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Isolation of Lytic Bacteriophage Against *Salmonella pullorum* from Layer Poultry Birds

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Abstract. The poultry market is troubled by Salmonellosis infections which has a large negative impact due to morbidity and decreased productivity. *Salmonella pullorum* causes a disease named Salmonellosis which affects the poultry industries and has long been a significant obstacle to the development of nations. Due to the occurrence of resistance to most of the antibiotics in the poultry, treatment of infection now becomes difficult and challenging. So, alternative therapy is needed to reduce the burden of bacterial load and to treat the infection. The main objectives of this study were to isolate *Salmonella pullorum* from the layer poultry birds with to check antibiotic resistance of *Salmonella pullorum* and to isolate lytic phages against *Salmonella pullorum.* Poultry fecal samples were collected from poultry farms in different areas. For isolation and purification of *Salmonella pullorum,* Salmonella Shigella agar (SS-agar) was used. Black centered colonies were observed on SS agar and non-lactose fermenter on MacConkey's agar. For the confirmation of bacterial isolates, gram staining yielded pink- red rods as observed microscopically. For citrate utilization isolates also +ve test, catalase production and methyl red reaction and negative for VP and indole reaction. The disk diffusion method on Mueller Hinton was performed to check the susceptibility pattern of *Salmonella pullorum.* Bacterium showed resistance to amoxicillin followed by tetracycline and ceftazidime, while sensitive to chloramphenicol and kanamycin. Sewage water was collected for the isolation of phages from different sewage lines of poultry farms. Bacteriophages against *Salmonella pullorum* were isolated through agar overlay method. Clear plaques were observed on petri plates.

Keywords: fecal, bacteriophage, sntibiotic resistance, Salmonellosis, sewage water, poultry

Introduction

The poultry industries in Pakistan are playing major role across the county to decrease the poverty among peoples by offering many opportunities to a huge number of workers. However, there is a significant in consumptions of poultry meat and egg as compared to other countries in the world. The major issue faced by the poultry industries in Pakistan is the infectious disease caused by *Salmonella* species (Shoaib *et al*., 2017). The poultry market is troubled by Salmonellosis infections, which have negative impacts due to morbidity and decreased productivity (Nair and Johny, 2019). Poultry meat and its products have served as major sources of *Salmonella* sp. that cause human and animal diseases as well as economic loss in poultry industries (Barbour *et al*., 2015). The two major infectious bacterial diseases that cause huge economic losses to poultry industry are fowl typhoid and pullorum diseases caused by *Salmonella gallinarum* and *Salmonella pullorum*, respectively (Rahman *et al*., 2004). These pathogens are gram negative rod shape facultative anaerobes having peritrichous flagella. Different serovars and biovars of *Salmonella* have different genome sizes ranging from 4460 to 4857 Kb. The Enterobacteriaceae family includes *Salmonella* strains that are of medicinal concern to both animals and humans. The *Salmonella* genus is a diverse group of bacteria comprising two main species and six subspecies containing more than 2579 serovars. Two of the currently known species are *Salmonella enterica* and *Salmonella pullorum* (Berhanu and Fulasa, 2020). Fowl typhoid and pullorum diseases are becoming an emerging threat due to worldwide spread because of expansion in poultry farming. The clinical symptoms of both Fowl typhoid and pullorum infectious diseases are similar and have abilities to infect all types of broilers, layers, young and adult chickens. *S. pullorum* is quite prevalent all over the

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commercial poultry farming countries including Pakistan. Due to the emergence of multidrug resistance, *Salmonella* is becoming a worldwide problem as most antibiotics to combat it have become ineffective (Shoaib *et al*., 2017). The antibiotic resistance developing in *Salmonella* can be difficult to control due to the utilization of antibiotics as a growth promoter. (Soomro *et al*., 2011). Due to antibiotic resistance mechanism the alternative method for control and treatment of *Salmonella* infections is phage therapy which has considered one of the most potential methods due to high specificity and without disturbing the normal flora of the host (Rizzo *et al*., 2020). Bacteriophages were determined by Twort (1915) as un-recognized molecules that inhibit bacterial growth but in 1917 D' Herelle become the first to isolate and characterize phages and identify the primary phage remedy for chicken typhoid such as *Salmonella gallinarum* in chickens. Beneficial effects of the usage of bacteriophages in combating bacterial infections have contributed to the development of research on the ability of using viruses that inhibit micro-organisms to treat diseases in both human and animals (Wernicki *et al*., 2017). Bacteriophage application is the main aim of phage therapy to reduce bacterial loads in human bacterial infections. The phage therapy used to treat infections depends on the type of infection, the specificity of these virus particles that provide a convenient way to fight bacterial diseases, and their specificity that is helpful in attacking the bacterial infected cells instead of normal body cells (Amenu, 2014). Due to these reasons, the scientific community has recently decided to pay attention to bacteriophages which also serve as novel tools to treat pathogenic drug-resistant bacteria.

Materials and Method

Samples collection. A total of 75 poultry fecal samples were collected aseptically from poultry farms and poultry shops in Faisalabad, Pakistan.

Isolation and identification of *Salmonella pullorum*. Various culture media such as xylose lysine deoxycholate agar, Salmonella Shigella agar and MacCkony Agar were used for isolation of *Salmonella pullorum* from poultry fecal samples. The samples were streaked on culture media and incubated at 37 °C for 24 h. For morphological characteristic gram staining was performed.

Biochemical test. Different biochemical tests including the "Voges Proskauer and Citrate Utilization Test", catalase, indole and methyl red were performed for identification of *Salmonella pullorum* (Manasa *et al*., 2017).

Antimicrobial susceptibility test. The Kirby-Bauer disk diffusion method was used to check antibiotic resistance of *Salmonella pullorum* against certain antibiotics. The antibiotics included amoxicillin, cefazolin, ceftazidime, kanamycin, chloramphenicol, ciprofloxacin and tetracycline and the results were assessed according to clinical laboratory standard institute guidelines (CLSI).

Isolation of bacteriophages. Bacteriophages against *Salmonella pullorum* were isolated by "Double Agar Overlay Method" as described by (Clokie and Kropinski, 2009).

Collection of sewage water samples. A total of 20 sewage water samples were collected in 40 mL Falcon tubes from different sewage systems present in the University of Agriculture Faisalabad, Pakistan. The temperature and moisture levels were also noted at the location where the sample was collected. The samples were brought to the microbiology lab for phage isolation.

Bacteriophage enrichment. The enrichment method of (Clokie and Kropinski, 2009) was used for the isolation of phages that is specific to *Salmonella pullorum.* The sewage samples were collected and centrifuged for 10 min at 10,000 x g and the supernatant was filtered with filter paper of 0.45 µm pore size. 0.7 mL centrifuged sewage water samples were mixed with 0.2 mL overnight culture of *Salmonella pullorum* in 1 mL of nutrient broth and incubated at 37 °C for 24 h. Bacterial cells were removed by centrifugation and the supernatant was filtered and processed to detect the presence of phages using agar overly method (Yildirim *et al*., 2018).

Spot test and plaque assay. Spot test was performed for the detection of phages in supernatant as described by (Chang *et al*., 2005). The titer of phage was determined by plaque assay by employing double agar overly method. The phage suspension was serially diluted. 1.5 mL of filtrate phage with 1.5 mL of *Salmonella pullorum* was mixed with 3 mL molten soft agar and poured on solidified nutrient agar plates. The plates were incubated at 37 °C for 24 h. The petri plates were examined for *Salmonella* plaque formation (Yildirim *et al*., 2018).

Table 1. Biochemical characterization of *Salmonella pullorum*

Bacteria	Biochemical tests							
	Oxidase	Catalase	MR	VP	Indole	Citrate	Coagulase	TSI
Salmonella pullorum	-	+	+	-	-	+	-	-

Results and Discussion

A total of 75 poultry samples were processed, 35 were positive for *Salmonella pullorum* which showed dark pink to red colour colonies with dark black center on SS agar, colourless colonies on MacConkey agar, while red colour colonies with black center on XLD agar after incubation at 37 °C for 24 h (Fig. 1).

Biochemical characterization. Various biochemical tests were performed. The isolate was citrate, catalase and methyl red positive, while VP and Indole negative as showed in Table 1.

Antimicrobial susceptibility test. Among the isolated bacteria, 86% of them were resistant to amoxicillin, 75% resistant to tetracycline 65% resistant to ceftazidime and 60% resistant to cefazolin, while 55% were susceptible to ciprofloxacin, 45% susceptible to kanamycin and 60% susceptible to chloramphenicol (Fig. 2 and Table 2).

Bacteriophage isolation. A total of 3 lytic phages against *Salmonella pullorum* have been isolated by using double-agar- overlay method. Clear plaques were observed in the petri plates that showed that the isolated phages were specific to *Salmonella pullorum* (Fig. 3). The bacteriophages against *Salmonella pullorum* showed clear and transparent zones, the size of the zones was 1-2 mm diameter.

Table 2. Antibiotic disc used against *Salmonella pullorum*

Antibiotic disc	Resistance (%)	Sensitive (%)
Amoxicillin	86%	14%
Cefazolin	60%	40%
Ceftazidime	65%	35%
Kanamycin	55%	45%
Chloramphenicol	40%	60%
Ciprofloxacin	45%	55%
Tetracycline	75%	25%

Salmonella pullorum causes extreme pullorum disease in domestic birds, which is increasingly becoming part of the economic implications for poultry. Pullorum disease is caused by *Salmonella pullorum*, a septic bacterial disease predominantly found in avian and it is the most significant disease of poultry, followed by high fatalities. In the present study the isolation of lytiv bacteriophage from sewage water and its antibacterial effect against *Salmonella pullorum* was evaluated. For

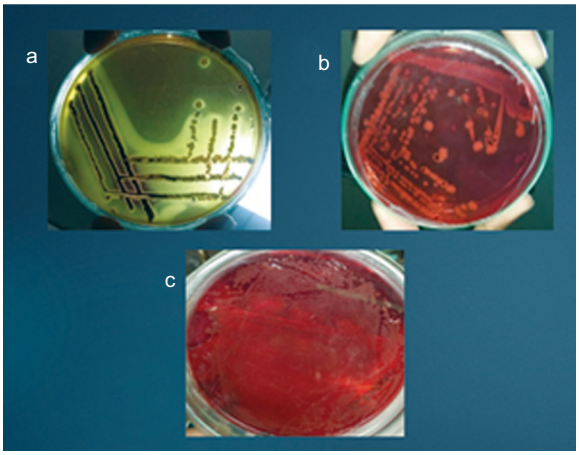

Fig. 1. Culture characteristics of *Salmonella pullorum* **(a)** growth on SS agar, **(b)** growth on XLD agar and **(c)** growth on MacConkey agar.

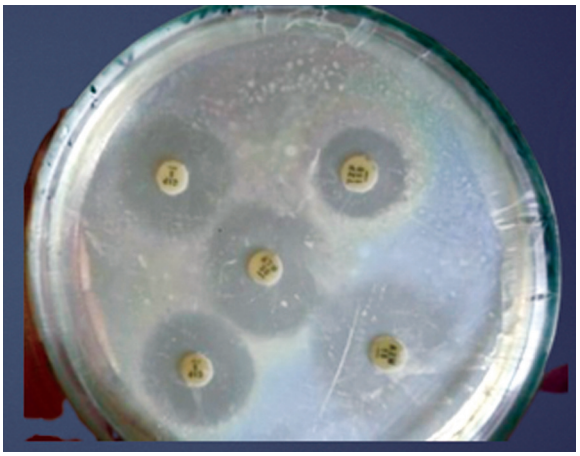

Fig. 2. Antibiotics sensitivity test for *Salmonella pullorum*.

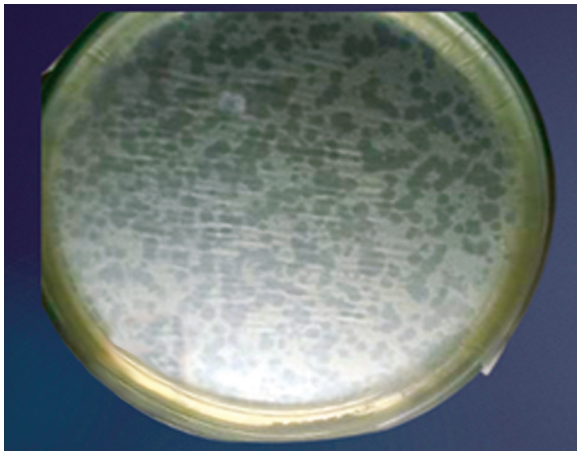

Fig. 3. Phage plaques show lytic activity.

isolation and purification of *Salmonella pullorum*, poultry fecal samples were collected from various poultry farms in Faisalabad. SS-agar was used to isolate the *Salmonella pullorum*. Black centered colonies were observed on SS agar and non-lactose fermenter on MacConkey's agar. The isolated bacteria were confirmed by gram stanning that showed pink- red rods observed under the microscope. *Salmonella pullorum* strains were identified based on biochemical tests. Out of 75 feacal samples 51 were positive for *Salmonella* and out of 51 positive samples 27 were positive for *Salmonella pullorum*. Differentiation of *Salmonella* was based on different biochemical tests including citrate, catalase and methyl red, VP and Indole tests. *Salmonella pullorum* is MR, catalase and citrate positive and negative for VP and Indole. The prevalence of *Salmonella* in intensively managed healthy chickens in Hadassah Ethiopia during 2008-2009 was 16.1% in sick and dead chickens (Aragaw *et al*., 2010). Another study was conducted to determine the prevalence and characteristics of *Salmonella* associated with layer eggs in Korea in 2013. They concluded that prevalence of *Salmonella* in farms was 59.3%, while 50.7% in flocks and 17.2% of eggs shells were contaminated with *Salmonella*. It's suggested that when the size of flock increases the incidence of *Salmonella* also increases (Min chin *et al*., 2015). The fowl typhoid group mainly includes two members, *Salmonella gallinarum* and *Salmonella pullorum*. The 27,000 *Salmonella* serotypes, only these two serotypes can cause high mortality rate in lying birds. They can be transmitted vertically from parents to offspring or horizontally from the environment to flock. Once the flock is infected the birds remain the carrier forever. When compared to broiler flocks, the prevalence of typhoid infection in layer chickens (eggs) is significantly higher globally. The primary cause is a dearth of effective bio-security. Most layer farms have a range of ages, which prevents all-in, all-out administration and jeopardises biosecurity (Shivaprasad and Barrow, 2008). Disk diffusion method on Mueller Hinton was performed to check the susceptibility pattern of *Salmonella pullorum* they showed resistance toward amoxicillin 86% followed by tetracycline and ceftazidime, while sensitive to chloramphenicol and ciprofloxacin. This tendency in antibiotic resistance demonstrates that ciprofloxacin and chloramphenicol can be used as drug of choice against *Salmonella pullorum*, while other drugs are resistant. To find out *Salmonella*'s antimicrobial susceptibility pattern, similar research was carried out in 2018. The multi-drug resistance (MDR) in *Salmonella pullorum* increases from (23.1%) in 2014 to (60.7%) in 2018. *Salmonella pullorum* was found to be resistant to nalidixic acid (78.5%) followed by gentamicin (52.3%) ciprofloxacin (26.9%) and penicillin (14.6%) (Seo *et al*., 2019). In the current research the prevalence and incidence of *Salmonella pullorum* in poultry was 40-50%. The prevalence of Salmonella in healthy poultry and its antimicrobial sensitivity was investigated by Parvej. They found 54% of isolated *Salmonella enterica* serovars were sensitive to ciprofloxacin, while 81.81% of these were resistant to ciprofloxacin, doxycycline, amoxicillin, kanamycin, tetracycline and gentamycin. *Salmonella enterica* serovars that are multidrug resistant were found in commercial poultry in Bangladesh and pulsed-field gel electrophoresis of the XbaI-digested genome showed identical banding patterns, suggesting that they are extremely clonal (Parvej *et al*., 2016).

Many developing countries use antibiotic prophylaxis in the poultry industry as a substitute for inadequate management practices but if withdrawal period requirements are not followed, this practice leads to an increase in antibiotic resistance and drug residues in the food chain. Resistance to available antibiotics has increased, which has revived scientists' interest in treating infection with lytic bacteriophages and their derivatives. Bacteriophages are an effective bacterial threat because they are both particular and adaptable. There are various studies on the use of bacteriophage to control the infections caused by bacteria which are resistant to commonly available antibiotics. For this purpose, sewage water was collected in this study for

the isolation of phages from various sewage lines of poultry farms. Bacteriophages against *Salmonella pullorum* were isolated through agar overlay method. Clear plaques were observed for *Salmonella pullorum.* The bacteriophages against *Salmonella pullorum* showed clear and transparent zones, the size of the zones was 1-2 mm in diameter. A great advantage to using lytic phage against bacteria is its high specificity which is guarantee of its accurate targeting. The findings of the present study are supported by literature where the bacteriophages against Salmonella were isolated. Bacteriophage f18SE infects a variety of hosts, including *Salmonella pullorum*, *S. enteritidis* PTs and *Salmonella typhimurium* serovars. This phage was isolated from the poultry wastewater channels in Olmue, of Chile. The prophylactic effect of f18SE has been effectively tested in chicks and *Caenorhabditis elegans*. It has a very high level of stability on inoculated eggs as well as under adverse circumstances (pH and T). Additionally, the oligo-polysaccharide of lipo-polysaccharide serves as its membrane attachment component. It is possible to foresee the use of phage f18SE in typing, vector development and biocontrol methods (Segovia *et al*., 2015).

Conclusion

The resistance of *Salmonella pullorum* to the most used antibiotic is increasing day by day due to the use of antibiotic as growth promoter in poultry industries. It's difficult to treat the infection caused by *Salmonella* due to its antibiotic resistance and affect the normal flora which leads to secondary infection. The purpose of this study was to isolate lytic bacteriophages that can be used to control or prevent Salmonella growth. So, it can further be used in the treatment of *Salmonella* in the poultry industries. Salmonella is highly susceptible to antibiotic resistance, and this issue is motivating researchers to develop antibiotic substitutes. Since lytic bacteriophages are extremely specific in their action and host specificity, they are thought of as the most reliable substitutes to antibiotics to inhibit growth of bacteria. Furthermore, phages that effectively multiply and lyse the host bacteria are preferred because they can quickly and effectively kill the target bacteria. The results of this study showed that isolated lytic phages inhibited the growth *Salmonella pullorum*. More extensive research is recommended to study and characterize these bacteriophages to control the bacteria in the poultry industry.

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Conflict of Interest. The authors declare that they have no conflict of interest.

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