Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Formation ability and drug resistance mechanism of *Klebsiella pneumoniae* biofilm and capsule for multidrug-resistant

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ARTICLE INFO

Original paper

Article history:
Received: April 10, 2023
Accepted: July 15, 2023
Published: October 31, 2023

Keywords:

Biofilm, Drug resistance, Klebsiella pneumoniae, Multidrug-resistant

ABSTRACT

This study was to explore the formation ability of biofilm and capsule and the drug resistance mechanism for multidrug-resistant *Klebsiella pneumoniae*. firstly, 55 strains of *K. pneumoniae* were screened out from the body fluid specimens of the laboratory. The strains were drug-resistant, and the characteristics of clinical infections of these strains were analyzed. Secondly, all strains were tested for the presence of biofilms and capsules, and then the deoxyribonucleic acid (DNA) genomes of the strains extracted were detected using polymerase chain reaction (PCR) technology. Finally, the serotype genes and virulence genes of the strains were screened, and the relationship between these two genes and the formation of capsules and biofilms was analyzed and compared. A new generation of sequencing technology was applied to analyze the genome structure of *K. pneumoniae*, comparative genomics technology was adopted to analyze the drug resistance plasmids, and molecular cloning and other methods were utilized to clone the drug resistance-related genes. of the 55 strains of *K. pneumoniae* isolated clinically, 61.8% came from blood with a total number of 34 strains; 8 strains were from secretion specimens (accounting for 14.5% of the total); and 7 strains were from drainage fluid (accounting for 12.7% of the total), including 2 strains from pus, bile, and pleural fluid, respectively. The strains were tested by PCR, of which iroN virulence genes were the most (34 strains), accounting for 61.8%, followed by wabG and fimH (33 strains, accounting for 60% of the total), followed by magA, K2, K20, K1, and K57. The positive rates of the two virulence genes (fimH and wabG) were higher in positive strains of biofilm. The drug susceptibility results showed that ampicillin and amoxicillin were more resistant to capsule-positive strains than the capsule-negative strains. *K. pneumoniae* had been able to form a complete capsule and biofilm, the formation rate of biofilm was higher than that of the capsule, and there was an increasing trend. The two serotype genes (K20 and K2) accounted for relatively high proportions, and *K. pneumoniae* carried relatively more virulence genes (wabG and fimH), which may be closely related to the capsule production of *K. pneumoniae*. In addition, resistance-related genes were also transferred horizontally in different strains of bacteria, forming a wide range of drug resistance, which brought great difficulties to clinical work.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.10.12

Introduction

Klebsiella pneumoniae belongs to the genus Klebsiella in the Enterobacteriaceae family and is the most important type of bacteria in the genus Klebsiella in the Enterobacteriaceae family. Infections with *K. pneumoniae* account for more than 95% of the total Klebsiella infections (1). It is the resident flora of the upper respiratory tract and intestinal tract of the human body. When the body's resistance is reduced, it enters the lung through the respiratory tract and merges with the adjacent large or small lobes of the lung, which is more common in the upper lobes. *K. pneumoniae* was first discovered by German pathologist E. Friedlander in 1882, and it was described as morphological characteristics, which was also known as Friedlanderella before. *K. pneumoniae* is a kind of gram-negative bacteria. It is a stubby bacterium, single or short chain, without movement ability, and capsule on the surface. Klebsiella has drug resistance to most antibiotics and has strong resistance to the external environment, making it difficult to treat in clinical work. However, under normal circumstances, Klebsiella does not infect the human body. It can only cause disease when there are inducements, such as host defense function defects (2). *K. pneumoniae* is a key pathogen of nosocomial infection, and it also has a high proportion in clinical isolation. In recent years, *K. pneumoniae* is prone to drug resistance with the introduction of broad-spectrum antibiotics, such as β-lactams and glycoside antibiotics. Studies have shown that its drug resistance is mainly due to the bacteria producing cephalosporinase (AmpC enzyme), aminoglycoside modifying enzymes (AMEs), β-lactamases (ESBLs), and other enzymes (3). Therefore, the bacterium is multi-drug resistant to the third-generation cephalosporins and aminoglycoside antibiotics commonly used in clinical practice. In recent years, it has been discovered in clinical work that among the pathogenic bacteria infected in the hospital, the infection rate of *K. pneumoniae* is slowly increasing, and more

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drug-resistant strains appear, which often leads to the failure of clinical treatment and affects the health of patients and the recovery of the disease. Studies have shown that the resistance mechanism of *K. pneumoniae* is also related to the active exclusion of antibacterial drugs and the horizontal spread of antibacterial drug resistance genes in different strains (4). *K. pneumoniae* also often causes urinary tract infections, and its infection rate is second only to *E. coli*. The clinical symptoms caused by *K. pneumoniae* are similar to those caused by *E. coli*, with positive results of urine culture and accompanied by urinary tract irritation. Patients with underlying diseases are more likely to be infected with *K. pneumoniae* than healthy people, causing severe sepsis, and a series of symptoms of infection and poisoning, such as chills, high fever, and profuse sweating (5). Some patients will experience cold shock with rapid pulse, cold limbs, and a drop in blood pressure. The incidence of this kind of septic shock in clinical patients is 63% (6). Some patients are also accompanied by mental changes, as well as abnormal blood clotting. A very small number of patients have migratory lesions, most of which migrate to the heart, kidney, brain, lung, and other parts. The cause of death of patients is generally uncontrolled infection or severe toxemia, and the case fatality rate is extremely high, which can be 50% (7). When the infection migrates to the meninges, there will be clinical manifestations of general purulent meningitis with high fever, headache, confusion, and stiff neck. The most characteristic is the purulent changes in the cerebrospinal fluid. The white blood cells and proteins in Antarctica also increase significantly, but the contents of sugar and chloride are reduced (8). It has been discovered in recent years that the capsule and biofilm formed by Klebsiella can carry many virulence factors, which can make clinically multidrug-resistant and severe infections. Therefore, it is extremely important to study the correlation between the formation ability of capsule and biofilm and the carrying status of virulence genes, so as to solve the problem of drug resistance of *K. pneumoniae* in clinical practice (9).

In 1992, Reid and other researchers used scanning electron microscopy to study the bladder epithelial cells of patients with spinal cord injury, so as to analyze the asymptomatic urinary tract infection caused by *K. pneumoniae*, and confirmed the fact that *K. pneumoniae* produces biofilm (10). In subsequent studies, *K. pneumoniae* isolated from urine, sputum, blood, and wounds can also produce biofilm (11). Studies have shown that biofilm is formed by the participation of capsule and lipopolysaccharide. It is a kind of bacterial aggregate that is tightly attached to the extracellular matrix of polysaccharides, proteins, enzymes, and nucleic acids. Capsules and lipopolysaccharides are conducive to the construction of bacterial biofilm. Some scholars have found that capsules and lipopolysaccharides can participate in the initial coverage of the matrix and the construction of the mature biofilm structure, and play an essential role (12). This is also the reason why the strains with capsules are more likely to produce biofilm. Foreign studies have shown that microbial infections rely on the biofilm of bacteria to a large extent, and the biofilm can prevent the body's immune system from killing strains. One of the mechanisms by which bacteria form drug resistance may be the production of biofilm. Biofilm can also prevent antibiotics from approaching the bacterial cells and protect the bacteria from the interference of antibiotics (13). When bacteria exist in biofilm, the drug resistance of the bacteria can be increased by tens to thousands of times. At this time, the use of antibiotics may cause the bacteria to produce extremely strong drug resistance so that it is not easy to kill the bacteria (14). This is also the reason why the infection occurs several times and is not easy to cure. The existence of *K. pneumoniae* bacteria in some medical devices is also due to the existence of biofilm.

In summary, this study aimed to explore the formation ability of biofilm and capsule of multidrug-resistant *K. pneumoniae*, and to explore the drug resistance mechanism of multidrug-resistant *K. pneumoniae*. 55 *K. pneumoniae* strains were selected as the research objects to analyze the characteristics of clinical infections and test the carrying rates of capsule and biofilm. In addition, a clinically isolated *K. pneumoniae* was analyzed using genome sequencing. The results of this study were intended to provide references for controlling the clinical drug resistance of *K. pneumoniae*, preventing the spread of drug resistance, prolonging the service life of antibacterial drugs, and the clinical treatment and prevention of *K. pneumoniae* with multidrug-resistant.

Materials and Methods

Collection of *K. pneumoniae* bacterial specimen with multidrug-resistant

The *K. pneumoniae* specimens were collected from the Microbiology Room of the Laboratory of Navy 905 Hospital from June 2019 to June 2022. These specimens were derived from aseptic body fluids of patients, including blood, secretions, sputum, and urine. A mass spectrometer and an automatic bacterial identifier were adopted to analyze the collected specimens, and a drug sensitivity analyzer was applied for identification. After multiple rounds of testing, a total of 55 *K. pneumoniae* strains were finally determined. This study was approved by the ethics committee of Navy 905 Hospital, and all patients had signed the informed consent.

Recovery, passage, and identification of strains

The *K. pneumoniae* bacteria in the microcentrifuge tube was taken and inoculated on the plate using the three-zone streaking method, and the inoculated plate was placed in a carbon dioxide constant temperature incubator for 18 hours. Then, a single colony was picked and transferred to other plates to continue subculture under the same conditions and for the same length of time.

Serotyping and virulence genes detection of *K. pneumoniae* by PCR

The deoxyribonucleic acid (DNA) of the *K. pneumoniae* strain was extracted and undertaken as a template for polymerase chain reaction. The primers, diethyl-nitrophenyl thiophosphates (dNTPs), and the enzyme system required for the reaction were placed in the gene amplification instrument. A 25 uL reaction system was composed of Premix Taq DNA polymerase, dNTPs, primers (F/R), and the extracted strain DNA template, with contents of 12.5 μL, 10 μM, 1 μL, and 2μL, respectively. Next, the sterile deionized water (8.5 μL) was added. The polymerase chain reaction (PCR) conditions were as follows. It was performed with pre-denaturation for 5 minutes at 94°C and then denaturation for 30 seconds at 94°C, the

annealing temperature was 53 - 63°C, the annealing time was half a minute or 90 seconds, and it was extended for 30 seconds. The operations should be repeated for a total of 25 cycles.

Drug susceptibility test

The drug susceptibility test was carried out using a bacteria automatic identification drug susceptibility analyzer and Mueller-Hiton medium. Antibiotics tested included ampicillin, ceftriaxone, ciprofloxacin, cefepime, ertapenem, amoxicillin, tetracycline, ceftazidime, amikacin, and cefuroxime. The detection indicators included the number of capsule and biofilm-positive strains and the influence of positive strains on antibiotic drug resistance. In addition, the relationship between drug resistance and the ability of strains to form the biofilm and capsule was analyzed.

Statistical analysis

The SPSS24.0 statistical software was used for statistical analysis, and $P<0.05$ was considered that the difference was statistically significant. The measurement data was expressed as x±s.

Results

Type distribution of 55 strains of *K. pneumoniae*

As shown in Figure 1 below, of the 55 strains of *K. pneumoniae* isolated clinically, 61.8% came from blood with a total number of 34 strains; 8 strains were from secretion specimens (accounting for 14.5% of the total); and 7 strains were from drainage fluid (accounting for 12.7% of the total), including 2 strains from pus, bile, and pleural fluid, respectively.

Subculture results of 55 strains

The strains were placed on a culture plate and inoculated using the three-zone streaking method. A single strain was transferred to another culture plate to continue the culturing to obtain a single strain, as shown in Figures 2 and 3.

The positive distribution of capsule and biofilm of 55 *K. pneumoniae* strains

As shown in Figure 4, among the 55 clinical isolates of *K. pneumoniae*, 10 strains were positive for capsule and biofilm, accounting for 18.2% of all strains; a total of 13 strains were negative for capsule and biofilm and 23.6% were negative for capsule (9 strains were negative for capsule but positive for biofilm, and 4 were negative for both capsule and biofilm, which accounted for 16.4% and 7.3%, respectively). Among them, 21 strains were biofilm-positive strains (including 10 strains that were both biofilm and capsule-positive; and 11 strains that were biofilm-positive but capsule-negative), accounting for 18.2% and 20.0%, respectively.

Distribution of 55 *K. pneumoniae* strains carrying serotypes and virulence genes

Among the 55 isolated strains, the carrying status of 11 virulence genes was different. Among 35 strains with capsule positive, K1, K2, K20, and K57 carried with serotype genes; and magA, fimH, wabG, rmpA, and iroN carried with virulence genes, and the positive numbers were 9,

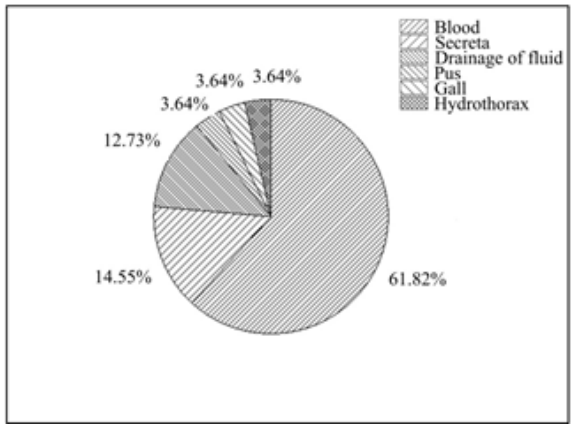

Figure 1. Type distribution of 55 strains of *K. pneumoniae*.

Figure 2. Inoculation of strains with three-zone drawing line method.

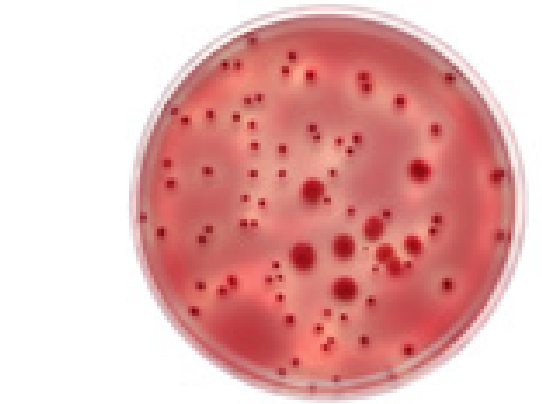

Figure 3. Culture of a single strain.

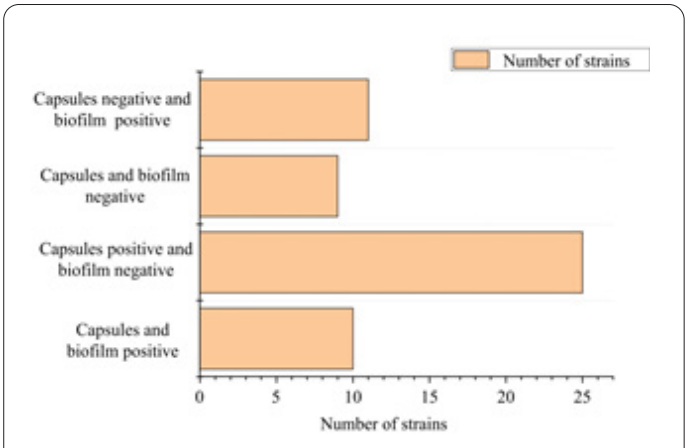

Figure 4. The positive distribution of capsule and biofilm of 55 *K. pneumoniae* strains.

17, 5, 10, 9, 18, 33, 33, and 34, respectively (as shown in Figures 5a and 5b). K5 and K54 genes were missed in capsule-positive strains. The serotype genes were found in K1, K2, and K20 and virulence genes were found in magA, fimH, wabG, rmpA, and iroN among the strains with biofilm positive. The positive numbers were 9, 2, 2, 6, 18, 17, 11, and 10, respectively. The missing genes were K5, K54, and K57 (as shown in Figures 5c and 5d). The results of PCR amplification of some genes are shown in Figure 6.

The relationship between capsule and biofilm and drug sensitivity of *K. pneumoniae*

As shown in Figure 7, the drug resistances of these ten types of drugs were not statistically different between biofilm-positive and negative *K. pneumoniae*, and the drug resistance of various drugs against capsule-negative strains and capsule-positive strains was basically the same. Compared with capsule-negative strains, the drug resistance of ampicillin and amoxicillin to the capsule-positive strains was lower. The drug resistance of ertapenem was stronger for capsule-positive strains, the drug resistance of tetracycline was strong against biofilm-positive strains, the drug resistance of ciprofloxacin in biofilm-positive strains was higher, and the drug resistance of other drugs was basically the same.

Discussion

K. pneumoniae is an opportunistic bacterium that resides in the respiratory and digestive tracts of the human body and is the main pathogen causing nosocomial infections. With the increase in drug resistance to *K. pneumoniae* in recent years, it has brought huge difficulties to doctors in the process of treating *K. pneumoniae* infections, and it also seriously threatens the lives and health of patients. A large number of studies have shown that *K. pneumoniae* strains are resistant to antibiotics after forming biofilm, and its drug resistance has also increased with the improvement of biofilm. In this study, 34 of 55 strains of *K. pneumoniae* were mainly derived from blood, accounting for 61.8%. The most important way of bacterial invasive infection is bloodstream infection. Due to the production of bacterial biofilm, *K. pneumoniae* can't be completely removed from medical devices during the sterilization process. In addition, catheter-related bloodstream infections also gradually increase as catheter inspections and treatments increase (15). In the previous epidemiological statistics of bloodstream infections, the most common bacteria are Staphylococcus aureus, which accounts for about 30% of the total infection rate, followed by *K. pneumoniae*, which accounts for about 10%. There were 8 strains from secretions, accounting for 14.5%, 7 strains from drainage fluid (12.7%), and 2 strains each from bile, pleural fluid, and pus. Although the proportions are relatively low, they are still It is one of the sources of speci-

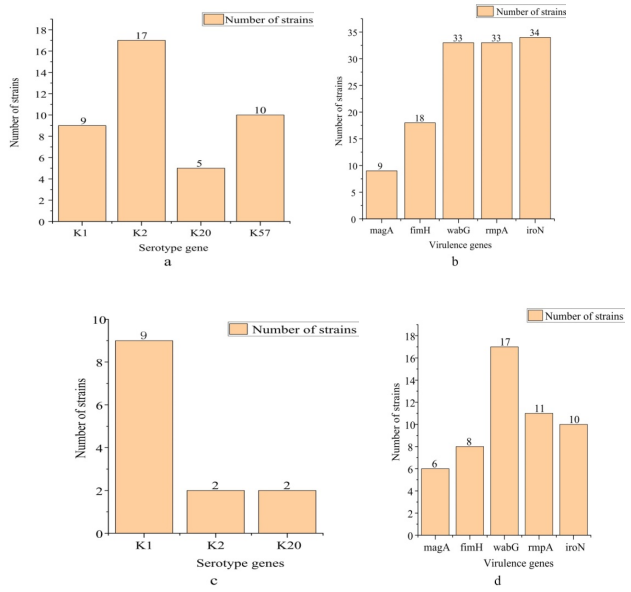

Figure 5. Distribution of 55 *K. pneumoniae* strains carrying serotypes and virulence genes. Note: Figure 5a illustrated the distribution of serotype virus genes among 35 capsule-positive strains, Figure 5b showed the distribution of virulence genes among the 35 capsule-positive strains, Figure 5c was the distribution of serotype virus genes among 20 biofilm-positive strains, and Figure 5d showed the distribution of virulence genes among 20 biofilm-positive strains.

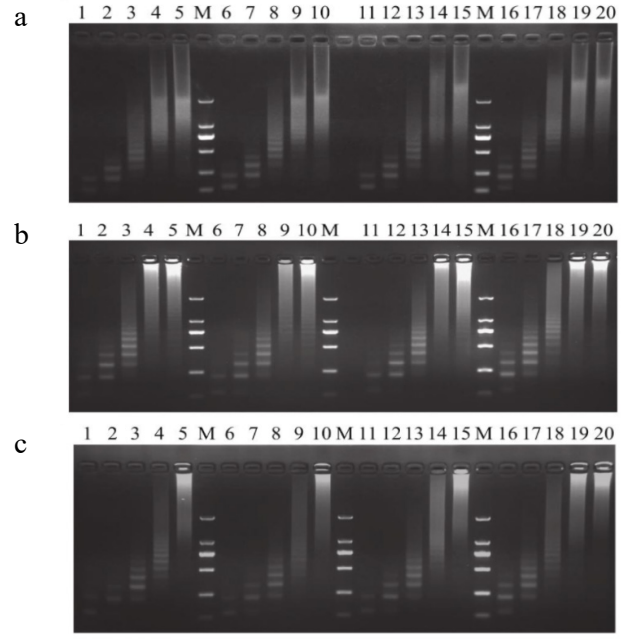

Figure 6. (a): The results of PCR amplification of some major virulence genes of clinical *K. pneumoniae*. (b): Partial amplification results of K2 serotype genes. (c): Partial amplification results of fimH virulence genes.

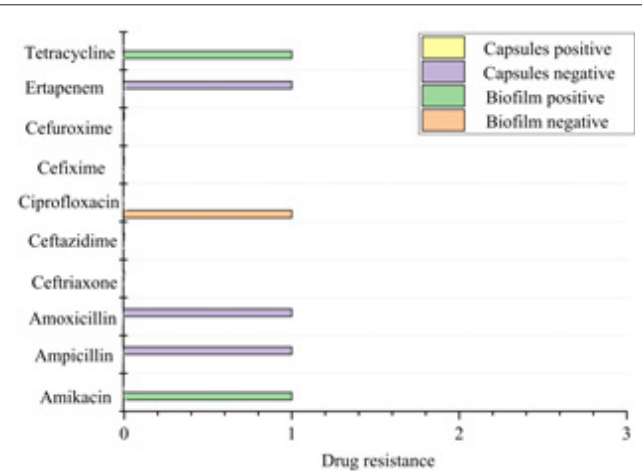

Figure 7. Comparison on drug resistance of ten types of drugs.

mens. Among the 55 *K. pneumoniae* clinical isolates studied in this study, 20 were biofilm-positive strains and 35 were biofilm-negative strains, accounting for 36.4% and 63.6%, respectively. At present, *K. pneumoniae* has strong capsule and biofilm-forming ability. In the past 5 years, the formation rate of biofilm has increased year by year (16). The drug resistance of its strains is closely related to the formation of biofilm. The increasing number of biofilm-positive strains is an important factor leading to the increasing resistance of *K. pneumoniae* (17).

Among 55 isolated strains, some serotype genes and some virulence genes were amplified by PCR technology, and the carrying status of 11 virulence genes was different. Among 35 strains with capsule positive, K1, K2, K20, and K57 carried with serotype genes; and magA, fimH, wabG, rmpA, and iroN carried with virulence genes, and the positive numbers were 9, 17, 5, 10, 9, 18, 33, 33, and 34, respectively. K5 and K54 genes were missed in capsule-positive strains. The serotype genes were found in K1, K2, and K20 and virulence genes were found in magA, fimH, wabG, rmpA, and iroN among the strains with biofilm positive. The positive numbers were 9, 2, 2, 6, 18, 17, 11, and 10, respectively. The missing genes were K5, K54, and K57. These types of virulence genes all have different degrees of carrying status, and the serotype gene K2 accounted for 31.0% of the total, which was the highest, followed by serotype gene K57 (accounting for 18.2% of the total), and then followed by K5 and K54. K5 and K54 were missed among the 55 specimen strains. Such data results were similar to those in Taiwan (18). In recent years, the two serotype genes, K1 and K2, show relatively high proportions. They are highly virulent, and they can cause invasive liver infections and may be accompanied by purulent liver abscesses, community-acquired pneumonia (CAP), and invasive liver abscess syndrome. The results of currently widely recognized studies show that among all serotype genes, there are 4 most common and higher pathogenicity types, namely K1, K2, K54, and K57 (19). The mechanism by which causes the recurrent infections may be closely related to their adhesion and invasiveness. *K. pneumoniae* can't be completely cured or cleared after extensive use of antibacterial drugs. In daily work, doctors should pay great attention to the capsule-positive and highly virulent *K. pneumoniae*, prevent the bacteria from being widely infected in the hospital, and make the protection well (20).

Conclusion

This study aimed to explore the formation ability of biofilm and capsule of multidrug-resistant *K. pneumoniae* and to explore the drug resistance mechanism of multidrug-resistant *K. pneumoniae*. Firstly, 55 strains of *K. pneumoniae* were screened out from the body fluid specimens of the laboratory. The strains were drug-resistant, and the characteristics of clinical infections of these strains were analyzed. Secondly, all strains were tested for the presence of biofilms and capsules, and then the DNA genome of the strains extracted from the strains was detected by PCR technology. Finally, the serotype genes and virulence genes of the strains were screened, and the relationship between these two genes and the formation of capsules and biofilms was analyzed and compared. Molecular cloning and other methods were utilized to clone the drug resistance-related genes. It was found that *K. pneumoniae* had been able to form complete capsules and biofilms. The formation rate of biofilm was higher than that of capsules, and there was a tendency to increase. K20 and K2 (two serotype genes) accounted for relatively high proportions, *K. pneumoniae* carried relatively more virulence genes wabG and fimH, and these two virulence genes may be closely related to the capsule production of *K. pneumoniae*. In this study, only 55 strains of *K. pneumoniae* were selected, which lacked the support of large samples. In addition, it only discussed the drug resistance of ten types of antibacterial drugs for *K. pneumoniae* and did not summarize most of the commonly used antibiotics in clinical practice. Therefore, it had to further explore the impact of more antibiotics on *K. pneumoniae* in follow-up studies. In conclusion, this study provided a reference for exploring the drug resistance mechanism of *K. pneumoniae* and the clinical treatment of *K. pneumoniae* infection with drug resistance.

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