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Evaluation of Phage-Antibiotic Synergism Against *Salmonella enteritidis* Isolated from Broiler

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Antibiotic, Bacteriophage, Broiler, Salmonella, Synergism

ABSTRACT

Salmonella enteritidis is a facultative intracellular zoonotic pathogen and has one health importance. Antimicrobial-resistant strains can be found in poultry, especially broiler, and can act as a carrier to spread these diseases to humans. Lytic bacteriophage therapy is increasing these days due to its less toxicity. Samples (n=25) were collected from a poultry farm in different areas of Faisalabad district. For the isolation of bacteria, Salmonella Shigella agar was used, and other biochemical tests were performed for confirmation of *S. enteritidis*. Then antimicrobial susceptibility was determined through Kirby Bauer disc diffusion method. Bacteriophages were isolated from sewage samples using the Double agar overlay method. In TEM, isolated phage SE-3 was observed as a *Siphoviridae* family member due to its non-contractile tail. It showed high stability at pH 6-9 and temperature 30-50 °C. Phage antibiotic synergism was performed by using the antibiotic gentamicin. Synergism of gentamicin with concentration 0.582 and phage with MOI 10 exhibit significant (p=0.02) results. The OD values of bacterial culture before the treatment and after the treatment were measured. Phage antibiotic synergism analysis indicated that phage showed synergism with antibiotics. After a comparison of OD values, a higher rate of bacterial killing was observed in synergistic treatments than in alone treatments. Our findings reveal that the potential for phage applications to reduce infections caused by bacteria increases when antibiotics are combined with phage.

INTRODUCTION

Salmonella enteritidis is a facultative intracellular bacterium capable of infecting both animals and humans (Sarrami *et al*., 2023). In broilers, *S. enteritidis* infection is a significant causing reduced productivity and compromised welfare. Moreover, this infection poses a hazard to public health through the utilization of contaminated chicken products. Salmonellae are not indigenous to the normal microbiota of the intestinal tract (Afshari *et al*., 2018). However, pre-exposed chicks with underdeveloped immune systems and immature intestinal microflora were found to be highly susceptible to colonization by *S. enteritidis* (Pan and Yu, 2014). *S. enteritidis* infections are known to induce significant changes in the gut microbiota and impair the integrity of the intestinal barrier, leading to detrimental effects on digestion, nutrient malabsorption, and immune system activation. These alterations collectively contribute to the development of inflammation (Rasigade and Vandenesch, 2014).

Broiler meat, as well as eggs and egg derivatives, is a common cause of human salmonellosis. The need to

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control the existence of *Salmonella* spp. in the poultry industry has been established. In this regard, commission regulation (Eurosurveillance Editorial Team) number 200/2012 has stipulated that a substantial proportion of broiler flocks, namely 1 percent or less, may test positive for *S. enteritidis* and *S. typhimurium* (Hugas and Beloeil, 2014). *Salmonella* is a commensal gut microorganism found in animals, particularly farm animals. The utilization of chicken meat and products represents a prevalent route of direct transmission of pathogenic bacteria to humans, thereby leading to the onset of illness.

The increase of antibiotic resistance in *S. enteritidis* is a significant problem because of the indiscriminate utilization of antibiotics in the food and livestock industry. Antimicrobial resistance strains have been identified in poultry, particularly in broiler chickens, which can serve as a source of transmission of these pathogens to humans (Asif *et al*., 2017). The acquisition of resistance genes in bacteria can occur through the transfer of moveable genetic elements, such as plasmids. This mechanism not only increases the flexibility of the host bacteria but also facilitates the proclamation and dispersion of these genes between bacterial populations. Multidrug-resistant bacteria, specifically *S. enteritids* and *S. typhimurium*, possess the capacity to invade human hosts, leading to systemic infection and potentially fatal outcomes in instances where treatment proves ineffective (El-Sharkawy *et al*., 2017).

In the present scenario of burning issues of MDR, the researcher focuses on bacteriophage therapy considered one of the best methods to overcome antibacterial resistance. Among bacteriophages, lytic bacteriophages are viruses that can divide independently by high-jacking host machinery hence killing the target bacteria (Jamalludeen *et al*., 2009). The understanding of Phage lytic functions is crucial for their use in the fight against bacterial pathogens. Bacteriophages with small RNA or DNA genomes encode single proteins which are called amurins and cause lysis by the inhibition of cell wall synthesis. Bacteriophages of double-stranded DNA genomes, which dominate in the environment, encode enzymes that are called endolysins and contribute to lysis by the cleavage of cell wall peptidoglycan (Woźnica *et al*., 2015). These lytic enzymes are generated in the cytoplasm and require the help of another enzyme called holin to cross the cytoplasmic membrane and reach their target. After holin disrupts the membrane, lysin degrades the peptidoglycan, and the phage progeny is then released (Dennehy and Abedon, 2021).

Bacteriophage therapy is considered a better therapy as compared to antibiotics against lethal bacterial infections. Most antimicrobial drugs have broad-spectrum activity and these can kill or inhibit the growth of many bacteria at one time, this is considered one of the big disadvantages of bacteria because due to this activity not only lethal but some beneficial bacteria are also affected with finally results in immune suppression of target individual. On the other hand, bacteriophages are quite limited in their action. They kill or target specific strains of bacteria (Domingo-Calap and Delgado-Martínez, 2018).

As of the present, scientists have investigated the synergistic impact of bacteriophages in conjunction with antibiotics that selectively target distinct strains of antibiotic-resistant bacteria (Tkhilaishvili *et al*., 2018). Several investigations have demonstrated synergistic outcomes, alongside contrasting reports indicating additivity and antagonism, when examining the combined effects of bacteriophages and antibiotics. The combined use of antibiotics and phage may result in decreasing the concentration of antibiotics required for individual antibiotic treatment and hence contribute to a reduction in the prevalence of both phage and antibiotic resistance. Moreover, phages may enhance the therapeutic efficacy of antibiotics by targeting antibiofilm effects when used together (Chang *et al*., 2019).

The objective of this study was to identify *S. enteritidis* from broiler chickens and to extract lytic bacteriophages from sewage water sources that target *S. enteritidis*. Additionally, assessed the combined effect of the newly identified phage and the particular conventional antibiotic, gentamicin, against *S. enteritidis.*

MATERIALS AND METHODS

This research was conducted at the Bacteriophage Lab of the Institute of Microbiology, Faculty of Veterinary Science, University of Agriculture Faisalabad. A total number of 25 broiler fecal samples were collected from broiler farms in different areas of Faisalabad district. The overall research was divided into major four steps. The first phase consisted of selecting sources, gathering samples, transporting them to the lab, and isolating and identifying *S. enteritidis* based on its colony form, staining ability, and biochemical properties. The second step involved determining the isolated bacteria's current antibiotic sensitivity and resistance patterns. The third step included the isolation of bacteriophages against *S. enteritidis*. In the fourth step, the evaluation of bacteriophage antibiotic synergism against the bacteria was determined.

Isolation and identification of S. enteritidis

Following the collection process, every sample was inoculated into the recently prepared nutrient broth (NB), appropriately placed into tubes, and incubated for 24 h

in an aerobic condition in a bacteriological incubator at 37 °C. The *Salmonella-Shigella* agar and McConkey agar were then individually streaked with a loop full of bacterial culture from incubated tubes. The plates were thoroughly checked for the presence of typical colonies of Salmonellae (Nesa *et al*., 2011).

For microscopic identification, Gram's staining of the pure culture was performed according to the method described by Thairu *et al*. (2014). For biochemical test confirmation different tests including; catalase, urease, oxidase, indole, methyl red, voges proskauer, citrate, and triple sugar iron test were performed (Hemraj *et al*., 2013).

Antimicrobial susceptibility test

The antimicrobial susceptibility of the isolates was assessed using the disk diffusion assay following the protocols established by the Clinical and Laboratory Standards Institute (CLSI) (Jorgensen and Turnidge, 2015). The bacterial strain was subjected to aerobic cultivation in 10 mL of nutrient broth at a temperature of 37 °C for 24 h. The culture was swabbed with a sterile, non-toxic cotton swab on Mueller-Hinton agar plates and allowed to dry for two to four min. Using tooth forceps, the antimicrobial sensitivity discs were then positioned on the culture plates and incubated. Antibiotic discs tested were amoxycillin (30 µg), ciprofloxacin (5 µg), tazobactam (110 µg), gentamicin (10µg), sulphamethoxazole (100 µg), chloramphenicol (30 µg), tetracycline (30 µg), ceftriaxone (30 µg) and ampicillin (10 µg).

Bacteriophage isolation

A total of 20 samples of sewage water were collected in 50 ml falcon from different sewage systems present in the University of Agriculture Faisalabad. The samples were brought to the microbiology lab for phage isolation. Collected sewage water samples were centrifuged for 10 min at 10000 rpm and the supernatant was passed through 0.22 µm syringe filters to remove solid and cellular materials. The phage isolation process was then started using the filtrate directly (Alharbi *et al*., 2023). Three things were mixed in an Eppendorf tube i.e. 1 ml of nutrient broth, 0.7 ml of filtrate was added, and 0.2 ml of overnight grown bacterial culture and incubated at 37 °C for 24 h. After that, centrifugation was performed at 8000 rpm for 10 min and the supernatant was collected. Filtration of supernatant was done with 0.22 µm filter paper syringe assembly. The filtrate was collected containing phage lysate and processed further for the isolation of bacteriophages. Then 1.5 ml of amplified phage lysate was mixed with 1.5 ml of *S. enteritidis*. The mixture was subjected to incubation at 37 °C for 1 h so that adsorption of phages with bacteria could occur. After that, 3 ml of molten soft agar (kept at 45 °C after melting) was mixed with this mixture. The mixture was gently swirled and poured on top of solidified Petri plates of nutrient agar. Then the petri plates were swirled to mix the suspension. The agar plates were subjected to an incubation period of 24 h at 37 °C. Petri plates were examined for plaque formation. These plaques were removed by using a sterile needle and placed in an Eppendorf tube with 100 µl SM buffer and 10 µl of chloroform. Then, the phage was stored at 4°C.

Characterization of isolated phages

Transmission electron microscopy

Purified phage pellets dissolved in SM buffer were used for TEM. Grid samples were prepared using a given phage lysate following a protocol of (Vongkamjan *et al*., 2012). Phage lysate was transported under sterile conditions, and 200-mesh grid was applied with two drops of phage stock and then let air-dried before being placed in an electron microscope. The magnification of photographed phages was done by using a single view camera having magnification × 100,000 and × 150,000, respectively (Pelyuntha *et al*., 2021).

Host specificity

Isolated *Salmonella* spp. was used for this experiment. A spot test was performed. 10 µL of phage 10^8 pfu/mL was spotted on bacterial lawn culture on the nutrient agar plate. Clear zones on spotted phage were observed after incubation at 37°C for 24h (Jensen *et al*., 2015).

pH and thermostability

For heat-stability tests, tubes containing phages were placed at various temperature ranges between 30-90 °C with intervals of 10 for 30 min. For pH stability testing, bacteriophage samples were combined in several tubes with a nutrient broth of varying pH (3-11) and incubated at 37°C for 2 h. Double-layer agar plate method was used to determine phage titer (Joe *et al*., 2022).

Determination of minimum inhibitory concentration (MIC) of antibiotics

MIC values of gentamicin were determined by the microdilution method (Moradpour *et al*., 2020). 100µl of sterile water was added up to the 10^{th} well. Then 100µl of 10% gentamicin was added to 1^{st} well and was mixed properly. After that, 100µl of bacterial culture from 1^{st} well was added to the second well and the procedure was repeated until the 10^{th} well (two-fold dilution). The plate was covered and incubated for 24 h. After the required incubation, OD was checked at 600 nm by a spectrophotometer.

Phage antibiotic synergism

Phage antibiotic synergism was evaluated by the modified broth microdilution method in flat-bottomed 96 multi-well microplates (Moradpour *et al*., 2020). A 96-well flat-bottom microtitration plate was used to assess the synergy between gentamicin and phages. Triplicate studies were carried out under four conditions: gentamicin alone, bacteriophage alone, gentamicin-bacteriophage combined, and bacteria alone as a control. Different antibiotic concentrations were combined with a set quantity of bacteriophage (MOI 10). Antibiotic concentrations were determined by a 2-fold serial dilution of MIC values and incubated on a microtitration plate at 37 °C for 24 h. To ensure clear findings, 100 µl of TTC (0.5%) was applied to the plates. The inhibitory impact of these combined antibiotic and phage doses on bacteria was investigated by measuring the optical density of various wells at 600nm.

Statistical analysis

The statistical analysis to evaluate the effectiveness of phage-antibiotic synergism was conducted using one-way analysis of variance (ANOVA) by comparing values of optical densities. The significance of the test was determined at $p \leq 0.05$.

RESULTS

Salmonella enteritidis *isolates*

Out of 25 samples that were collected, 10 samples were positive while 15 were negative for *S. enteritidis*. The positive samples showed colorless colonies with a dark black center on SS agar after incubation at 37 °C and for 24 h. For further confirmation, the bacteria were grown on MacConkey agar. They showed colorless colonies on MacConkey agar due to non-fermentation of lactose after incubation at 37 °C for 24 h (Fig. 1). Under the microscope the bacteria looked Gram-negative dark pink rods. During biochemical analysis, *S. enteritidis* were found positive for catalase, citrate utilization test, and methyl red positive and were negative for oxidase, urease, Voges-Proskauer, and indole tests. In the TSI test, it showed an alkaline slant and acidic butt with H_2S production.

Antibiotic susceptibility test

Antimicrobial resistance was investigated against *Salmonella enteritidis* isolates (Fig. 1). The high number of *S. enteritidis* isolates showed high resistance to ampicillin (85%), tetracycline (80%), and ceftriaxone (70%) and lower resistance to chloramphenicol (65%), gentamicin (55%), and sulphamethoxazole (50%). *S. enteritidis* isolates showed susceptibility to ciprofloxacin (75%) and tazobactam (60%) which can be used as drugs of choice in *S. enteritidis* infection (Fig. 2).

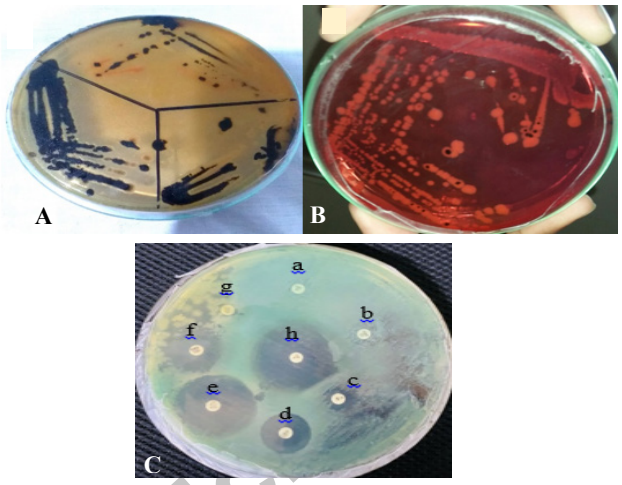

Fig. 1. *S. enteritidis* showed black growth on SS agar (A), NLF (non-lactose fermenter) growth on MacConkey agar (B), C shows AST against Tetracyclin (a), Ampicillin (b), Ceftriaxone (c), Gentamicin (d), Ciprofloxacin (e), Tazobactam (f), Sulfamethoxazole (g), and chloramphenicol (h).

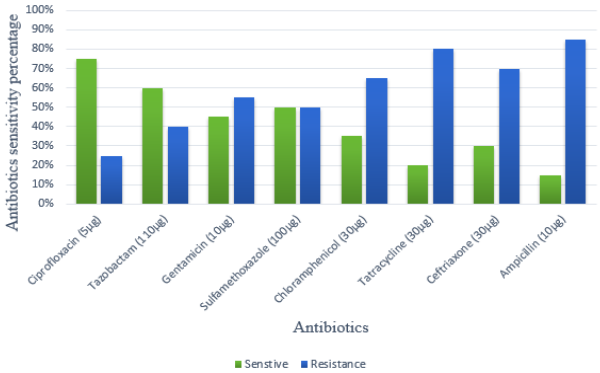

Fig. 2. Antibiotic susceptibility testing of *S. enteritidis* isolates.

Bacteriophage in the sewage samples

Phage presence in the sewage samples was assessed using the double agar overlay method. Phages exhibited lytic activity by generating clear and uniformly-sized 5mm plaques on lawns of *S. enteritidis* isolates (Fig. 3A). Three phages were isolated against 10 bacterial strains. Out of these three only one phage showed high lytic activity with almost 70% of host range. This isolated phage also exhibits lytic activity against other species of *Salmonella* and some *E. coli* (Table I). Isolated phage name as SE-3. Under TEM isolated phages have a non-contractile tale, which belongs to the family *Siphoviridae* with a diameter of 86.24nm of

capsid and 196.28 nm length of the tail (Fig. 3B). Phage titer was maintained at 5×10^7 pfu/ml to determine the pH and thermostability of isolated phage. High phages were observed between pH ranges 6-8. And after 8 phage titer decline (Fig. 4A). For thermostability, phages were stable between 30-50° C. After 60 no phage was observed (Fig. 4B).

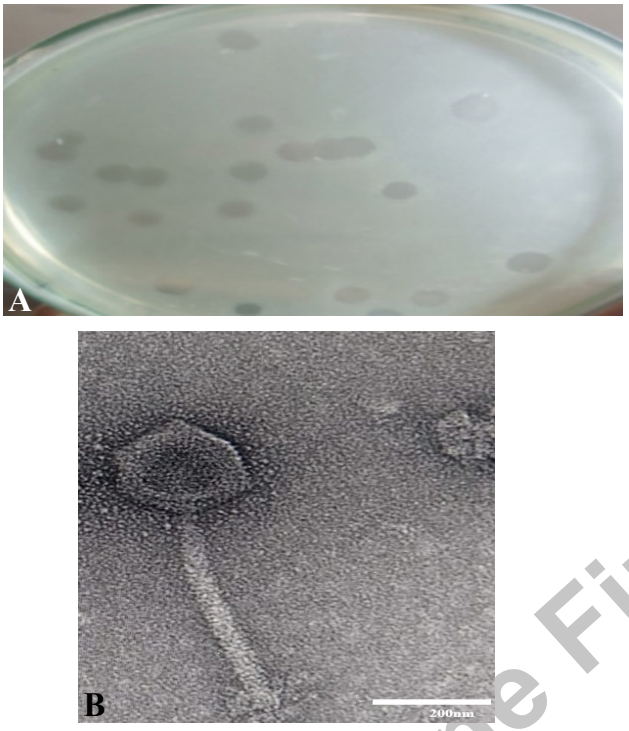

Fig. 3. Bacteriophages: A, phage plaques showing lytic activity. B, Isolated phage belongs to Siphoviridae with 86.24nm of capsid and 196.28 nm length of tail. as seen under TEM.

Table I. Host range of *S. enteritidis.*

S. No.	Bacteria	Positive
1	*S. enteritidis* (SE1, SE3, SE4, SE5, SE6, SE8, SE9, SE10)	+ve
2	*S. enteritidis* (SE2, SE7)	-ve
3	*S. pullorum* (SP16)	+ve
4	*S. pullorum* (SP23)	-ve
4	*Salmonella typhimurium*	+ve
6	*E. coli* (EC 5)	+ve
7	*E. coli* (EC 8)	-ve

Minimum inhibitory concentration

MIC of gentamicin was determined using the microdilution method. All 10 isolates of *S. enteritidis* were uniformly susceptible to gentamicin with MIC 4.5 µg/ml.

Phage-antibiotic synergism

For gentamicin alone, absorbance was recorded between 4.5, 2.25, 1.125, 0.562, 0.281, 0.14, and 0.07µg/ml and for alone SE-3 application (MOI=10) and for gentamicin-SE-3 mixture. Bacterial growth inhibition was not effective at low gentamicin conc. i.e. at 0.14 the inhibition was 39%, at 0.281 it was 58%, and at 1.125 it was 83% (Fig. 5). Bacterial growth inhibition showed no significant difference in gentamicin alone (89% for 0.562, 93% for 2.25, and 94% for 4.5). At 1.125 µg/ml of gentamicin, the combination of SE-3-gentamicin significantly inhibits more bacterial growth (93%) than phage alone (82%) and antibiotic alone (85%). Synergistic action was significant (p-value 0.02) at MIC 0.562µg/ml of gentamicin, which is higher than alone.

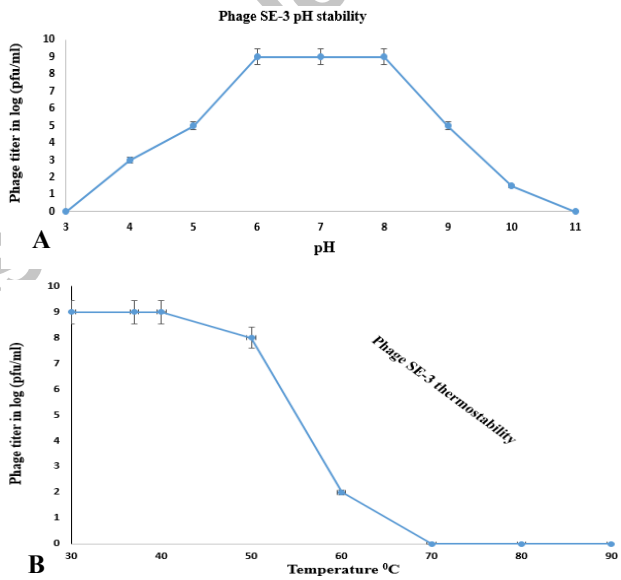

Fig. 4. A, pH stability of phage SE-3 at different pH. B, Temperature stability of Phage SE-3 at different temperature.

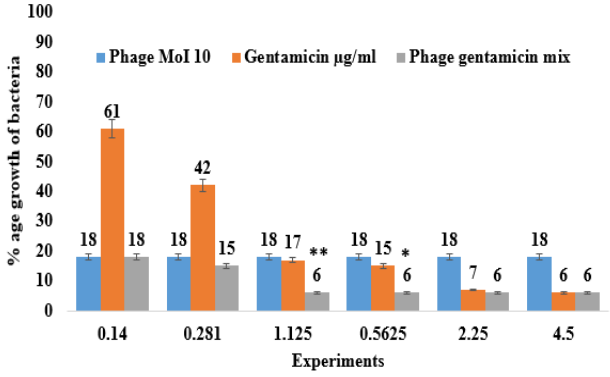

Fig. 5. Phage SE-3 and gentamicin synergism action.

DISCUSSION

Salmonella species are well-known among bacterial pathogens that cause foodborne infections and result in a large number of infections and deaths worldwide. One of the most common serotypes is *S. enterica* serovar *Enteritidis*, which is responsible for this problem in human beings. They are present in nature as primary reservoirs of humans and animals (Andino and Hanning, 2015). The second most commonly reported bacterial zoonosis is salmonellosis. The European Union (EU) has classified salmonella as a prominent etiological agent of foodborne illnesses (Eurosurveillance Editorial Team, 2012). In the current study, 25 fecal samples were collected from different poultry in Faisalabad district. Out of 25 samples, 10 samples (40%) were positive for *S. enteritidis* and 15 samples (60%) were negative for *S. enteritidis*.

Multiple drug-resistant *S. enteritidis* has become a persistent problem worldwide. At this time, antibiotic resistance is an important health, economic and social issue. A possible reason for the increased prevalence of *Salmonella* is its resistance to antimicrobial agents due to the indiscriminate use of antimicrobial agents in food animals, including poultry. Therefore, it is needed to be treated and controlled immediately. In the current study, *S. enteritidis* showed resistance to all antibiotics except Tazobactam and ciprofloxacin. Owing to the increase in antibiotic resistance, bacteriophages have gained significant importance as a promising substitute for antibiotics to reduce and overcome bacterial infections (Keen, 2012).

The successful isolation of bacteriophages yields observable evidence in the form of a distinct region characterized by varying levels of turbidity. The isolated phage ST-1 was studied for its morphological characteristics, along with physical and thermal stability. According to the findings of our investigation, the ST-1 phage has a shorter incubation time and a low multiplicity of infections, as well as higher stability at different temperatures and pH levels. The concentration of this phage ST-1 remained unchanged throughout variations in temperatures (20 °C to 40 °C) and pH values (6 to 8). These results were similar to a previous study (Zhao *et al*., 2022), in which phase against *Salmonella* showed high stability at pH (5–8) and temperature (20–40 °C). Some phase even exhibited lytic activity at a temperature of 60–70 °C. This high stability at adverse temperatures increases the lytic potential (Elbediwi *et al*., 2020).

The presence of plaque development indicates that bacteriophages have the potential to infect bacteria. Bacteriophages demonstrate a broad host specificity and can infect more than two-thirds of the strains tested, showing an extensive range of hosts (Kawasaki *et al*., 2016). Bacteriophages that target specific pathogenic bacteria linked with the disease show the possibility for natural control strategies in combating foodborne infections. Bacteriophages may exhibit varying degrees of selectivity towards different bacterial strains, ranging from highly targeted to more generalized, depending on the number of bacteriophages available (Allen *et al*., 2014). Bacteriophages with a wide host range show potential for use in combating bacterial illnesses and as an intervention in food treatment (Bielke *et al*., 2007). The plaque produced in this study had a somewhat opaque hue. Bacteriophages' effectiveness in targeting receptor molecules on the host cell's surface influenced the formation of plaques. The varying amounts of turbidity seen in plaques might indicate the specific life cycle shown by the bacteriophage being studied (Narulita *et al*., 2018). The study discusses the possibility of using bacteriophage and antibiotics together to reduce bacterial resistance. Scientists have enhanced monophage treatment by combining phages with antibiotics to reduce the potential development of resistance to certain antibacterial drugs. Phage-antibiotic therapy has effectively reduced the emergence of phage and antibiotic-resistant strains. An example often cited is when antibiotic sensitivity is restored because phages degrade cell surface receptors, which let the effluence of antibiotics (Chan *et al*., 2016). Several *in vitro* studies confirm that antibiotics may regain effectiveness when used in combination with bacteriophages, leading to a reduction in the amount of bacteria present (Petsong *et al*., 2018). Phage antibiotic synergism (PAS) in lytic bacteriophages is caused by a direct relationship with bacterial filamentation, leading to an increase in bacterial size and cellular surface area (Davis *et al*., 2021). Phage antibiotic synergy is linked to both quick and delayed lysis. Shortening the latent period or speeding up lysis increases the frequency of completing the infective cycle repeatedly. Conversely, delaying lysis allows more time for phage assembly, leading to a larger phage burst size (Abedon and Yin, 2009). Bacteriophages may reduce the minimum inhibitory concentrations of resistant bacterial strains under certain circumstances (Liu *et al*., 2020).

A study by Comeau *et al*. (2007) displayed synergy among several antibiotics such as aztreonam and cefixime and the phage MFP when subjected to a uropathogenic *E. coli* strain. The combined antibiotic and phage therapy displayed an increased incidence of phage lysis on the host bacterium. Adding antibiotics sequentially after phage treatment can also control the development of phage mutants. This is according to Valerio *et al*. (2017) who further showed that upon the addition of sub-lethal concentrations of ciprofloxacin when combined with the

phage, the occurrence of phage mutants was lesser than that observed when antibiotics were not added.

In the current study, a large amount of killing was seen with MIC gentamicin plus phage SE-3, this describes that phage replication was at a higher ratio in MIC of gentamicin. A higher MIC of antibiotic, phage could have an interference effect of the drug on its replication. Phage and antibiotics combined treatment inhibited the reach of control levels of bacterial counts. A large amount of killing was detected in phage and gentamicin combination.

CONCLUSION

In this study we isolated and identified antimicrobial-resistant *Salmonella enteritidis* from poultry, confirming its potential to spread zoonotic infections to humans. The isolation and characterization of bacteriophages, specifically the Siphoviridae family member SE-3, demonstrated their stability under varying pH and temperature conditions. The research further highlighted the synergistic effects of combining gentamicin with phage therapy, leading to a significantly higher bacterial killing rate than antibiotics or phages alone. These findings underscore the potential of phage-antibiotic synergism as a promising strategy to combat antibiotic-resistant bacterial infections, particularly in the context of zoonotic pathogens such as *S. enteritidis*. This approach could serve as an effective alternative or adjunct to conventional antimicrobial treatments, reducing the burden of resistant infections in both poultry and human populations.

DECLARATIONS

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Ethical statement

The study was conducted according to the ethical approval provided by the International Biosafety Committee (IBC) of the University of Agriculture, Faisalabad and were approved by the Intitutional review board of University of Agriculture, Faisalabad, experimental animals/laboratory animals.

Statement of conflict of interest

The authors have declared no conflict of interest.

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