinese Medicine, Beijing, 100105, China

³Department of Emergency, Beijing Hospital of Traditional Chinese Medicine Affiliated to Capital Medical University, Beijing, 100010, China

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ABSTRACT

This study was to explore whether *Streptococcus pneumoniae* would form biofilms and the formative factors of biofilms, as well as the drug resistance mechanism of *S. pneumoniae*. In this study, a total of 150 strains of *S. pneumoniae* were collected from 5 local hospitals in the past two years, and the minimum inhibitory concentrations (MIC) of levofloxacin, moxifloxacin and penicillin were determined by agar double dilution method to select the drug-resistant strains. The polymerase chain reaction (PCR) amplification and sequencing were performed on specific genes of drug-resistant strains. In addition, 5 strains of *S. pneumoniae* with penicillin MIC ≤ 0.065 $\mu\text{g/mL}$, 0.5 $\mu\text{g/mL}$, 2 $\mu\text{g/mL}$, ≥ 4 $\mu\text{g/mL}$ were randomly selected, and the biofilms were cultured on two kinds of well plates for 24 hours. Finally, whether the biofilms were formed was observed. Experimental results revealed that the resistance rate of *S. pneumoniae* to erythromycin in this area was as high as 90.3%, and the strains that were resistant to penicillin account for only 1.5%. The amplification and sequencing experiment revealed that one (strain 1) of the strains, which was resistant to both drugs, had a GyrA mutation and ParE mutation, and strain 2 had a parC mutation. All strains generated biofilms, and the optical density (OD) value of penicillin MIC ≤ 0.065 $\mu\text{g/mL}$ group (0.235 ± 0.053) was higher than that of 0.5 $\mu\text{g/mL}$ group (0.192 ± 0.073) ($P < 0.05$) and higher than the OD value of the 4 $\mu\text{g/mL}$ group (0.200 ± 0.041) ($P < 0.05$), showing statistically great differences. It was confirmed that the resistance rate of *S. pneumoniae* to erythromycin remained high, the rate of sensitivity to penicillin was relatively high, and the moxifloxacin and levofloxacin-resistant strains had appeared; *S. pneumoniae* mainly showed QRDR mutations in gyrA, parE, and parC; and it was confirmed that *S. pneumoniae* can generate biofilms *in vitro*.

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Introduction

Streptococcus pneumoniae is a common pathogen of community-acquired respiratory infections. Recent studies have found that *S. pneumoniae* can also cause low immunity, bacterial meningitis, and sepsis. According to the data issued by World Health Organization (WHO) (1), about 2 million people die from *S. pneumoniae* infection every year in the world, second only to tuberculosis, and the incidence of related diseases in China ranks second in the world (2). With the large number of various antibacterial drugs used in clinical treatment, the drug resistance and multiple drug resistance of bacteria are becoming more and more serious. At present, it is generally believed that the enhancement of antibiotic drug resistance by the *S. pneumoniae* strain and the gradual increase of related drug resistance strains are the root cause of the increasing incidence of infectious diseases of this strain (3). Especially after the 1980s (4), *S. pneumoniae*, which has drug resistance, became popular worldwide, especially *S. pneumoniae*, which has high drug resistance to penicillin, also began to appear in many countries. It has brought great difficulties to traditional treatment and has seriously threatened the health of people all over the world (5). Therefore, studying the drug resistance mechanism of *S. pneumoniae*

and clarifying its drug resistance model is of great significance for the development of new antibiotics and the guidance of clinical medication.

In recent years, the new definition of biofilm is a biological community formed by microorganisms in a fixed state. The main feature of biofilm after the updated definition is the irreversible adhesion of microbial cells to the substrate, interface or mutual adhesion (6); and it will produce a considerable amount of extracellular polymer matrix and bury itself in it, and change the genotype with the change of growth rate and gene expression (7). Biofilm mainly has the ability to reduce the sensitivity to antibacterial drugs and evade host immune defenses. Many chronic infections in the history of human diseases are related to the formation of biofilm. Infection and colonization of *S. pneumoniae* in the human body are related to the formation of biofilm (8). According to previous research results, whether *in vitro* or *in vivo*, both invasive and normal-free strains have the ability to form a biofilm (9). Many factors affect the formation of biofilms, such as nutrients *in vitro*, pH, osmotic pressure, antibiotics, bacterial typing, and extracellular matrix. The impact of antibiotics on biofilm varies greatly depending on the concentration greatly (10).

In summary, understanding the mechanism of *S. pneumoniae* drug resistance and mastering the formation pro-

* Corresponding author. Email: dingkezai4059@163.com

cess of *S. pneumoniae* biofilm are not only conducive to the clinical treatment of *S. pneumoniae* infectious diseases and the control of the epidemic of drug resistance strains, but also of great significance for exploring the bacterial drug resistance and the discovery of new targets for drug effects.

Materials and Methods

Experimental strains

In this study, 150 strains of *S. pneumoniae* from 5 local hospitals in the two years from 2019 to 2020 were collected, and the corresponding clinical information (patient gender, age, specimen collection location, etc.) was collected and recorded. The Colombian blood plates were adopted to transfer the strains. The inclusion criteria were defined as follows: patients diagnosed with a respiratory critical illness at the time of admission. The exclusion criteria were defined as follows: patients diagnosed with asthma or bronchiectasis, or patients with other life-threatening diseases.

Identification of strains

The collected strains were immediately inoculated on a Columbia Petri dish. After the strains were cultured in an incubator at 35°C and 5% CO₂ for 24 hours, the morphology of the strains was observed, and the colonies that were umbilical concave and suspected of alpha hemolysis were collected for further bile lysis and Optochin test. The one that passed the experiment was *S. pneumoniae*, which was stored in a magnetic bead strain preservation tube at -80°C.

Determination of minimum inhibitory concentration (MIC)

According to the MIC breakpoint of the latest version of the drug susceptibility test standard (The Clinical and Laboratory Standards Institute, CLSI) (11), the MIC was determined by using the agar dilution method recommended by the National Committee for Clinical Laboratory Standardization (NCCLS). Firstly, the cryopreserved bacteria were resuscitated and inoculated on the blood plate. It was estimated that 50 strains of bacteria can be measured on each plate and cultured in the incubator for 19 hours. Secondly, the MH agar was prepared according to the method of the product specification and autoclave, and a considerable proportion of the concentration of antibacterial drugs was prepared. According to the purpose of the experiment, a total of 5 concentration gradients were prepared this time. When the MH agar was cooled to about 50°C, it was mixed with 5% defiberized sheep blood, and a drug agar plate was prepared in a ratio of 1:19. Then, the MH agar mixed with sheep blood was added to the antibacterial drugs of different concentrations, and mixed thoroughly. The mixed agar was poured on a sterilized plate to ensure that the thickness of the agar was about 3 mm. Thirdly, a bacterial suspension with a concentration of 0.5 McDonald's standard was prepared, a sterile cotton swab was adopted to spot bacteria on the surface of the plate, and the inoculated agar plate was placed in an incubator at 35°C and 5% CO₂ for 20 hours. Fourthly, after the incubation, the plate was taken out of the incubator and placed on a dark, non-reflective table to determine the end of the test. The lowest drug concentration that inhibited bacterial

growth was taken as the MIC.

DNA extraction of *S. pneumoniae*

Based on the previously measured drug susceptibility results, *S. pneumoniae* that had drug resistance to levofloxacin, and moxifloxacin were selected, the strains were resuscitated, and the instructions on the bacterial genomic DNA extraction kit had to be followed.

Firstly, 2 mL of bacterial suspension was prepared with physiological saline (containing as many bacteria as possible), which was placed in a centrifuge tube and centrifuged at 10,000 rpm for one minute to remove the supernatant as completely as possible. Secondly, 20 mM Tris (pH 8.0) was mixed with 2 mM Na₂-EDTA, 1.2% Triton X-100, and lysozyme with a final concentration of 20 mg/mL to configure the Enzymatic Lysis Buffer. Then, 180 µL of Enzymatic Lysis Buffer was added to resuspend the bacteria. Thirdly, the bacteria were incubated at 37°C for half an hour. Fourthly, they were added with 20 µL of Proteinase K and mixed well, added with 200 µL of Buffer GL and mixed well, and then incubated again at 56°C for half an hour. Fifthly, after 200 µL of absolute ethanol and mixed thoroughly, all the mixed solution was added to the adsorption column and centrifuged at 10,000 rpm for one minute. The unnecessary waste liquid in the collection tube was poured out, and then the solution was placed back into the collection tube. Sixthly, after 500 µL of Buffer GW1 was added, the mixture was centrifuged at 10000 rpm for one minute to discard the waste liquid. The above actions were repeated and the adsorption column was put back into the collection tube. Seventhly, after 500 µL of Buffer GW2 was added to the adsorption column, the mixture was centrifuged at 10000 rpm for one minute to discard the waste liquid, and the adsorption column was put back into the collection tube. Eighthly, the mixture was centrifuged at 12000 rpm for two minutes to discard the waste liquid, and the adsorption column was placed at room temperature until it was completely dried. Ninthly, the adsorption column was placed in a new centrifuge tube, added with 100 µL of Buffer GE to the middle of the adsorption column, and placed at room temperature for five minutes. Then, the solution was centrifuged at 10,000 rpm for one minute to collect the DNA. Finally, a microplate reader was applied to detect the concentration, and the DNA was stored at -20°C.

Gene Sequencing

The PCR primers (Shanghai Jierui Company) needed in this study were as follows:

parC: (Forward 5'-TGGGTTGAAGCCGTTCA-3'; Reverse 5'-TGCTGGCAAGACCGTTGG-3'), pare: (Forward 5'-AAGGCGCGTGATGAGAGC-3'; Reverse 5'-TCTGCTCCAACACCCGCA-3'), gyrA: (Forward 5'-CCGTCGCATTCTTTACG-3'; Reverse 5'-AGT-TGCTCCATTAACCA-3') and gyrB: (Forward 5'-TTCTC-CGATTTCTCATG-3'; Reverse 5'-AGAAGGGTAC-GAATGTGG-3')

After PCR amplification was completed, the amplified products were taken, and the sequencing results were compared with the sequence of the standard strain D39 published by GenBank.

Culture of biofilm

The frozen strains were resuscitated and 5 strains of

S. pneumoniae with penicillin MICs ≤ 0.065 $\mu\text{g}/\text{mL}$, 0.5 $\mu\text{g}/\text{mL}$, 2 $\mu\text{g}/\text{mL}$, and ≥ 4 $\mu\text{g}/\text{mL}$ were randomly selected according to the results of previous drug resistance experiments. They were inoculated on the Columbian blood tablet in an incubator at 37°C and 5% CO₂ for 20 - 24 hours.

Then, it should cultivate the biofilm, and the specific procedures can refer to foreign literature for specific cultivation methods. Firstly, an inoculating loop was adopted to transfer the colony into a sterile test tube containing Todd Hewitt Broth (THB) medium, and a turbidimetric meter was applied to adjust the bacterial solution to 0.5 wheat concentration. The 20 strains were divided into 3 equal parts and inoculated on a 96-well plate, and the THB medium without the inoculated strains was undertaken as a blank control group, and all were cultured in an incubator at 35°C and 5% CO₂ for 24 hours. Secondly, the liquid in the 96-well plate was slowly poured out and washed 3 times with phosphate buffer saline (PBS). In addition, the 96-well plate was dried at 60°C for 1 hour. Thirdly, after it was dyed with 1% crystal violet dye for 15 minutes, the excess dye was dried and dried for 15 minutes. Finally, the well plate was cleaned with ethanol with a purity of 95% and measured the optical density (OD) value at a single wavelength of 570 nm with a microplate reader. The measurement was repeated 3 times and the average value was taken and recorded.

Determination of biofilm

In this experiment, FITC-labeled concanavalin A (FITC-ConA) and propidium iodide (PI) solution were selected for double staining. The former can bind to the polysaccharide in biofilm and emit green fluorescence, and the latter can bind to the DNA in the bacteria, emitting red fluorescence. The concentration of FITC-ConA was 5 $\mu\text{g}/\text{mL}$, and the concentration of the PI solution was 100 $\mu\text{g}/\text{mL}$.

One strain from each of the four groups of strains with different MICs was selected for Confocal laser scanning microscope (CLSM) detection. The selected strains were cultured on a blood plate and placed in an incubator at 35°C and 5% CO₂ for 18-24 hours. An inoculating loop was adopted to transfer the cultured colonies into a sterile test tube containing THB culture solution, and the turbidity meter was applied to adjust the concentration of the bacterial solution to 0.5 wheat concentration. A cover slip was put in a 24-well plate, which was added with 2 mL of adjusted concentration of *S. pneumoniae* solution to each well. Then, the 24-well plate was placed in an incubator at 35°C and 5% CO₂ for 24 hours, and then the cover slides were taken out. The 24-well plate was rinsed with PBS and fixed with 2.5% glutaraldehyde solution for 1.5 hours; the coverslip was rinsed with PBS solution again and stained with FITC-CONA 4°C in the dark for half an hour. The PI was stained at 4°C for 15 minutes in the dark, and rinsed with PBS for 5 minutes on a horizontal shaker. After the above operations were repeated 3 times, it was finally mounted with 40% glycerol-PBS and placed on a laser confocal scanning microscope (Olympus Co., Ltd., Japan) to observe and take the photo.

Statistical analysis

All data in this experiment were expressed by (mean \pm standard deviation), and comparison between groups was performed by analysis of variance. $P < 0.05$ was considered

that the difference was statistically significant.

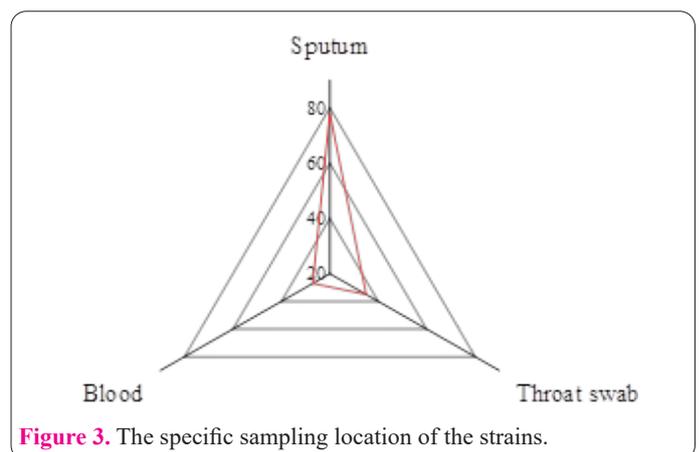
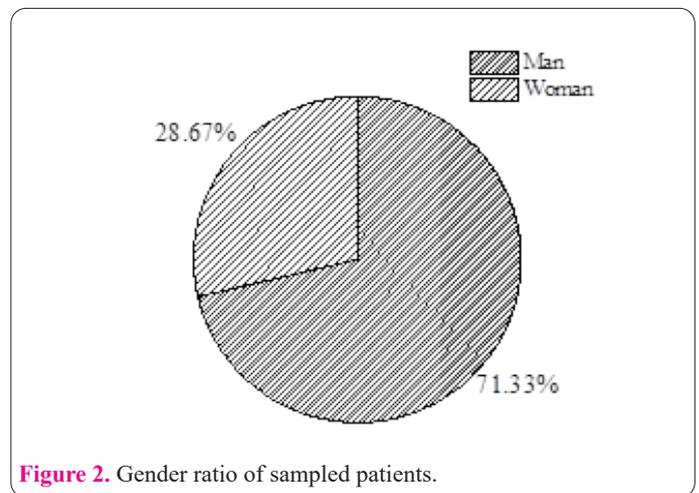
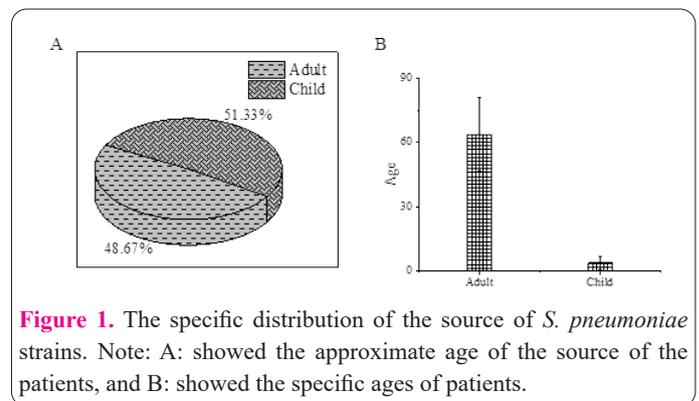
Results

Sources of *S. pneumoniae*

Among the 150 isolates of *S. pneumoniae* collected this time, 73 were from adults with an average age of 63.7 ± 17.3 years old, and 77 were from children with an average age of 3.7 ± 3.3 years old (as shown in Figure 1). There were 107 male patients and 43 female patients (as illustrated in Figure 2). The main source of isolates was sputum, the rest were from throat swabs and blood, and meningitis isolates did not appear, as given in Figure 3 below.

Results of drug resistance

Based on the experimental results, the strains of *S. pneumoniae* selected in this study that had drug resistance



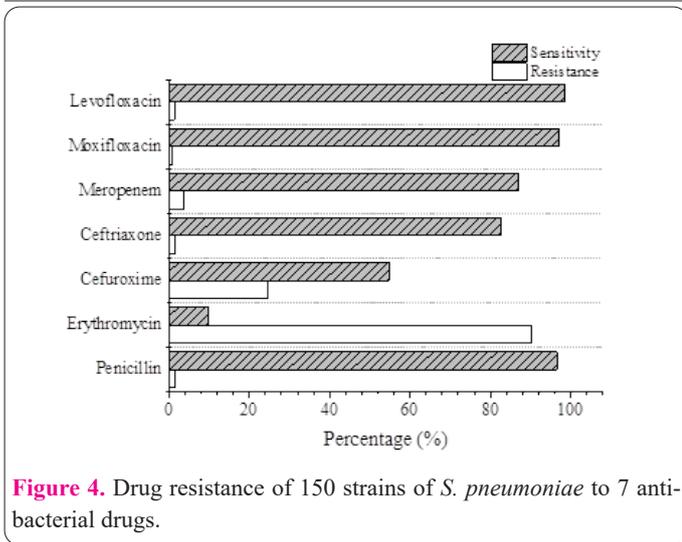


Figure 4. Drug resistance of 150 strains of *S. pneumoniae* to 7 antibacterial drugs.

to penicillin accounted for only 1.5%, and the drug resistance rate to cefuroxime was 24.7%; the rate of sensitivity to ceftriaxone and meropenem was 82.7% and 86.9%, respectively; and the drug resistance rates to these two were only 1.7% and 3.7%, respectively. *S. pneumoniae* was famous for its high drug resistance rate to erythromycin. It can also be seen from the results of this experiment that its drug resistance rate to erythromycin was as high as 90.3%. However, there was still a high rate of sensitivity for moxifloxacin and levofloxacin, which were 97.2% and 98.4%, respectively. The specific drug resistance results were shown in Figure 4.

Gene sequencing results

The amplification and sequencing experiment on QRDR of parC, parE, gyrA, and gyrB genes of *S. pneumoniae*, which had drug resistance to levofloxacin and moxifloxacin revealed that one (strain 1) of the strains, which was resistant to both drugs, had GyrA mutation and ParE mutation, while the remaining gene points did not show amino acid mutations, and the other strain No. 2 showed parC mutations. The other gene points also did not appear as amino acid mutations. Figure 5 below showed the electropherogram of the drug-resistance gene of *S. pneumoniae*.

The drug resistance test results of *S. pneumoniae* against penicillin

According to the latest version of the MIC breakpoint of CLSI, only 1.5% of strains were drug-resistant to penicillin, strains that were not sensitive to penicillin account for 3.4% of all strains, and the MIC of penicillin was mainly distributed in 0.5 - 2 µg/mL, as shown in Figure 6.

Detection of biofilm of *S. pneumoniae*

The adhesion index (average OD value × 0.5/average increase (OD 600)) was introduced in this study to show the final result, and because the OD 600 in this study was the McGill concentration (0.5), the OD value was used directly to express the result.

The OD values of all strains in each group were compared by analysis of variance and showed that the differences were statistically significant. In addition, the differences between the groups were further compared. It was found that the OD value of penicillin MIC ≤ 0.065 µg/mL group (0.235 ± 0.053) was higher than that of 0.5 µg/mL group

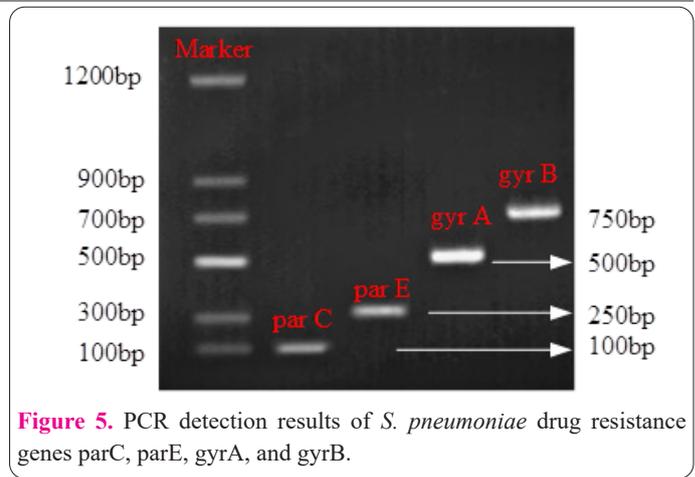


Figure 5. PCR detection results of *S. pneumoniae* drug resistance genes parC, parE, gyrA, and gyrB.

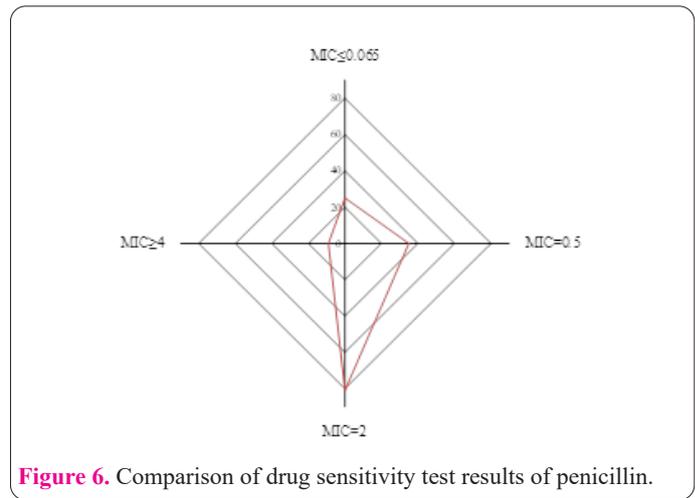


Figure 6. Comparison of drug sensitivity test results of penicillin.

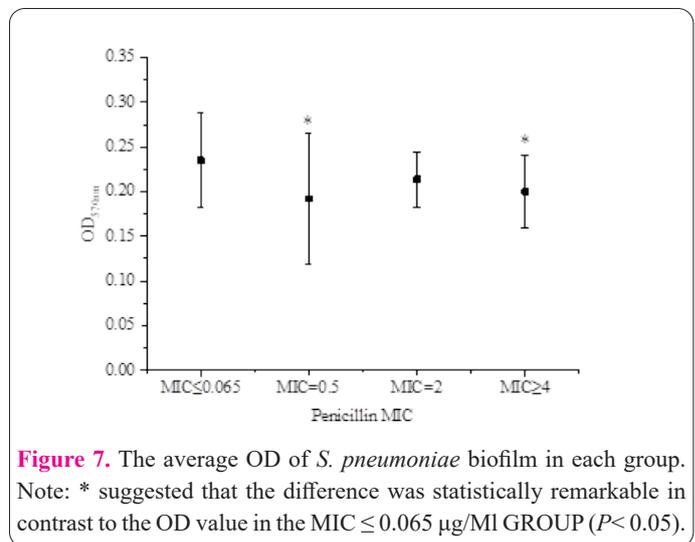


Figure 7. The average OD of *S. pneumoniae* biofilm in each group. Note: * suggested that the difference was statistically remarkable in contrast to the OD value in the MIC ≤ 0.065 µg/MI GROUP ($P < 0.05$).

(0.192 ± 0.073) ($P < 0.05$) and higher than the OD value of the 4 µg/mL group (0.200 ± 0.041) ($P < 0.05$), showing statistically great differences. However, the OD value (0.214 ± 0.031) of the 2 µg/mL group was not much different, and it was not statistically significant ($P > 0.05$); the OD of the strains in the 0.5 µg/mL group, 2 µg/mL group, and ≥ 4 µg/mL group showed no statistically great difference. The comparison of the OD values of each group was shown in Figure 7.

Images of *S. pneumoniae* biofilm under a laser confocal scanning microscope

After CLSM, it can be observed that *S. pneumoniae* initially began to form biofilm after 24 hours of culture.

The combination of FTTC-ConA and the polysaccharide matrix in a biofilm can emit green fluorescence, which proved that bacteria produce polysaccharide matrix and biofilm was formed. In addition, the combination of PI and bacterial DNA showed red light, and the green light emitted by the polysaccharide matrix and the red light emitted by bacterial DNA overlapped each other to show yellow (as shown in Figure 8).

Discussion

S. pneumoniae is a common gram-positive bacterium that usually colonizes the upper respiratory tract of healthy humans. Under normal circumstances, there will be no adverse reactions. This state is called "carrying". But when the carrier's immunity declines, *S. pneumoniae* will migrate along the upper respiratory tract to the middle ear, lungs, brain, and other parts, easily causing otitis media, pneumoniae, or life-threatening meningitis and other diseases (12). In recent years, *S. pneumoniae* has gradually developed drug resistance to various antibiotics due to a large number of antibiotics being abused, especially the drug resistance of macrolide antibiotics (13), leading to changes in the treatment of *S. pneumoniae* infection, which is very tricky. In order to explore the latest drug resistance of *S. pneumoniae* to commonly used antibiotics in the region in the past two years, *S. pneumoniae* strains provided by five hospitals within two years were collected to conduct in vitro drug resistance experiments.

Macrolide antibiotics are the main antibacterial drugs for the traditional treatment of *S. pneumoniae* infectious diseases. However, with the emergence of a large number of *S. pneumoniae* isolates with high drug resistance to macrolide antibiotics, people are also eagerly looking for new effective drugs. Among them, fluoroquinolone antibacterial drugs have entered the scope of clinical treatment due to their strong antibacterial activity (14). However, as the amount of clinical treatment gradually increased, *S. pneumoniae* strains that produced drug resistance to fluoroquinolones also appeared and began to increase. The drug resistance of this type of drug is usually formed gradually, and its sudden mutation often occurs first in *parC* or *gyrA*, with a frequency of about 10^{-6} to 10^{-9} (15). The formation of *S. pneumoniae* biofilm also increases the tolerance of *S. pneumoniae* to fluoroquinolones. The mechanism of action of fluoroquinolone antibacterial drugs is mainly through specific binding with bacterial DNA, blocking bacterial nucleic acid replication to achieve the purpose of sterilization (16). Therefore, the reason why *S. pneumoniae* has drug resistance to fluoroquinolone drugs is the gradual accumulation of spontaneous mutations in its genes, which leads to specific binding region mutations and enhances the drug resistance of the overall strain (17). In this experiment, two selected strains of *S. pneumoniae*, which had drug resistance to fluoroquinolones, showed mutations in the QRDR binding region and found mutations in the *gyrA*, *parE* and *parC* genes that lead to drug resistance. One (strain 1) of the strains, which was resistant to both drugs, had a *GyrA* mutation and *ParE* mutation, while the remaining gene points did not show amino acid mutations, and the other strain No. 2 showed *parC* mutations. The other gene points also did not appear as amino acid mutations.

According to relevant literature research, most bacteria

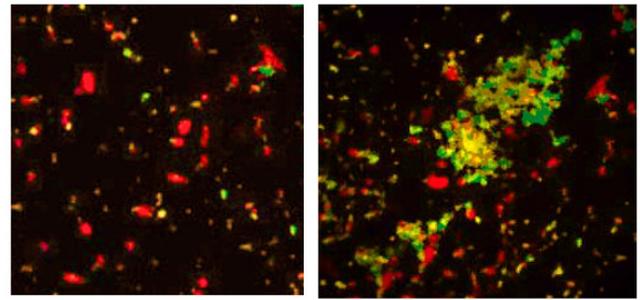


Figure 8. CLSM images of biofilm produced by *S. pneumoniae*. Note: the left image showed the adhered cells and polysaccharide matrix under a 30-fold microscope, and the right image showed the cell aggregation and adhesion under a 60-fold microscope.

can not only improve drug resistance to antibiotics after forming biofilm but also protect themselves from the "tracing" of the host's immune system. Therefore, it can be speculated that the drug resistance of *S. pneumoniae* is also closely related to the formation of biofilm (18). Researchers have found *S. pneumoniae* biofilm in the nasal mucosa of patients with chronic sinusitis, and some studies have found that the antibiotic-sensitive *S. pneumoniae* strain is thicker than the biofilm formed by the drug resistance strain, and studies have also reported that drug resistance caused by the gene level may cause the production of bacterial biofilm to weaken. This experiment also confirmed through CLSM images that the clinically isolated *S. pneumoniae* can form biofilm in vitro. In addition, the biofilm formation of *S. pneumoniae* strains under different penicillin MICs was compared. The experimental results suggested that with the increase of penicillin MIC, the tendency of strains to produce biofilm gradually weakened. For example, in the experimental results, the biofilm-forming ability of strains with penicillin MIC ≤ 0.065 $\mu\text{g/mL}$ was stronger than that of strains with MIC ≥ 4 $\mu\text{g/mL}$.

In summary, the drug resistance of *S. pneumoniae* to common drugs and the drug resistance mechanism in this region were explored in this study. The results suggested that *S. pneumoniae* in this region showed a high rate of drug resistance to erythromycin and a high rate of sensitivity to penicillin, and the moxifloxacin-and levofloxacin-resistant strains had appeared. Regarding moxifloxacin and levofloxacin drug resistance strains, PCR sequencing found that these *S. pneumoniae* had target mutations, mainly QRDR mutations of *gyrA*, *parE*, and *parC*. Finally, it was confirmed that *S. pneumoniae* can produce biofilm in vitro, and it was found that the biofilm production rate of *S. pneumoniae* strain with high penicillin MIC was much lower than that of other groups. This investigation on the drug resistance mechanism of *S. pneumoniae* and whether it would form biofilm provided a new direction for the future clinical treatment of *S. pneumoniae* disease that already had drug resistance, and helped to understand the effect of biofilm on mutation and drug resistance of *S. pneumoniae*.

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