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# ISOLATION OF KLEBSIALLA PNEUMONIA FROM POST COVID INFECTIONS AND RAISING BACTERIOPHAGES

**Syeda Sumaiyya Fatima [1&2] Shaik Muzammil Pasha[1], Y.S.Goutham[1], V.Nikhil[1], Zainab Kausar[1], A. Sanjay [1] ,V.Meghana[1], B.Keerthi[1] and Chand Pasha[1]***

[1]Department of Microbiology, Nizam College, Osmania University, Hyderabad, Telangana, India
[2]Department of Microbiology, Mumtaz Degree and PG College , Hyderabad, Telangana.

## ABSTRACT

Coronavirus disease 2019 (COVID-19) co-infections have been reported as a public health concern worldwide. The presence of *Klebsiella pneumoniae* in COVID-19-infected patients is a major problem due to its multidrug resistance (MDR). The excessive use of antimicrobials in COVID-19 patients leading to spread of antimicrobial resistance. *Klebsiella spp*. are commensals, and a leading cause of opportunistic and nosocomial infections. The MDR strains of *K. pneumoniae* causing increased infections. Alternative strategies to handle infections caused by these bacteria are required as strains become resistant to almost all antibiotics. Bacteriophages are viruses that can infect and kill bacteria by cell lysis. Phages are now being considered as alternatives to antimicrobial therapies. Several in vitro and in vivo studies have shown the potential for lytic phages to combat MDR *K.pneumoniae* infections. In the present study, 15 *K.pneumoniae* strains were isolated from COVID-19 patients and found to be resistant to majority of antibiotics. Five phages were isolated against *K.pneumoniae* isolates. Isolated phages are specific to all isolated 15 strains of *K. pneumoniae*, having a burst size of 221 plaque forming units per cell and being found to be tadpole shaped measuring a diameter of 95 nm and long non-contractile tail of 120 nm. The isolated phages are specific to only *K.pneumoniae* and can be used in COVID-19 cases to control *K. pneumoniae*.

**KEYWORDS:** COVID-19, *Klebsiella pneumoniae*; antimicrobial resistance; bacteriophage, phage therapy.

**INTRODUCTION:**

The *Klebsiella* sps causes pneumonia, urinary tract infection, and sepsis, particularly in immune compromised patients, also causing secondary infections in ventilated or catheterized patients, in both the home and nosocomial settings (Dhesi et al., 2020; He et al., 2020; Zhu et al., 2020). Subclinical carrier of *Klebsiella sps* is observed in cardiovascular (Jameson et al., 2018; Yan et al., 2017) and inflammatory bowel diseases (Atarashi et al., 2017). *Klebsiella pneumoniae* is the most dangerous species transforming to hypervirulent clones with extended virulence factors (Bengoechea and Sa Pessoa, 2019; Choby et al., 2020; Holt et al., 2015; Shankar et al., 2018). Post Covid fungal and bacterial infections are causing big threat to patients. *Aspergillus* in Fungi and *Klebsiella* in bacteria are major microorganisms during Covid and post Covid-19 infections. The presence of *K. pneumoniae* in covid-19 and post Covid-19 patients is a major problem due to its resistance to multiple antibiotics and it can possibly make the treatment of covid-19 patients more complicated. Antimicrobial resistance (AMR) has a threat to global health. *Klebsiella* readily gains and transfers AMR genes, made it a World Health Organization priority pathogen (Tacconelli et al., 2018). The multi-drug resistant *Klebsiella* has made useless to most available antimicrobial drugs (Sanchez et al., 2013; Elemam et al., 2019; Sonnevend et al., 2017; Turner et al., 2020). MDR *Klebsiella* infections have an increased risk of mortality (Ben-David et al., 2012; Mollers et al., 2017) and are difficult to treat (Patro and Rathinavelan, 2019) to cause outbreaks economically costly (Mollers et al., 2017). Bacteriophages offer one of potential alternative treatment (El Haddad et al., 2019). Phage therapy is a potential weapon against MDR bacterial infections (Dedrick et al., 2019; Schooley et al., 2017). Phage therapy depends on availability of sensitive, lytic and specific phages against AMR bacteria (Brussow et al., 2004). Bacteriophages could be better choice to control *K. pneumoniae* in Covid patients and hospital environment as they are high specific to host bacteria and lysing the bacterial cells. In the view of above *K. pneumoniae* were isolated from aspirations of Covid-19 patients and raised specific and lytic bacteriophages.

**MATERIALS AND METHODS:**

**Samples collection:**The 50 aspiration and sputum samples were collected randomly from 50 patients which included 25 male and 25 female and their age in the range (16 – 80) years during March-April 2021. Specimens were collected from ICU wards at KIMS hospital, Hyderabad, Telangana from the patients diagnosed with COVID-19 positive. Phages were isolated from environment samples (drainage, ponds, lakes, stagnant water, and hospital wastes)

**Isolation of *Klebsiella* Spp:** The samples transported to laboratory are inoculated on MacConkey agar and incubated at 37°C for 24 hours. The essential identification of *K. pneumoniae* was done by culturing, morphology (microscopy, motility - by hanging drop method) and bio-chemical tests (Catalase, Indole, Oxidase, Citrate utilisation, Urease and Glucose fermentation).

**Bio-Chemical Characterisation:**

Biochemical characterisation of *Klebsiella* was done using Catalase test, Indole test, Oxidase test, Citrate utilization test, Urease test and Glucose fermentation test.

**Catalase Test:** $H_2O_2$ is broken down by the catalase enzyme present in them in to water and oxygen. The oxygen evolved here causes bubbles (effervescence arise) within seconds,

indicating a positive test (catalase +ve) and absence of bubbles is a negative result (catalase -ve)

**Indole Test:** The amino acid, tryptophan present in the medium is converted into indole, pyruvic acid and ammonia by the enzyme tryphophanase. The indole reacts with kovacs reagent to form a red colour, at the interface between the top of the broth and reagent, in the indole positive bacteria.

**Oxidase Test:** The oxidase test strip is moistened slightly with sterile water and the growth is rubbed into the moistened paper of the strip. If the microbe has cytochrome oxidase, it will add electrons to the reagent, changing it from colorless to deep indigo blue in a matter of 10-20 seconds.

**Citrate Utilisation Test:** The *Klebsiella* here reacts with the enzyme citrase which metabolises citrate and generates $CO_2$ which combines with sodium and water to form sodium carbonate. Production of alkaline byproducts increases pH, causing bromothymol blue (green) to turn blue, indicates a positive result for *Klebsiella* utilising citrate.

**Urease Test:** Urea, a diamide of carbonic acid, is broken down by the urease which is produced by *Klebsiella* into ammonia and $CO_2$. A rise in pH causes a color change. Development of pinkish red colour is a positive result for *Klebsiella* utilising urea.

**Glucose Fermentation Test:** Inoculum is transferred aseptically to a tube of OF glucose medium with oil overlay. The agar is stabbed to place the bacteria in the butt of tube. The tube was incubated at 35-37°C for 24hrs. If glucose is fermented, acidic end products are accumulated and pH decreases. pH indicator in medium changes colour to indicate acid production.

**Antibiotic Susceptibility Test:** Antibiotic susceptibility test of *Klebsiella* was determined by kirby-bauer disc diffusion method. Eight different antibiotics were used in the test obtained from merck India. Zones of inhibition were measured in millimetre.

**Isolation of the bacteriophages:**

Bacteriophage enrichment: Bacteriophage enrichment was done by taking 4mL of 0.2 µ filtered sample water suspension (phage source), 1mL of 10x Luria broth and 1mL of exponentially growing *Klebsiella* sps and incubated at 37°C for 24hr. Then the suspension was centrifuged at 15000 rpm for 5min and filtered through a 0.2 µ syringe filter. The filtrate was mixed with pure culture and overlayed using double agar layered-based plaque assay method (Kropinski et al., 2009).

**Detection of bacteriophages/ Plaque assay:**

In a sterile Eppendorf, 0.2 µ syringe filtered 100 µL of bacteriophage source and 100 µL of exponential *Klebsiella* bacterial culture were added and incubated at 37°C for 15 min, then it was mixed with 5mL low melting agar (0.8%) and poured onto Luria agar plate. Allowed the low melting agar to solidify for 30 min at room temperature and then plates were incubated inverted at 37°C for 24h.

**Purification of phage:**

Using a sterile scalpel, an isolated plaque was picked from the overlayed Luria agar plate and suspended with 500 µL of phage buffer and diluted. A dilution was mixed with exponential bacterial culture; incubated, and undergone double agar layered-based plaque assay (Kropinski et al., 2009). Individual plaque obtained in this method was selected.

**Host Inactivation studies:**

Pure *K. pneumoniae was* inoculated into flask containing luria broth and incubated at 37°C for 24hr. Then the flask was infected with 0.2 µ filtered phages and incubated at 37°C with gentle shaking. The sample was collected from flask every 1 hour, till 8 hours

consecutively. Hourly samples of the flask were spread on the Luria agar plates respectively for the viability of host cells. The numbers of colonies in the hourly samples were counted by using colony counter Multilab India. The time required to kill 90% of initial cells was measured.

**Burst size determination:**

An isolated plaque was picked into a sterile Eppendorf containing 500 µL of phage buffer and then it was added to 500 µL of bacterial culture in an Eppendorf and 100 µL of the mixture was undergone double-layered agar based plaque assay (Kropinski et al., 2009).

**Purification of phages:**

**Phage purification with Centrifugation**

Phage lysate was made cell-free by centrifuging at 5000rpm for 10min and clear lysate was again centrifuged at 15000rpm for 5h to precipitate phages. The pellet was suspended in the phage buffer.

**Chloroform**

The phage lysate was centrifuged at 5000rpm for 10 minutes and the cleared phage lysate was taken into phage buffer and treated with 15% chloroform. The top layer was taken and centrifuged at 5000rpm for 10 minutes and the supernatant was filtered through a 0.2 µ syringe filter.

**Poly Ethylene Glycol**

By centrifugation of 15000rpm for 5min, the cells were removed and the supernatant was collected. PEG 8000 was added to the supernatant solution to make a 2% concentration and stirred at 4°C overnight to precipitate the bacteriophages. Then the solution was centrifuged at 15000rpm for 10min, bacteriophages were collected as pellets and suspended in phage buffer, and dialyzed.

**Transmission electron microscopy of phages:**

One drop of the purified PK1 phage suspension was placed on a copper grid with carbon-coated Formvar film for 10 min at room temperature. 4% aqueous phosphotungstic acid was used for staining at pH 7. The sample was air-dried overnight and examined with a Zeiss TEM 900 electron microscope, Carl Zeiss AG, it was operated at 50 kV. The phage particles were visualized using the Image SP software and a CCD camera.

**Determination of host range:**

The host range of obtained phages was determined by *E. coli, Salmonella*, *K. pneumonia, Staphylococcus aureas and Campylobacter* isolates. 1mL of pure *E .coli, Salmonella*, *K. pneumonia, S. aureas and Campylobacter* isolates were spread on Luria agar plates respectively. 50 µL of phages was sprayed on the Luria agar plates with pure *E .coli, Salmonella*, *K. pneumonia, S. aureas and Campylobacter* cultures. These plates were incubated at 37°C for 24hrs. Then plates were observed for plaques.

**Bacteriophage efficacy studies:**

The plaque formation ability of phages on host bacterial strains or the effectiveness of phage on host bacterial strains was determined by 100 µL of phage and 100 µL of pure isolates of different strains respectively, mixed with low melting agar, overlayed on a Luria agar plate and incubated at 37°C for 24hr. The number of plaques was counted. The highest efficacy was considered in that bacterial strain, where the highest number of plaques was produced.

**RESULTS**

**Isolation and identification of *Klebsiella*:**

Fifteen pure *K. pneumonia* were isolated from samples collected from Covid 19 patients. *K. pneumonia* was identified by growth on Media, microscopy, and Biochemical characteristics, and the results were presented in Table 1.

**Morphology**

Fifteen *K. pneumonia* strains were isolated from patients suffering with Covid 19. They

were identified as *Klebsiella* based on large, mucoid and pink colonies on MacConkey agar. They were gram negative, straight rod, and 0.3–1.0 µm in diameter and 0.6–6.0 µm in length. They were non motile as tested by hanging drop method. In the biochemical tests, it was Catalase positive, Indole negative, Oxidase negative, Citrate positive, Urease positive and Glucose fermentation positive confirming *K. pneumonia*.

**Antibiotic susceptibility test:**

Out of eight antibiotics tested, all strains showed little clear zones of inhibition around the disc indicating the high resistance.

Results showed a high rate of resistance against ampicillin, amoxicillin, and tetracycline, followed by amikacin, norflaxacin, streptomycin, ciprofloxacin, Kanamycin. Resistance was observed for fifteen *Klebsiella* strains. *Klebsiella sps* were highly resistant to all the antibiotics used in this test (Table 2).

**Table 1**: Identification of Isolated *Klebsiella* by growth, microscopy, and biochemical characterstics.

| Strain | Growth on specific media | Microscopic morphology | Biochemical tests | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Catalase test | Indole test | Oxidase test | Citrate test | Urease test | Glucose fermentation test |
| *Klebsiella* 1 | Mucoid and pink colonies | non-spore-forming, non-motile, capsulated, Gram-negative, straight rod, 0.3–1.0 μm in diameter and 0.6–6.0 μm in length. The rods are arranged singly. | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella* 2 | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella* 3 | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella* 4 | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella* 5 | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 6* | Mucoid and pink colonies | The rods are arranged in pairs. | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 7* | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 8* | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 9* | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 10* | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 11* | Mucoid and pink colonies | The rods are arranged in short chains. | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 12* | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 13* | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 14* | do | do | Positive | Negative | Negative | Positive | Positive | Positive |
| *Klebsiella 15* | do | do | Positive | Negative | Negative | Positive | Positive | Positive |

**Table 2**: The Antibiotic–sensitivity profile of *Klebsiella* as Zone of inhibition (mm) with 100µg concentration:

| Bacteria | Ampicillin | Amoxicillin | Norflaxacin | Streptomycin | Tetracycline | Ciprofloxacin | Kanamycin | Amikacin |
|---|---|---|---|---|---|---|---|---|
| *Klebsiella 1* | 0.0 | 02 | 04 | 05 | 02 | 05 | 04 | 02 |
| *Klebsiella 2* | 01 | 03 | 02 | 04 | 00 | 04 | 03 | 03 |
| *Klebsiella 3* | 00 | 00 | 01 | 03 | 05 | 03 | 05 | 04 |
| *Klebsiella 4* | 02 | 01 | 02 | 05 | 05 | 04 | 02 | 03 |
| *Klebsiella 5* | 00 | 02 | 02 | 02 | 04 | 05 | 04 | 04 |
| *Klebsiella 6* | 02 | 02 | 04 | 04 | 03 | 04 | 04 | 02 |
| *Klebsiella 7* | 00 | 03 | 03 | 05 | 01 | 03 | 03 | 04 |
| *Klebsiella 8* | 02 | 01 | 04 | 04 | 02 | 06 | 04 | 03 |
| *Klebsiella 9* | 01 | 03 | 02 | 05 | 03 | 02 | 04 | 02 |
| *Klebsiella 10* | 02 | 01 | 01 | 02 | 04 | 03 | 06 | 01 |
| *Klebsiella 11* | 02 | 02 | 03 | 04 | 05 | 05 | 04 | 03 |
| *Klebsiella 12* | 01 | 01 | 03 | 04 | 01 | 04 | 02 | 02 |
| *Klebsiella 13* | 02 | 03 | 04 | 03 | 06 | 04 | 06 | 04 |
| *Klebsiella 14* | 01 | 02 | 04 | 05 | 04 | 03 | 04 | 02 |
| *Klebsiella 15* | 01 | 01 | 03 | 04 | 03 | 03 | 03 | 03 |

**Bacteriophage enrichment:**

Phage enrichment filtrate contained numerous phages and formed plaques of varying sizes specific to *K. pneumonia* strains.

**Detection of bacteriophages/plaque assay:**

In Plaque assay, after incubation, bacteriophage plaque formation was determined and plaques were counted as plaque forming units (PFU). *K. pneumonia*. phages were small and round as in Fig. 1 .

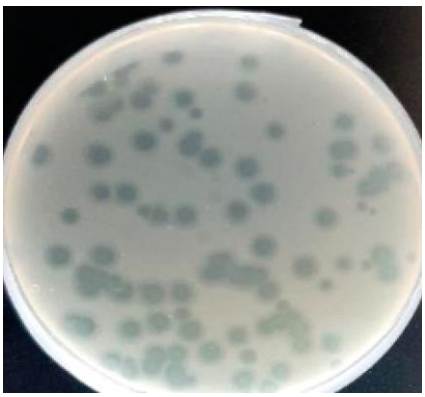

Figure 1: Plaques of *K. pneumonia* on luria agar plate.

**Phage purification:**

The Plaque was purified and used in the plaque assay method which produced plaques specific to *K. pneumonia* on luria agar plates. The single and isolated plaque was selected as pure phage.

**Host Inactivation Studies:**
The number of colonies in hourly samples was counted using the colony counter. The viable cell count was more till 1 hour, from the 2nd hour, the number of viable cells started decreasing in descending order. 90% of *K. pneumonia cells* were inactivated in 7 hours.

**Burst size determination:**
Plaques were observed on luria agar plates. The plaque with the largest burst size was the bacteriophage with higher effectivity. KP1 phage produced a burst size of 221 PFU per cell.

**Transmission electron microscopy of phages:**
Five *K. pneumonia* bacteriophages (KP1, KP2, KP3, KP4, KP5) were isolated from drainage, ponds, lakes, stagnant water and hospital wastes. KP1 phage was used for electron microscopy. *K. pneumonia* PK1 phage was like lambda phage in morphological appearance, having an icosahedral heads with a diameter of 95nm and long non-contractile tails of 120nm as in Fig. 2.

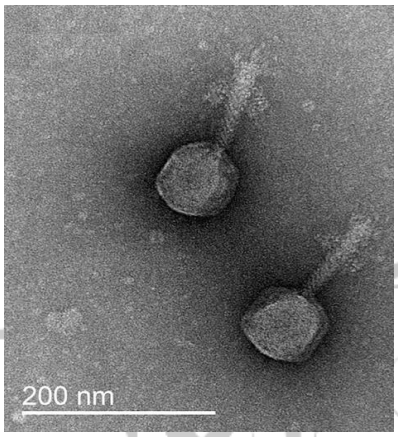


Figure 2: Electron microscopy of *K. pneumonia* phage KP1.

**Host ranges:** Host ranges were determined using *E. coli, Salmonella, K. pneumonia, S. aureas and Campylobacter sps.* The phages infected all fifteen *K. pneumonia* isolates. The bacteriophages didn't infect *E. coli, Salmonella, S. aureas* and *campylobacter* isolates.

**Bacteriophage efficacy studies:**
Plaque formation efficiency of five *Klebsiella* phages on fifteen *Klebsiella* strains were obtained. *Klebsiella* phages lysed all fifteen *Klebsiella* strains. Plaque formation eficiency was more in ten strains (*Klebsiella* 1, *Klebsiella* 3, *Klebsiella* 4, *Klebsiella* 6, *Klebsiella* 9, *Klebsiella* 10, *Klebsiella* 12, *Klebsiella* 13, *Klebsiella* 14, *Klebsiella* 15).

**DISCUSSION**
From 50 Covid-19 hospitalized patients, collected aspiration/sputum samples have shown a positive *K. pneumonia* growth in 15 samples (30%). Among the positive samples, 9 of 15 samples were of male patients (60%) whereas 5 of 15 (40%) of females, suggesting the respiratory system of men are more susceptible to secondary bacterial growth and bacterial pneumonia than women (Habeeb and Hussein, 2023; Barbagelata et al., 2020; Sharifipour et al., 2020). Secondary bacterial infections with multidrug resistant microbes are serious health issue and challenges to the entire medical field. The results confirm that, 15 out of 15 *K. pneumonia* isolates were

tested by the antibiotic resistance. This is due to plasmid-borne β-lactamases (Garcia-Menino et al., 2021). It is worth mentioning that in most of the cases of multidrug resistant *K. pneumoniae* in hospitals and healthcare centers post the Covid-19 pandemic were substantially higher than the pre-pandemic time (Habeeb and Hussein, 2023). MDR *K. pneumoniae causing* the severity of the disease and the increased mortality rates among incubated patients (Li et al., 2021; Ramadan et al., 2020).

As all the *K. pneumonia* are resistant to tested antibiotics there is a need of alternative therapy for combating of MDR *K. pneumonia*. *Klebsiella*-infecting phages, were isolated from water samples sourced from different environments. The 5 phages were obtained by using 15 isolated strains of *K. pneumonia*. This confirms that the propagation host is influencing the host range (Jensen et al., 1998). This is a vitally important consideration for phage therapy, and we cannot generalize phage behavior based on genome similarity, but we must also consider prevailing culture conditions (Townsend et al., 2020). Although multiple features of strain-specific bacterial immunity can protect against phage replication, in *Klebsiella* the primary defense against both phages and antibiotics is a protective polysaccharide capsule (March et al., 2013; Whitfield et al., 2006). This capsule forms the outermost layer of the *Klebsiella* cell and acts as an important virulence factor (Simoons-Smit et al., 1986). *Klebsiella* phages have shown to be specific to host capsule types (Hsu et al., 2013; Bhetwal et al., 2017, Hoyles et al., 2015) and this is often linked to phage sugar-degrading enzymes called depolymerases that target specific capsule types. (Majkowska-Skrobek et al., 2016; Solovieva et al., 2018; Rieger-Hug et al., 1981). The broad host ranges observed in our studies in phages may be due to multiple depolymerises as reported by Townsend et al., (2021). Phage specificity for target and lysis of bacteria should be high to prevent non-specific bacterial targeting (Loc-sCarrillo and Abedon, 2011). Complete *K. pneumonia* 15 hosts inactivation was achieved in 7 hrs where as it took 12hrs as reported by Townsend et al. (2021). Phage cocktails were used to improve the impact of phages on *Klebsiell*a populations and are considered crucial for the efficacy of phage therapy. (Kakasis and Panitsa, 2019; Chan and Abedon, 2012; Chan et al., 2013). But in present study phages are potential and can kill all the studied *K. pneumonia* strains. These phages may be tested on Covid 19 patients and hospital to control multidrug resistant *K. pneumonia* strains.

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