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Isolation of Bacteriophages against *Enterobacteria spp.* Isolated from Poultry Waste

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ABSTRACT

The increasing occurrences of antibiotic-resistant bacterial infections in poultry poses a significant challenge to the poultry industry, necessitating alternative therapeutic approaches. This study explores the potential of bacteriophage therapy as a biocontrol measure against *Enterobacteria spp* infections in poultry. *Enterobacterium* is a major pathogen responsible for economic losses due to increased morbidity and mortality. In this study, two *Enterobacter* isolates were obtained from poultry waste samples, characterized using cultural, morphological, biochemical characters and confirmed by 16S rRNA sequencing. Four lytic bacteriophages were isolated from poultry waste samples and were studied to determine their lytic ability against host *Enterobacter.* The most potent lytic phage was selected, purified and enriched for further studies. The potent lytic ability of selected phage demonstrated that these phages specifically target and lyse their corresponding *Enterobacteria spp* hosts, suggesting their potential as a substitute to antibiotics for managing bacterial infections in poultry. This study highlights the viability of phage therapy as a sustainable and active approach to combating antibiotic-resistant *Enterobacteria spp* strains in poultry farming.

INTRODUCTION

Enterobacteria spp is one of the prevalent pathogens that negatively impact poultry. Infection of *Enterobacteria spp* causes several extra-intestinal pathological disorders such as septicemia, bronchitis, and other respiratory infections to poultry birds that seriously impair poultry production economically (2). The poultry infections are difficult to control by antibiotics as poultry pathogens are becoming multidrug resistant and thus needs alternative therapy. Bacteriophages are specialized viruses that precisely target and eliminate such bacteria. There is a lot of interest in using *Enterobacteria spp* bacteriophages as biocontrol agents. Within the source of their particular bacteria, bacteriophages are widely distributed. Bacteriophages are viruses that infect and replicate within bacteria. They are precise to their host bacteria, meaning a particular phage will only infect a specific type or strain of bacteria. The word "bacteriophage" comes from the Greek words "bakterion," meaning small rod-shaped bacterium, and "phagein," meaning to eat. Essentially, bacteriophages "eat" or infect bacteria. Phages are crucial in the natural regulation of bacterial populations and are abundant in various environments, including soil, water, animals, and birds. Scientists are interested in phages because of their host specificity, self-replication, and environmental ubiquity. Researchers have examined the possible uses of bacteriophages in industry, agriculture, and medicine. One area of focus has been the progress of phage therapy as a treatment for bacterial infections instead of antibiotics. Bacteriophages are a desirable alternative for targeted bacterial control because of their specificity, which keeps them harmless to non-target organisms (1).

The overuse of antibiotics caused by the increased demand for poultry products is leading to resistant microbiological diseases. Phage therapy is becoming more and more popular as an alternative therapy. An attractive way to manage infections in poultry is to use bacteriophage to eradicate microorganisms that are resistant to treatment. Numerous phages are known to regulate pathogenic *Enterobacteria spp*.

Many antibiotics are utilized intensively to manage the microbial load in commercial poultry, which is the cause of the rise in development of antibiotic-resistant bacteria. Antibiotics, notably tetracycline, have been employed in animal husbandry for health maintenance, disease prevention, and enhancement of growth, particularly in poultry farming. Tetracycline, being the predominant antibiotic used on farms, serves additional functions beyond microbial control, including anti-inflammatory properties, inhibition of tumour growth, and regulation of growth processes. Excessive utilization of antibiotics and other chemical interventions has exacerbated the proliferation of antibiotic-resistant bacterial strains (15,16). It is important to use alternative tactics to prevent the spread of antibiotic resistance among microorganisms. In place of antibiotic treatment and effective management of microorganism's resistant to many drugs, phage therapy may be a viable option. Bacteriophage therapy is a key substitute to antibiotics in the recent era of multidrug-resistant bacterial illnesses as phages are employed topically, orally, or systemically in research examined. These investigations yielded effectiveness rates ranging from 80-95% and

few adverse effects related to the digestive system or allergies. Significant efficacy of phages against *Enterobacteria spp*, *Acinetobacter spp.*, *Pseudomonas spp.*, and *Staphylococcus aureus* was also established by British examinations (3). Due to their high specificity and ability to lyse target bacteria, bacteriophages have the potential to be used as therapeutic agents for bacterial diseases. Although *Enterobacteria spp* is naturally found in poultry environments and the intestinal tract's microbiota, specific strains of the bacteria must have particular virulence qualities to induce disease. The virulence-associated genes (VAG). of *Enterobacteria spp.* strains encode a wide range of virulence components that aid in their ability to infect. These substances play a part in colonization, adhesion, invasion, and survival against host defences. They include toxins, adhesins, invasins, and protectins(4).Given the aforementioned, the current study was conducted to isolate *Enterobacteria spp* species from various poultry farms and develop bacteriophages to combat them (1).

Phage Therapy to control bacterial infections

Phage therapy in human's traces back to its first documented use in 1919, coinciding with their discovery. However, the roots of phage therapy date back even further to 1896 when Ernest Hankin detected antibacterial activity against *Vibrio cholera*, the causative mediator of cholera. This discovery gained further momentum in 1915 when Frederick Twort speculated that this antibacterial activity could be attributed to viruses, but his findings were not extensively pursued. Bacteriophages were officially discovered in 1917 by Félix d'Hérelle, who later reported successful treatment of plague using antiplaque phages in 1925, sparking interest in phage therapy. His work in India, particularly at the Haffkine Institute in Bombay
(now Mumbai), further advanced phage therapy research. While the concept of phage therapy faded in the Western world with the initiation of antibiotics in the 1940s, it persisted in the former Soviet Union and is still practiced today. The Eliava Institute in Tbilisi, Georgia, is recognized as a pioneer in phage therapy research and application. Despite initial scepticism and unreliable early trials, phage therapy gained attention in the United States. William Smith and colleagues reported successful phage therapy in mice, contributing to renewed interest in phage therapy as a potential alternative or supplement to antibiotic treatment. (19, 25)

Phage therapy has emerged as an effective therapeutic approach in combating poultry infections like *Enterobacteria* infections.

1. Materials and Methods

1.1. Sample collection

A poultry waste samples was collected from local commercial poultry shops in Satara, India in sterile zip-lock bags and kept in the refrigerator until use.

1.2. Isolation of *Enterobacteria* host

Pure strains of the host *Enterobacteria were* isolated from poultry samples collected from poultry shops located in Satara. The sample was suspended in sterile distilled water. The mixture was then filtered by using the Whatman filter paper. The filtrate was then serially diluted (10-fold serial dilution) in sterile saline water. 0.1ml of appropriate dilution was spread plated on MacConkeys agar medium. The plates were incubated for 24 hours at 37℃. The MacConkeys agar is helpful in the isolation and differentiation of different enteric and gram-negative bacteria. After incubation plates were observed for red/pink coloured colonies having mucoid consistency. Well isolated colonies were further sub-cultured and preserved on MacConkeys agar slants as it supports the growth of bacterial species belonging to *Enterobacteriaceae* family (7).

1.3. Identification of the host bacterium

1.3.1 Study of Cultural and Morphological Characteristics of *Enterobacteria spp*.

The medium MacConkey agar medium was used to for isolation and purification of *Enterobacter*. A sterile strain *of Enterobacteria spp.* was streaked on the media using the four-quadrant streak method. The cultural and morphological characters of host bacterium were studied.

1.3.1.1. Gram Staining of the *Enterobacter*

A colony was selected from a MacConkeys agar plate and suspended in a tube containing saline. A smear was prepared on a clean glass slide, heat-fixed, and air-dried. The smear was stained with crystal violet, a basic dye, for 30 seconds. The slide was then flooded with Gram's iodine for approximately one minute and rinsed with tap water. Subsequently, 95% ethanol was applied for 10 seconds to decolorize the sample. The slide was then counterstained with safranin for 30 seconds. Excess stain was drained off and the slide was blotted dry with blotting paper. The prepared slide was examined under an oil immersion lens. Cells displaying a purple hue are identified as gram-positive, while those exhibiting a red or pink hue are categorized as gram-negative bacteria (27).

1.3.1.2. Motility of the *Enterobacteria spp*.

Numerous methodologies have been devised to investigate bacterial motility. One of the simplest techniques for observing living microorganisms involved suspending them in a fluid medium such as water, saline, or broth. This suspension was then used to create a "hanging drop" preparation, utilizing a cover slip and a hollow-ground slide also known as a cavity slide. The slide featured a concave cavity at its centre, into which a drop of the microbial suspension prepared in saline water was placed. Subsequently, the coverslip was secured in place using either clay or Vaseline gel. Upon inversion of the coverslip over the well, the drop of microbial suspension would hang from the glass within the concave region of the slide. The slide was initially observed under 45X and then under 10X objective lenses. Microscopic examination of such a preparation yielded valuable insights. This method was employed to ascertain the motility of organisms. Furthermore, the slide could be observed for extended durations as the drop resisted rapid desiccation.

1.3.1.3. Study of biochemical characteristics

Biochemical assays were employed for microbial classification, leveraging variations in the metabolic activities demonstrated by bacterial strain. A range of biochemical tests was utilized to ascertain the identity of the host; they are as Indole (20), Urease (23), Nitrate reduction (29), Methyl red [MR] and Voges Proskauer [VP] (30), Citrate Utilization Test, Lactose utilization test, Glucose utilization test.

1.3.1.4. 16 S rRNA and Phylogenetic analysis

Host isolate was subjected to 16 S rRNA sequencing and phylogenetic analysis for its identification.

1.4. Determination of mid-logarithmic phase of *Enterobacteria spp*. by Growth Curve

The actual number of phages that will enter any given cell is a statistical process that requires a mid-log growth phase of cells. Therefore, information about the mid-log growth phase of the host bacteria is necessary. This experiment aimed to determine the growth curve of *Enterobacteria spp.* bacteria. For this optical Density [OD] was measured at 620 nm by using a spectrophotometer. Spectrophotometry is employed to measure the increase in cellular mass. Growth of bacteria results in turbidity of the growth medium; turbidity or optical density serves as a proxy for the amount of light absorbed by a bacterial suspension. A sterile side arm flask containing 150 ml of phage broth was inoculated with a loopful of an overnight culture of the host bacteria, *Enterobacteria spp*. An uninoculated flask with phage broth was used as a negative control. Both flasks were incubated at 37^{0}C. The optical density (O.D) of the inoculated side arm flask at 10-minute intervals for the first 1-2 hours, then at 30-minute intervals up to 5 hours was measured at 620 nm using a colorimeter, with the uninoculated phage broth as the blank. The growth curve of the bacterial pathogen was plotted as a time against $OD_{620,}$ and the lag, log, mid-log, and stationary phases were determined from the plot (17).

1.5. Isolation of lytic phages against *Enterobacteria spp*.

1.5.1. Sample collection

The poultry sample utilized for isolating the host culture was also employed for the isolation of phages. The collected sample was suspended in distilled water and agitated, followed by filtration using Whatman filter paper (No.1) to eliminate suspended particulate matter. The resulting filtrate was consequently consumed for the isolation of phages.

1.5.2. Isolation of Phages

For the isolation of phages, 10 ml of the filtrate was extracted from the remaining filtrate and subjected to chloroform

treatment. Chloroform treatment was employed to lyse host cells containing intracellular phages. This procedure involved adding 0.5 ml of chloroform to 10 ml of filtrate and allowing it to settle for ten minutes. Once settled, the supernatant was carefully collected into sterile centrifuge tubes. The sample treated with chloroform was then centrifuged for 15 minutes at 3000 rpm, a speed selected to effectively separate the desired components without causing sample damage. The resulting supernatant was collected in sterile dilution tubes and placed in a water bath to remove any residual chloroform odour, with the temperature adjusted to between 40°C and 50°C. Following the elimination of the chloroform odour, the sample underwent an enrichment procedure. Susceptible bacterial host, the *Enterobacteria spp.* isolates were mixed with a filtrate in phage broth tubes. Phage broth is a specialized liquid medium formulated for the enrichment and propagation of bacteriophages infecting bacteria, including phages that target *Enterobacteria spp.* bacteria. The purpose of using phage broth for the enrichment of phages includes:

a) Selective growth

The nutrients in phage broth usually encourage the growth of host *Enterobacteria spp* bacteria, which creates an atmosphere that is favourable for phages reproduction. The broth may also include inhibitors or selective agents to suppress the growth of non-target bacteria, allowing phages to proliferate selectively.

b) Increased phage concentration

Since the phage broth supports the growth of phages it also leads to the increased concentration of phages.

In this protocol, a 24-hour-old well-isolated host culture was obtained from a MacConkey agar plate, and a dense culture suspension was prepared. Subsequently, 1 ml of the culture suspension was combined with 1 ml of the chloroform-treated sample and inoculated into phage broth tubes. These tubes were then incubated for 24 hours at 37°C. After the incubation period, 0.2 ml of chloroform was introduced into each tube, followed by centrifugation for 15 minutes at 3000 rpm (8).

1.5.2.1 Double agar overlay method (DAL)

The 5-7 ml of sterile soft agar-containing tubes were inoculated with aliquots of diluted enriched filtrate and mid-log phase culture *Enterobacteria spp.* host bacterial culture, which was subsequently poured out onto agar plates holding medium. Dilutions were performed in triplicate form. Soft agar was used as it aids in easy diffusion of the phage throughout the media, resulting in more uniform plaque development. Additionally, it solves the issue of uneven bacterial-phage solution absorption into the hard agar, which frequently results in uneven plate formation. The plates were then incubated for 24 hours at 37℃. During the incubation period, the phage attaches itself to the host bacterial cells, lyses, and infects the cells, and it starts to proliferate to nearby bacterial cells. This subsequently results in the forming of Plaques or clear zones after 6 to 24 hours. After incubation, the plates were observed for such clear zones (9,11).

1.5.3. Purification of Plaques

For purification drives, well-isolated plaques were repeatedly proliferated until a single phage type was obtained. The purification process involved the inoculation of individual plaques of different morphology in phage broth medium along with the host suspension. The mid-log phase culture of host suspension was inoculated with the plaques of given sizes in these phage broth tubes and incubated for 24 hours at 37℃. After the incubation period, the sample was gently shaken and centrifuged at 3000 rpm for 15 minutes. Plaques with varying morphologies had their supernatant collected in two distinct sterile flasks. The supernatant was then filtered using syringe filter having 0.22 µm pore size (Hi Media) in different sterile flasks respectively. Bacteria, fungi, and particulate matter were successfully removed from the supernatant that passed through 0.22 µm filters. The filtrate obtained was known as phage lysate. The phage lysate was then serially diluted up to 10^{-7} dilutions. Dilutions were performed in triplicate format. The 0.5 ml of each dilution was mixed with 1 ml of host suspension and then were added to molten soft agar butts and these butts were again overlayed on Bactotryptone agar plates. The plates were incubated for 24 hours at 37℃. Post incubation period, the plates were observed for pure plaques having the same morphology. The entire purification process was repeated until plaque with same morphology was obtained, determined by plaque size (10).

1.5.4. Enrichment to get desired Plaque forming Units (PFU)

The lysate of purified phage was amplified to carry out further experimental processes. For the amplification, two sterile flasks containing 50 ml phage broth were prepared. In these flasks, 2ml of pure phage lysate of different morphology of plaques and 4ml of the bacterial host were combined respectively. These were incubated for the overnight at 37°C. The concentrated lysate samples underwent centrifugation at 3000 rpm for 15 minutes, followed by filtration through a sterile membrane filter assembly. This apparatus also recognized as a vacuum flask or suction flask, constitutes specified laboratory glassware employed in filtration systems. It comprises a flat-bottomed flask featuring a sidearm and a perforated plate, commonly called a Buchner funnel, which securely fits into the flask's neck. Typically, the flask's sidearm is linked to a vacuum source for filtration (13). The filtrates obtained were further propagated to obtain amplified phages and high-titter lysate samples. For propagation, 4 ml of each lysate sample and 6 ml of the suitable host were added to 100 ml of sterile phage broth respectively, and the mixture was incubated for an overnight at 37°C. Steps involving centrifugation and filtration were repeated. The amplified lysate samples were stored at 4°C for further use (10).

1.6. Determination of host cell density and titter of phage lysate

1.6.1. Determination of Total Viable Count (TVC/ml) of host *Enterobacteria spp*.

The procedure comprises spreading the sample over an agar plate, leading to the growth of distinctive colonies evenly distributed across the agar surface when the optimal cell concentration is plated. In this technique, a 24-hour-old well-isolated colony was picked up and inoculated in a sterile phage broth tube. Initial optical density was measured at 620 nm and the tube was incubated for approximately 4-5 hrs at 37°C. After incubation, the mid-log phase culture was serially diluted up to 10 folds. 0.1 ml of each dilution was spread on the Bacto tryptone agar plates. The sample was allowed to absorb for 5-7minutes before incubation. The plates were then incubated at 37°C for 24 hours. After incubation discrete colonies were observed on each plate.

The colonies were counted and the colony-forming unit was calculated by the following formula (13).

$$\textbf{CFU/ml} = \frac{\text{no. of colonies X dilution factor}}{v\text{olume of culture plated}}$$

1.6.2. Determination of Plaque forming Unit Pfu/ml

Viral titters in the cell culture supernatant were strongminded using a modified plaque-forming unit (PFU) assay. In this assay, each plaque formed on plates with varying dilutions was counted. These counts, represent the visible manifestations of viral infection, Subsequently, the plaque-forming units (PFU) were calculated using the formula mentioned below. This quantitative method provides a precise assessment of viral infectivity within the cell culture supernatant, facilitating insights into viral propagation dynamics and potency. Such information aids in understanding the pathogenic potential and virulence characteristics of the viral strains under investigation. Moreover, it serves as a crucial parameter for assessing the efficacy of antiviral treatments or vaccines targeted against these isolates (26).

$$\textbf{PFU/ml} = \frac{\text{no. of bacteriophages plaques}}{dilution\ factor\ X\ v\text{olume of bacteriophage lysate used}}$$

2. Result

2.1. Isolation of host bacterium

After the four-quadrant streaking of appropriate dilution of sample on MacConkey agar medium and 24 hrs of incubation, a well isolated pink coloured, mucoid colony was selected and further purified on same medium and preserved on MacConkey's broth.

2.2. Characterization of host

By studying cultural, morphological, biochemical characterization and 16 S rRNA sequencing with phenotyping analysis, host bacterium was identified as *Enterobacteria spp.*

2.2.1 Cultural and Morphological Characteristics of *Enterobacteria spp*.

The medium used for isolating *Enterobacteria spp* was MacConkeys agar. After a 24-hour incubation period at 37°C, the morphological characteristics were recorded. The *Enterobacteria spp* colonies appeared as pink, circular, measuring 1-2 mm in size with a moist consistency (Table No.1). The isolate was found to be Gram-negative short rods and motile.

Table No.1 Colony Characteristics of *Enterobacteria spp*. on MacConkey Agar

isolates	Size	Shape	Colour	Margin	Opacity	Elevation	Consistency
Isolate 1	1-2mm	Circular	Pink	Entire	Opaque	Convex	Moist

2.2.2. Biochemical characterization of *Enterobacteria spp*.

Biochemical tests were conducted the isolated host bacterium, revealing their distinct metabolic and enzymatic properties. Isolate demonstrated a high ability to produce indole but a low ability to produce urease. Additionally, they exhibited strong fermentation abilities for glucose and lactose. The isolate *Enterobacteria spp.* showed a lower nitrate reduction rate, while *Enterobacteria spp.* displayed a higher nitrate reduction rate. Isolates tested positive for the methyl red test and negative for the Voges Proskauer test as shown in Table No. 2.

Table No: 2 Results of biochemical Tests of isolated host bacterium

Isolates	Indole	Urease	Nitrate Reduction	Methyl red [MR]	Voges Proskauer [VP]	Citrate	Lactose	Glucose
Isolate 1	+	-	+	-	+	+	-	+

'+' = Positive test, '-' = Negative test

2.3. 16S rRNA sequencing and Phylogenetic Analysis host bacterium isolate

The isolated host bacterium was identified as *Enterobacteria spp* by 16S rRNA sequencing technique, a widely recognized method for precise bacterial identification. The obtained sequences were compared with reference databases to determine phylogenetic relationships and assign accurate scientific names. As shown in Fig No.1, the 16S rRNA analysis identified the *Enterobacteria spp*. These results provided definitive taxonomic classifications and facilitated the building of phylogenetic trees, offering insights into the evolutionary connections among the isolates and their respective taxa.

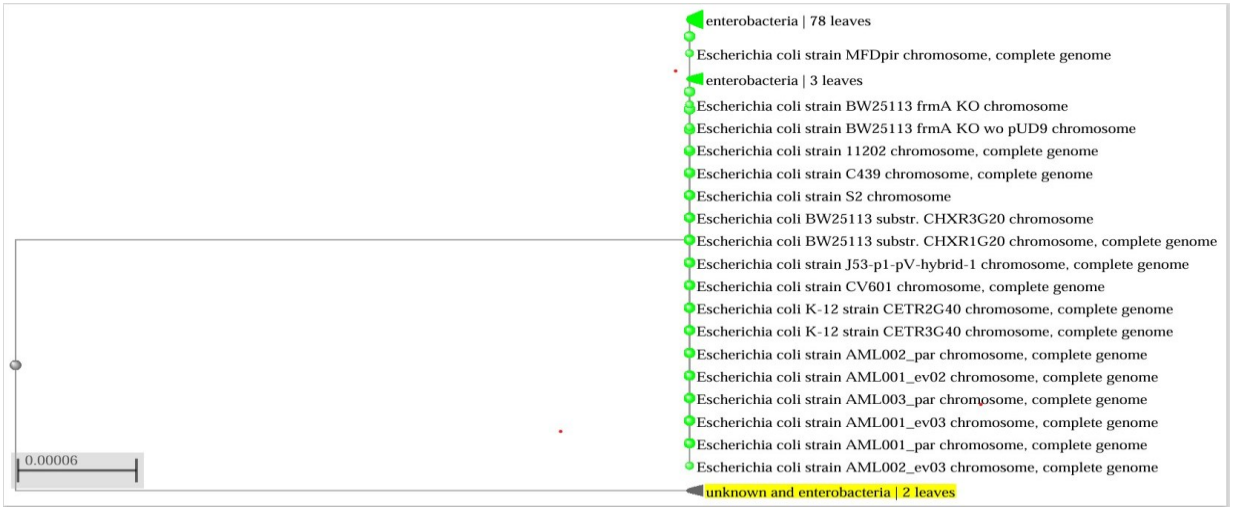

Fig 1. Phylogenetic tree of host bacterium

2.4. Determination of mid log phase of host *Enterobacteria spp*.

The growth curves of host *Enterobacteria spp.* was plotted to obtain a mid-log phase culture. The actual number of phages that will enter any given cell is a statistical process and that requires a mid-log growth phase cell. Therefore, the information about the mid-log growth phase of the host bacteria is necessary. After 3 hours, a mid-log phase culture was achieved for host bacterium *Enterobacteria spp.*, which was then used to calculate the total viable count. (Fig No 2)

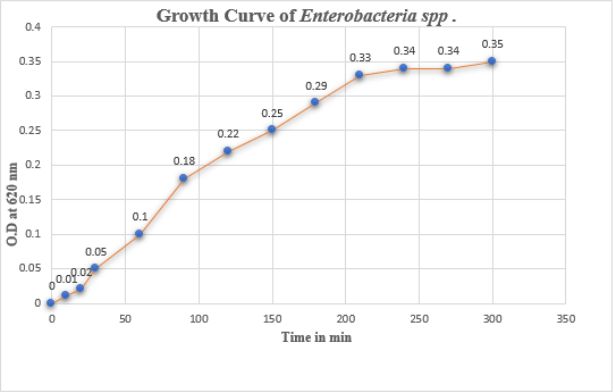

Fig.No.2 Growth curve of *Enterobacteria spp.*

2.5. Determination Total Viable Count (TVC/ml) of host *Enterobacteria spp*

The total viable count (TVC/ml) of the mid-log phase culture of isolate *Enterobacteria spp* was calculated as $72.48*10^{8.}$ This information was crucial for understanding the growth dynamics, assessing the viability of *Enterobacteria spp.*, and standardizing experiments that require a specific number of active cells.

2.6. Isolation of Phages against host *Enterobacteria spp*

A total of four phages were isolated from *Enterobacteria spp.* The plaques exhibited a distinct morphology, appearing as well-defined, clear and circular formations on BTA plates(Fig.No.2). For *Enterobacteria spp*, the average plaque diameter was approximately 1mm or less than 1mm for minute plaques and roughly 2mm to 4 mm for larger plaques. A single clear plaque with 3 mm was selected, purified and enriched for further studies.

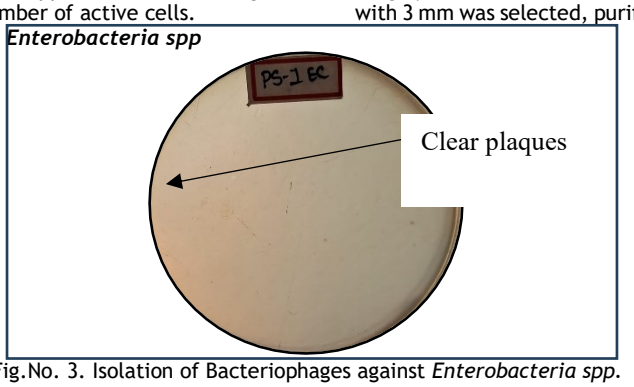

Fig.No. 3. Isolation of Bacteriophages against *Enterobacteria spp.*

2.7. Determination of phage titer (Plaque forming Units/ml)

The phage titer of purified phage lysate in term of Plaque forming Units/ml (pfu/m)l was calculated as $44.16*10^{6}$. This quantitative data offers valuable insights into the host's comparative infectivity and replication capabilities.

DISCUSSION

Our study underscores the potential of bacteriophages as a targeted biocontrol strategy against *Enterobacteri*a infection in poultry. The specificity of the isolated phages to their respective host strains highlights their suitability for phage therapy, aligning with previous research advocating for the use of strictly lytic phages in combating bacterial infections. Given the growing concerns over antibiotic resistance and the limitations of conventional antimicrobial treatments, phage therapy emerges as a promising alternative for managing colibacillosis in poultry. Furthermore, the application of 16S rRNA analysis provides valuable insights into bacterial taxonomy and evolutionary relationships, strengthening the characterization of the isolated strains. By advancing our understanding of bacteriophage-host interactions, this study contributes to the ongoing exploration of phage therapy as a viable solution for bacterial infections in commercial poultry farming.

CONCLUSION

This study focused on utilizing bacteriophages to reduce bacterial pathogen populations and mitigate the severity of diseases caused by these pathogens. The escalating prevalence of drug-resistant bacteria has posed significant challenges in the effective treatment of diseases. Bacteriophage therapy stands as a promising solution to overcome this obstacle. Lytic phages, much like antibiotics, demonstrate remarkable antibacterial efficacy.

In this study, a host *Enterobacteria spp.* was obtained from poultry waste sample. Host isolate was identified as *Enterobacteria* by 16 S rRNA sequencing and analysis of phylogenetic tree. Further, these isolates were used for isolation of lytic bacteriophages. Based on the outcomes of the investigation, it was inferred that the four bacteriophages obtained from diverse poultry reservoirs exhibit the capacity to induce lysis in their respective *Enterobacteria spp.* strains. These phages were circular and distinctive, with a clear appearance. Further investigations must be focused on the determination of morphological and genomic characterization of the bacteriophages, identification of the bacteriophages, in vivo assessments of phage efficacy, stability of phages in poultry gut environments, and potential synergistic effects with existing antimicrobial strategies for potential use of isolated phages for phage therapy in poultry to control *Enterobacteria spp.* infections.

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