

ISOLATION AND CHARACTERIZATION OF ESCHERICHIA. COLI FROM POULTRY WITH BACTERIOPHAGE THERAPY PROSPECT

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ABSTRACT

Common bacteria known as *Escherichia coli* are naturally present in birds' digestive tracts and, consequently, in their surroundings. Clinical condition known as colibacillosis, is thought to be among the most prevalent and significant illnesses affecting poultry. Avian Pathogenic *E. coli* (APEC) is the causative agent of colibacillosis. All age groups of birds as well as commercial layers, broiler chickens, and breeders are susceptible to colibacillosis. For many decades, antibiotics have been the main offensive against colibacillosis. However, the need for alternative treatments for colibacillosis and a focus on disease prevention measures has arisen due to the rise in antibiotic resistance. In order to isolate *E. coli* from five broiler chickens various organs were collected. In the current study total of 2 bacterial strains EC328 and EC329, were isolated from chicken. Standard biochemical and cultural procedures were used to identify the isolates as *E. coli*. The biochemical test results show that isolates don't react with urease test and did react with lactose fermentation and indole test. To determine the pattern of antibiotic sensitivity in the isolated isolates, sensitivity testing was conducted. The antimicrobial sensitivity rates of tetracycline, enrofloxacin, and azithromycin were the highest and were 90%, 100%, and 90%, respectively. Two bacteriophages against *E. coli* were discovered, identified, and given the names ECP8 and ECP9 during this study. It is suggested that due to their superior

bacterial growth reduction, both of these bacteriophages (ECP8 and ECP9) may be possible phage treatment options in the future after the necessary animal models and clinical studies are completed.

Keywords: Colibacillosis, Poultry, Antimicrobial Resistance, Phage therapy

INTRODUCTION

Colibacillosis, disease is caused by the *Escherichia coli* bacterium, is also known as cellulitis and mushy chick sickness. Both birds and poultry are affected by the illness. In poultry, *E. coli* is the most prevalent bacterial infection. Fecal contamination of eggs, feed, and water is how it spreads. It is characterized by pericarditis, perihepatitis, air sacculitis, and other diseases in its subacute form and by septicemia that causes mortality in its acute form. Serum, fibrin, and inflammatory cells (pus) are among the exudations that are present in the peritoneal (abdominal) cavity (Panth, 2019). Chickens' inflammatory reaction produces fibrin, which is found covering the surfaces of several organs, including the oviduct, ovary, gut, alveoli, heart, lungs, and liver (Kika *et al.*, 2023).

However, as other opportunistic bacteria might behave similarly to *E. coli* in secondary infections, we shouldn't rely just on the lesions to diagnosis an infection with *Escherichia coli* without also using the descriptors "coli" or "coliform". It has been noted that avian colibacillosis is a prevalent infectious illness that affects birds of all ages globally and has a substantial financial impact on the production of poultry (Nolan *et al.*, 2013). Due to the death and reduced productivity of impacted birds, losses are minimal and occur mostly during the late lay stage and during the peak egg production period (Linden, 2015).

Furthermore, when an egg is contaminated by feces, *E. coli* can break through the shell and infect the yolk sac. This can cause significant early chick mortality and spread to the chicks during hatching. Birds' digestive tracts naturally contain the *E. Coli* bacterium, and the majority of strains are not harmful. In young chicks, they manifest as colisepticemia (blood infection) and yolk sac infection; in adults, they manifest as peritonitis and egg coligranuloma (Indra *et al.*, 2022).

Potential risk factors for APEC include things like dietary composition, flies, insects, water, and rodents. By exposing birds to more pathogens and stress, the intensification of poultry production and the rapid extension of poultry production systems raise the prevalence of colibacillosis (Baranwal *et al.*, 2019). Clinical symptoms may not be present in situations when the infection is minor or prolonged. When a bird has colisepticemia, they may cease eating and drinking and become sluggish. The extent of decreased water intake may be a sign of the disease's severity. Birds with chronic illnesses exhibit unthriftiness and limited development (Sapkota and Pandey, 2019).

Antimicrobials have been used to combat colibacillosis for many years. But when antibiotics are used excessively, *E. Coli*, like many other bacteria, can develop antibiotic resistance. This resistance is brought on by either overuse of antibiotics on a preventative

basis or improper use of antibiotic therapy with an underdosage (Sargeant *et al.*, 2019). The rise of antibiotic resistance necessitates the implementation of new tools and techniques as well as alternative ways to manage bacterial infections. Viruses that destroy bacterial cells, or bacteriophages, are used as a means to control *E. coli* infections (Luppi, 2017). Naturally occurring in the environment, bacteriophages have been shown to be effective in controlling colibacillosis. Food items, wastewater, soil, and forest undergrowth are just a few of the diverse settings that are home to bacteriophages, or simply "phages" (El-Gohary *et al.*, 2014). Phages need bacterial cells as hosts in order to survive, thus they are frequently seen with bacteria (El-Gohary *et al.*, 2014). The processes of phage-host interaction harm some bacteria that function as hosts for phage replication. The bacterial host's physical and biological characteristics are impacted by these processes. Nowadays, phage treatment is acknowledged as a substitute approach to bacterial illnesses, potentially reducing the requirement for antibiotics (Huff *et al.*, 2013).

MATERIALS AND METHOD

SAMPLE COLLECTION

5 samples from broiler chicken's cloacal swabs, visceral organs were collected across five farms from district Abbottabad.

SAMPLE PROCESSING

For *E. coli* isolation, samples were processed aseptically and was suspended in saline buffer. Using a tissue grinder or sterile mortar and pestle, the cleaned specimens were further physically homogenized to a fine particle fineness in order to ensure equal microbial

dispersion. This homogenate was then floated in a pre-measured volume of nutrient-rich liquid culture medium, such as Luria-Bertani (LB) broth, in sterile 50mL Falcon tubes. For optimal growth conditions, the sample-to-media ratio was carefully maintained at 1:10. Then, for a standard 24-hour incubation period, the inoculation tubes were put in a shaking incubator set to 37°C ($\pm 1^\circ\text{C}$) and agitated at 180–200 rpm. The samples were cultured on LB agar and incubated aerobically at 37°C for 24 hours before the bacterial growth was assessed (Saidi *et al.*, 2013).

MORPHOLOGY BASED CHARACTERIZATION OF ISOLATED *E. COLI*

GRAM STAINING

A small amount of distilled water was put to a clear slide in order to carry out the Gram staining. A small quantity of pure culture was applied to the slide using a sterile needle. The needle was used to distribute the culture uniformly on the slide. A drop of crystal violet was applied to the slide smear using sterile water. After achieving a consistent dispersion, the crystal violet was swirled in and allowed to dry for around 30 seconds. After applying crystal violet stain, the slide was gently washed with distilled water that had been sterilized. To get rid of any remaining crystal violet color, a droplet of Lugol's iodine was added to the smear after the slide had been thoroughly cleaned with pure distilled water. Crystal violet and Lugol's iodine work together to hold the stain in place. Acetone was used to clean the slide following the application of Lugol's iodine. Acetone is a decolorizer that helps get

rid of extra stains on the slide. A drop of safranin was applied to the slide to hide the smudge. Gram-negative bacteria acquire their characteristic hue from the counterstained safranin stain. The slide was eroded clean after being properly cleaned with water to remove any remaining safranin. The extra liquid was carefully removed from the slide using blotting paper. To preserve the discolored smear, a drop of mounting agent Canada balsam was applied to the slide. A 100X magnification microscope was used to view the slide with the plated smear (Greenwood *et al.*, 2012).

BIOCHEMICAL CHARACTERIZATION

Biochemical assays, including lactose fermentation test, indole test, test urease test were carried out. Briefly described as follow:

LACTOSE FERMENTATION TEST

Tests of lactose utilization determine if bacteria can ferment or oxidize a specific carbohydrate lactose to produce acid. The way an organism consumes lactose varies depending on its complement of enzymes. The first step was making a liter of nutrient broth and adding a little sprinkle of phenol red indicator, which changes the broth color as bacteria grow in it. Each test tube was filled with nutrient broth, Durham tubes were then placed inside, and the tubes were autoclaved. Following autoclaving, carbon source lactose was added to the test tubes. Test tubes were filled with 48-hour-old cultures of *E. coli* strains, and the test tubes were monitored for any changes in the culture (Reddy *et al.*, 2022).

UREASE TEST

Urea was created when amino acids underwent decarboxylation. The breakdown of urea

produced CO₂ and ammonia. The presence of ammonia, which made the solution more alkaline, was indicated by phenol red, which changed from a light orange hue at a pH of 6.8 to a magenta (pink) tint at a pH of 8.1. Phenol red was employed to detect pH variations in solutions. Urease-positive bacteria colored the medium pink after a day. Because of the generation of acid, negative organisms either produced a yellow color change or no color change at all. A urea agar slant can be inoculated with one or two drops of an overnight brain-heart infusion broth culture, or a piece of a well-isolated colony can be applied to its surface. Additionally, incubate the tube at 35 to 37 degrees for 48 hours to 7 days. After then, observe for the emergence of a pink hue at least seven days (Brink, 2010).

INDOLE PRODUCTION TEST

An indole test is used to determine out if an organism can convert tryptophan to indole. A culture of the bacterial strains was injected into a tube filled with tryptophan broth and kept at 37 °C for 24 to 48 hours in order to perform the Indole test. Add 0.5 ml (5 drops) of Kovac's reagent and mix gently. Observe the topmost layer of the liquid; if purple or red rings appear there, a favorable result is shown; if yellow rings appear, an unsuccessful outcome is shown (KOMAL, 2019).

ANTIMICROBIAL SUSCEPTIBILITY TESTING

The disk diffusion susceptibility test identify the sensitivity or resistance of facultative anaerobic and pathogenic aerobic bacteria to different antibiotic drugs (Forbes *et al.*, 2007). In this investigation, Mueller-Hinton agar

(Merck, Germany) was subjected to disk diffusion (Kirby Bauer's) antimicrobial susceptibility testing. To ascertain the susceptibility of agents, this approach was carried out in compliance with the recommendations set forth by the Clinical and Laboratory Standards Institute (CLSI). The antibiotic disks (which include imipenem, moxifloxacin, ciprofloxacin, meropenem, azithromycin, tetracycline, enrofloxacin and cefixime) was utilized (Forbes *et al.*, 2007).

ISOLATION OF BACTERIOPHAGES FROM SEWAGE

To isolate bacteriophages, the gathered sewage samples were taken to Abbottabad University of Science and Technology's Microbiology Laboratory. First, the water samples were shaken for two minutes. To get rid of the big particles, and silt, they were centrifuged for ten minutes at 10,000 rpm. 40ml of clear sewage supernatant was added to 10ml of sterile 5x nutritional broth in a conical flask. 200ul of an overnight culture of *E. coli* was added to the flask as an inoculant. Overnight, the inoculated flask was shaken (at 120 rpm) at 37°C. Following incubation, the flask's contents were centrifuged for five minutes at 10,000 rpm. The clear supernatant was collected in a fresh, clean falcon tube and stored at 4°C for later use after being filtered with a 0.22 ul syringe filter. By using a spot test, the presence of bacteriophages in the filtrate was identified (Asif *et al.*, 2018).

LYTIC SPECTRUM DETERMINATION BY SPOT TEST

The spot test method was used to assess the phages' capacity to induce lysis in various

bacterial strains. The nutrient agar plate was covered with 100ul of an overnight-grown culture of *E. coli* for the spot test. After applying 5 ul of the filtrate, the plates were allowed to dry for nearly ten minutes. The plates were then incubated for the whole night at 37°C. After that, the plates were examined for a distinct lysis zone produced by bacteriophages. Any bacterial lawn showing a lysis zone at the end of the incubation time is considered vulnerable to the phages (Asif *et al.*, 2018).

QUANTIFICATION AND PURIFICATION OF BACTERIOPHAGES USING DOUBLE LAYER AGAR ASSAY

Bacteriophages from the lysate were quantified and purified using the double layer agar overlay technique. The first step included serially diluting the lysate (1:9). Log phage bacteria (100µl) were introduced to each dilution. After pouring the liquid onto an LB plate, 3-5 milliliters of LB semisolid agar were added, and the mixture was stirred to ensure adequate mixing. Overnight, the plates were incubated at 37°C. Following incubation, the plaque morphology was examined and tallied. Plaque forming units per milliliter (pfu/ml) were used to count the plaques that developed on the plate. After the phage was purified, their pfu was ascertained. Plates with unique plaque were chosen for phage purification. Carefully, a sterile micropipette tip was used to tap the plaque surface. For phage propagation, the tip was placed in a test tube with 10 ml of nutrient broth and 1 ml of fresh *E. coli* culture. It was then cultured for 24 hours at 37 °C, after which plaque was visible and purified. Up to

ten repetitions of the purification stage were carried out. The lysate titer was then determined (Alvi *et al.*, 2020).

RESULTS

SAMPLE COLLECTION AND PROCESSING

A total of 5 samples were collected from broiler chickens across five farms. Samples included cloacal swabs (2), visceral organs (3). Each sample was stored in sterile containers and

transported under cold chain conditions to maintain bacterial viability. Samples were surface sterilized in a saline buffer solution for one minute. They were then rinsed three times with sterilized water and immediately transferred to LB broth medium (Figure 1).



FIGURE 1: SAMPLE PROCESSING

IDENTIFICATION OF BACTERIAL ISOLATES

Samples were pre-enriched in buffered peptone water and incubated at 37°C for 18–24 hours. Enrichment was done by using LB broth. The

processed sample were cultured on the surface of LB agar, where white or creamy colonies show typical *E. coli* morphology. Out of five samples two *E. coli* strains was isolated from chicken (EC328 and EC329) (Figure 2).

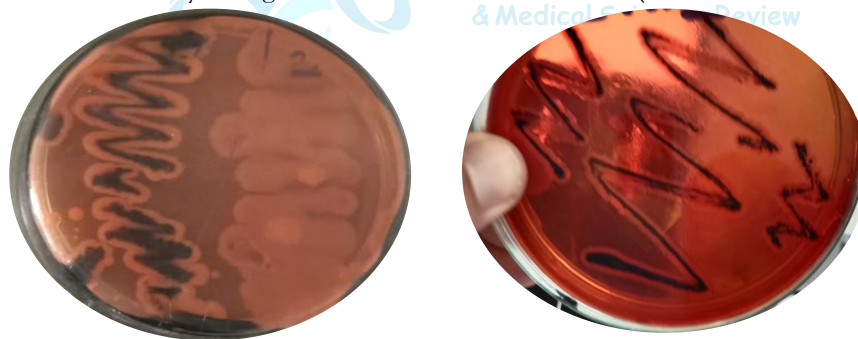


FIGURE 2: MORPHOLOGY OF BACTERIAL ISOLATES EC328 (A), EC329 (B)

GRAM STAINING RESULTS

Gram staining revealed that an isolated species of *E. coli* which had been grown for the overnight, was a rod-shaped, Gram-negative bacterium and appear pink color under

microscope. The Gram staining result and cellular arrangement offered the initial evidence that these isolates were *E. coli* before doing confirmatory biochemical tests.

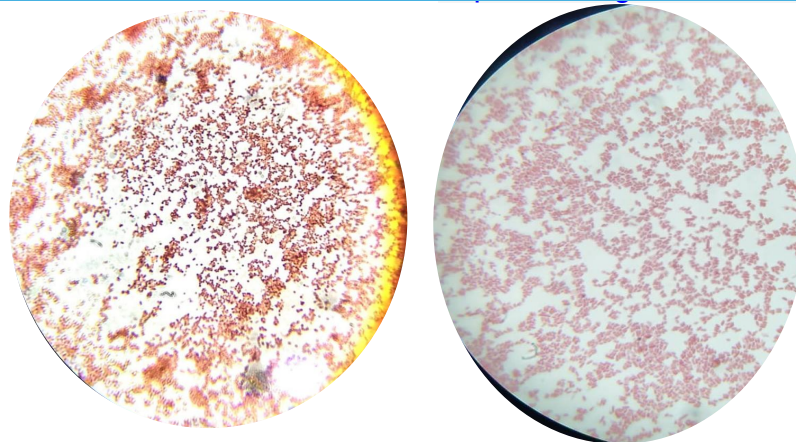


FIGURE 3: MICROSCOPY OF BACTERIAL ISOLATES

BIOCHEMICAL CHARACTERIZATION

LACTOSE FERMENTATION TEST

The ability of bacteria to ferment or oxidize a particular lactose in order to create acid is

determined by lactose fermentation tests. *E. coli* stains (EC328 and EC329) gave a positive result to lactose utilization test red color show positive result (Figure 4).

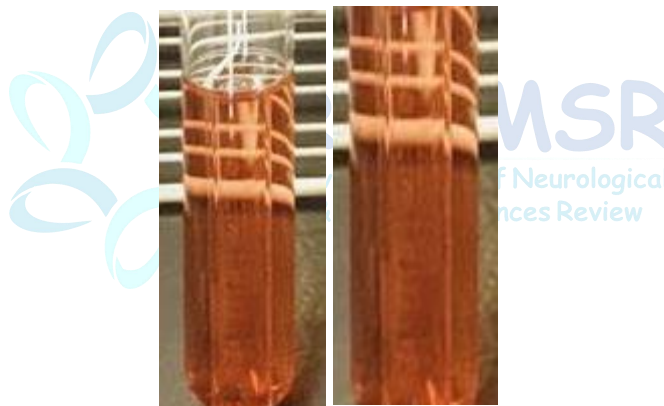


FIGURE 4: LACTOSE TEST RESULT FOR *E. COLI*

UREASE TEST

Bacterial isolates EC328 and EC329 were urease negative, which mean that does not

break down urea into ammonia and carbon dioxide (Figure 5).



FIGURE 5: UREASE TEST FOR BACTERIAL ISOLATES

INDOLE TEST

A reddish-colored ring appeared on the glass tube surface as soon as the Kovac's reaction was injected, indicating a successful indole test.

Upon adding five to six drops of Kovac's reagent, bacterial isolate (EC328 and EC329) gives a positive indole test result (Figure 7).

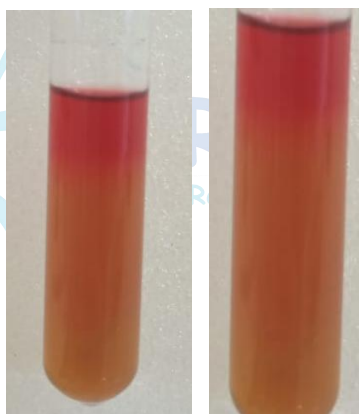


FIGURE 7: INDOLE TEST RESULTS FOR BACTERIAL ISOLATES

TABLE 1: BIOCHEMICAL CHARACTERIZATION OF BACTERIAL ISOLATES

TESTS	EC328	EC329
Gram staining	-	-
Lactose fermentation test	+	+
Urease test	-	-
Indole	+	+

ANTIMICROBIAL SUSCEPTIBILITY TESTING

To determine the antibiotic sensitivity pattern of isolated strains, sensitivity testing was

performed. The highest antimicrobial Sensitivity rate was substantial, Tetracycline 90%, Enrofloxacin 100%, Azithromycin 90%.

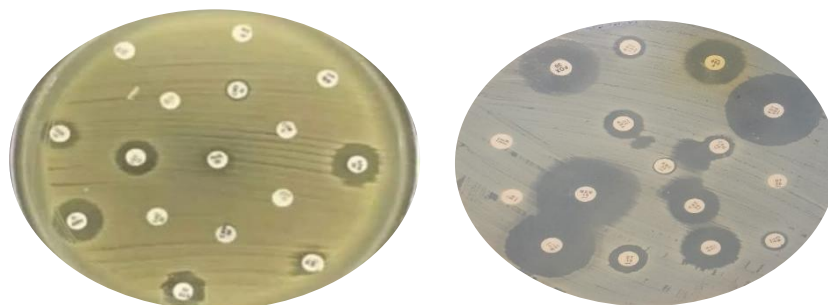


FIGURE 8: ANTIBIOTIC RESISTANCE PATTERN OF BACTERIAL ISOLATES
SPOT TEST FOR THE DETECTION OF *S. AUREUS* SPECIFIC BACTERIOPHAGES
Bacteriophages against *E. coli* (EC328 and EC329) was detected in two of five sewage samples. ECP8 and ECP9 was the name of the bacteriophages that was acquired from DHQ Shangla.



FIGURE 9: DETECTION OF BACTERIOPHAGES THROUGH SPOT TEST ECP8 AND ECP9

ISOLATED BACTERIOPHAGES PRODUCED CLEAR TRANSPARENT PLAQUES

On a double layer agar plate, the isolated phage produced a circular, clear plaque that was antagonistic to EC328 and EC329. The plaque is composed of two circular layers, with an outer circle around the inner, completely transparent center. The existence of this hazy

layer surrounding plaque is a sign that bacteriophages are producing the depolymerase enzyme. ECP8 and ECP9 were a diameter of 2 mm. The halo surrounding the plaque indicates that the bacterial host cell was decapsulate by soluble enzymes such depolymerase, which was produced by phages ECP8 and ECP9.

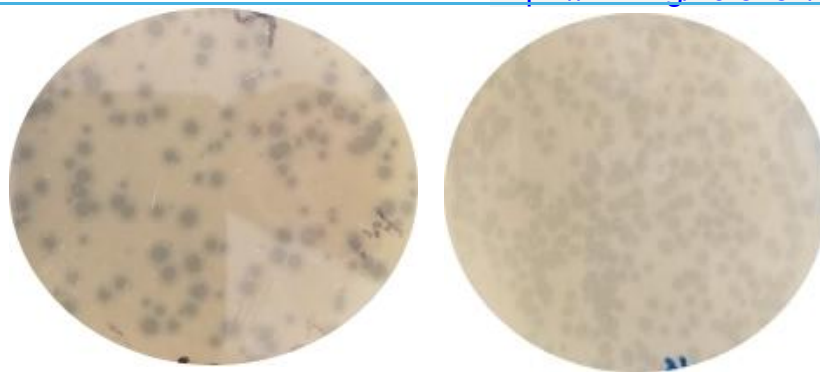


FIGURE 10: ISOLATED BACTERIOPHAGES HAVE TRANSPARENT PLAQUE ECP8 AND ECP9

DISCUSSION

One of the major pathogens causing intestinal and systemic illnesses in humans and other animals are bacteria belonging to the Enterobacteriaceae family. One of the more well-known members, *Escherichia coli*, and the possible issues with strains of this organism, such as Colibacillosis in chickens, have drawn public attention due to recent outbreaks of gastrointestinal illnesses. In this investigation, five samples were taken from broiler chickens in five farms in the Abbottabad region that had respiratory and digestive symptoms (diarrhea, respiratory rales, tracheitis, periorbital edema with fibrinous air sacculitis, pericarditis, and perihepatitis). Cloacal swabs (2) and visceral organs were among the samples. For one minute, the samples were surface sterilized in a saline buffer solution. They were then rinsed three times with sterile water and immediately placed to LB broth medium. On LB agar, where white or creamy colonies exhibit typical *E. coli* morphology, culture was carried out. Two *E. coli* strains named as EC328 and EC329, were isolated from chicken out of five samples. Similar study was reported by Fri *et al.*, (2015) which shows 20 boiler

chicken samples were collected and after processing *E. coli* was isolated from 12 samples. An isolated strain of *E. coli* that had been cultured overnight was shown by Gram staining to be a rod-shaped, Gram-negative bacterium that appeared pink under a microscope. Rehman *et al.*, (2018) reported a similar investigation that demonstrated *E. coli* was a rod-shaped, Gram-negative bacterium. Biochemical characterization is the another aspect of study. Indole and urease tests for lactose fermentation were carried out in current investigation. The lactose consumption and indole tests gave positive findings for all strains of *E. coli*, although the urease test yielded negative results. Similar study by Ruya *et al.*, (2015) which shows of *E. coli* were positive towards lactose utilization test and indole test while negative towards urease test. Sensitivity testing was done to ascertain the isolated isolates' pattern of antibiotic sensitivity. Tetracycline, Enrofloxacin, and Azithromycin had the greatest antimicrobial sensitivity rates, which were significant at 90%, 100%, and 90%, respectively. According to a similar study by Ishan *et al.*, (2018), *E. coli* strains shown

high antimicrobial sensitivity to azithromycin and tetracycline.

In a spot test, two out of five sewage samples possessed bacteriophages against *E. coli* (EC328 and EC329). The bacteriophages that was obtained from DHQ Shangla was known as ECP8 and ECP9. This is because bacteriophages rely on their host bacteria for development, making them obligatory parasites. One possible cause of this illness is variations in the quantity of host bacteria at sample collection sites (Alvi *et al.*, 2018). It's possible that the three water samples' lack of phage identification was caused by the collection of water without sediments since phage particles progressively adhere to the sediment that has accumulated at the bottom of the water body. Depolymerase, one of the soluble enzymes produced by the phages EC328 and EC329, broke down the bacterial host cell, as evidenced by the halo surrounding the plaque. As the bacterial capsular polysaccharide (CPS) penetrated the agar layer, the halo showed that the depolymerase enzyme had broken it up into separate oligosaccharide components (Alvi *et al.*, 2018).

CONCLUSION

The most prevalent bacterial disease in chicken, colibacillosis, has significant financial implications for the producers. Antibiotic resistance has emerged as a result of disease management using antimicrobials, raising serious concerns for both human and animal health. It is imperative that a new strategy for managing colibacillosis, including different preventative measures and alternative therapies, be used in conjunction with the prudent use of

antibiotics. Bacteriophages remain a potent alternative to conventional medicinal approaches, despite the existence of various alternatives. They are capable of being isolated from the bacterial environment. Bacteriophages EC328 and EC329 seem to be promising candidates for phage therapy after the necessary animal modeling and clinical trials because of their *in vitro* efficacy in inhibiting bacterial growth.

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