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RESEARCH ARTICLE

Efficacy of Lytic Bacteriophages against Multidrug Resistant *Salmonella enteritidis* from Milk and Meat

Abu Baker Siddique[1*], Zeeshan Nawaz[1], Muhammad Asif Zahoor[1], Azhar Rafique[2], Bilal Aslam[1], Nida Nazia[1] and Riaz Hussain[3]

[1]Department of Microbiology, Government College University, Faisalabad, 38000- Pakistan
[2]Department of Zoology, Government College University, Faisalabad, 38000- Pakistan
[3]University College of Veterinary and Animal Sciences, The Islamia University of Bahawalpur, 63100- Pakistan
*Corresponding author: absiddique@gcuf.edu.pk

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Salmonellosis is major food borne illness caused by *Salmonella enteritidis*. The emerging multidrug resistance has forced the scientists to investigate alternative control strategies including phage therapy to combat salmonellosis. Therefore, the current study was carried out to isolate multidrug resistance *S. enteritidis* from raw milk (n=40) and meat (n=30) samples. A total of 10 *S. enteritidis* isolates were recovered from raw milk whereas 8 isolates were from meat samples. These isolates were confirmed by Polymerase Chain Reaction using *spvC* gene specific primers yielding 571bp product. The anti-biogram studies were carried out on all isolates using different antimicrobials. The results showed 11 (61%) isolates were susceptible to most of the antibiotics while 7 (39%) isolates were found resistant and hence declared as multidrug resistant *S. enteritidis*. In the second step, sewage water was processed for the isolation of bacteriophages and was used for bacteriophages enrichment against a susceptible strain of *S. enteritidis*. Both clear and turbid plaques were isolated and enumerated. The results of bacteriophages isolation showed a total of 7 different bacteriophages isolated out of 10 processed samples. Lytic potential was measured by co-cultivation of *S. enteritidis* with isolated bacteriophages. The results showed the significant lytic potential of isolated bacteriophages against multidrug resistant *S. enteritidis* isolates from raw milk and meat samples. Altogether, the current study highlighted the significance of *S. enteritidis* as an important multi drug resistant food borne pathogen, further the bacteriophages therapy could be an alternative control remedy.

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INTRODUCTION

Salmonella enteritidis is the major enteric pathogen of human and animals. The bacterium is responsible for several foods borne diseases ranging from mild illness, diarrhea and other gastric disturbances which may lead to deaths among humans. *S. enteritidis* is the main cause of bacterial gastroenteritis among industrialized and developing countries (Hendriksen *et al*., 2004) and is considered pathogenic among human and animals (Hassan *et al*., 2017; Wotzka *et al*., 2017). There is different clinical manifestation caused by Salmonella spp. i.e. enteric fever, bacteremia, gastroenteritis and asymptomatic carrier state (Feasey *et al*., 2012; Ansari *et al*., 2017). Different foods and sources are held responsible for transmission of *S. enteritidis* among humans including fruits and vegetables (Woolston *et al*., 2013), carrier animals and humans (Wegener *et al*., 2003) and animal origin food products including poultry meat and milk etc (Marin *et al*., 2015; Ansari *et al*., 2017). Non typhoidal Salmonella shows broad host range and often involves food animals and in immunocompromised patients (Gordon, 2008). In human, salmonellosis caused by domestic food products mainly table eggs and chicken (Hald *et al*., 2007; Ozbey *et al*., 2017). The incidence of human salmonellosis is greatly increased over the year in some countries. Non typhoid serotypes of Salmonella affects about 2 to 3 million persons and mortality rate is

about 500 to 2000 deaths each year. The two main serotypes of Salmonella isolated in 2000 from humans were *S. typhimurium* and *S. enteritidis* (Chiu *et al*., 2004).

Fluoroquinolones are considered as effective antibiotics for the treatment of *S. enteritidis.* However, an increased incidence of multidrug resistance *Salmonella* strains has been observed due to inappropriate utilization of antibiotics (Eguale *et al*., 2015). At present, the antibacterial resistance among bacterial pathogens is a global concern and development of next generations of antibacterial agents is also more focused. However, the process of developing next generation antibacterial agents is time consuming and expensive. Therefore, alternative and new strategies are suggested to control the infection and drug resistance (Preisner *et al*., 2010; Wibow *et al*., 2017).

Novel strategies like phage therapy are developed to treat bacterial infections other than antibiotics and chemicals (Joerger, 2003). A wide range of foodborne pathogens including *Salmonella spp.* could be controlled by phage therapy. Bacteriophages are considered as most abundant group of viruses and are present in environment mainly in water, soil, sewage and different foodstuffs (Sajjad *et al*., 2004; Parasion *et al*., 2014). The use of lytic bacteriophages to control the drug-resistant bacteria without harming the eukaryotic cell is known as bacteriophage therapy (Sulakvelidze *et al*., 2001). Bacteriophages mainly fall in order *Caudovirales* with tail of different length and icosahedral head. Most common bacteriophage families are *Myoviridae, Podoviridae* and *Siphoviridae* (Sepúlveda-Robles *et al*., 2012). The antibacterial activity of phages is due to the phage attachment on bacterial cells followed by changes in bacterial metabolism which leads to bacterial lysis (Rasool *et al*., 2016). Most of the bacteriophages are bactericidal in nature and the bacteria are incapable of recovering their viability following phage therapy along with minimum risk of secondary bacterial infection due to high specificity of bacteriophages against bacteria (Skurnik *et al*., 2007; Zhang *et al*., 2017). By considering the vitality of *S. enteritidis* and importance of bacteriophages isolation of bacteriophages specific to *S. enteritidis* was carried out from sewage water. Further, the lytic activity of isolated phages was also evaluated.

MATERIALS AND METHODS

Sample collection: Raw milk samples were aseptically collected in sterile falcon tubes (n=40) while the chicken meat samples were collected sterile plastic bags (n=30) and transferred to Post Graduate research laboratory, Department of Microbiology, Government College University, Faisalabad, Pakistan using standard protocol as described by FDA bacteriological analytical manual (Lesmana *et al*., 2016). The samples were stored at 4^{o}C in refrigerator till further processing.

Isolation and identification of *Salmonella enteritidis*: Samples were cultured on selective medium MacConkey`s agar (Difco, USA), brilliant green agar (Difco, USA) and Salmonella Shigella agar (Difco, USA) using streak plate method followed by incubation aerobically at 37°C for 24-48 hours. Identification of *Salmonella enteritidis* was carried out on the basis of colony characteristics, morphology and various biochemical tests including motility, citrate utilization test, methyl red test, triple sugar iron and indole test as stated by Akthar *et al.* (2010).

Molecular identification of the isolates: DNA from isolates was extracted using standard phenol chloroform technique (Hussian *et al.*, 2017). To confirm the isolates, amplification of *spvC* gene using forward primer and reverse primers (Table 4) was carried out yielding 571bp PCR product (Amini *et al*., 2010). DreamTaq Green PCR Master Mix (Thermo Scientific, USA) Amplification was done at 94°C for 4 min followed by 25 cycles each 1 min at 94°C, then at 59°C for 45sec and 72°C for min. Final extension was carried out 72°C for 5 min. The amplified PCR products were visualized using 1% agarose gel electrophoresis and visualized under Gel Documentation System (Dolphin Doc, USA).

Anti-biotic susceptibility testing: All the isolates were tested for antibiogram studies using Kirby–Bauer disk diffusion assay and Broth microdilution method according to Clinical Laboratory Standard Institute (CLSI) guidelines. The isolates turbidity was adjusted to 0.5 McFarland turbidity standard. Different antibiotic disks such as amikacin (30 µg), ceftriaxone (30 µg), piperacillin and ciprofloxacin (100/10 µg) were tested. Zones of inhibition were noted after the incubation for 24 hours at 37°C and compared with CLSI scale (Beige *et al*., 2015; Rasool *et al*., 2016). While minimum inhibitory concentration was determined by Broth microdilution method according to the guidelines of Clinical Laboratory Standard Institute (CSLI) (Humphries *et al*., 2012).

Molecular detection of Fluoroquinolone genes: Polymerase chain reaction was performed for the detection of chromosomal quinolone resistance determining region (QRDR) in *Salmonella enteritidis* while for the detection of plasmid-mediated quinolone resistances (PMQR) all the isolates were subjected to the multiplex PCR. Various Fluoroquinolone resistance genes were investigated which include *gyrA, gyrB, qnrA, qnrB* and *qnrS*. Amplification was done at 94°C for 5 min followed by 35 cycles each of denaturation at 94°C for 1 min, annealing at 56°C for 50sec and extension at 72°C for 1 min, Final extension was done at 72°C for 5 min. Primer sequences of all the genes which were investigated are given in Table 4. Following the agarose gel electrophoresis gel PCR products were visualized under gel documentation system (Dolphin Doc, USA).

Isolation of bacteriophages: For isolation of phage samples were collected from sewage water from different areas of Faisalabad. Sewerage water collected from 7 different locations while hospital waste water was collected from 3 locations (Olszak *et al*., 2015). Multidrug resistant *Salmonella enteritidis* were nominated to isolate bacteriophages. For the isolation of bacteriophages Agar overlay method was used. Enrichment of phages was separately carried out against a susceptible strain of *S. enteritidis* by selecting single distict plaque picked from plate (Henry *et al*., 2013).

Assessment of phages efficacy by spot assay: Spot assay was used to check the lytic activity of isolated phages and the best sewage filtrate and susceptible host were further used to check the plaque morphology using agar overlay method (Rasool *et al*., 2016). Well isolated plaques were selected for purification and phage count (Sangha *et al*., 2014).

Effect of salt on plaque morphology: Different divalent salts are considered to enhance the attachment of bacteriophages to the bacterial cell as well better intracellular progression. $CaCl_2$ and $MgSO_4$ were tested for their effect on plaque morphology (Rasool *et al*., 2016).

Statistical analysis: Bacterial and phage counts were determined by duplicate plating and all food experiments was independently performed at least twice. Results were presented as mean values, and standard deviation of the mean is indicated by error bars.

RESULTS

Isolation antimicrobial susceptibility of *Salmonella enteritidis:* Out of 40 raw milk samples 10 were found positive and 30 were negative while from 30 meat samples 8 were positive for the presence of *Salmonella enteritidis.* The isolation was carried out on Salmonella Shigella agar (Oxoid, UK) and all isolates were confirmed through species specific primers yielding 571bp PCR product (Fig. 1). Eighteen isolates were subjected to commonly used antibiotics for the antibiogram studies. Zones of inhibition around the discs were measured in mm while minimum inhibitory concentration was measured in µg/ml. Out of 18 strains, 11 (61%) were susceptible to most of the drugs while 7 (39%) strains were resistant against the drugs tested. Fluoroquinolones resistance genes (QRDR and PMQR) were also detected in the isolates (Table 4 and Fig. 2). These MDR strains were numbered as MDR1, MDR2 and MDR3, MDR4, MDR5, MDR6 and MDR7 (Table 1 and Table 2).

Table 1: Resistance pattern of *Salmonella enteritidis* against various drugs

Strain #	CHL (mm)	AMP (mm)	CRO (mm)	CIP (mm)	AK (mm)
MDR1	R	R	R	R	19
MDR2	R	R	R	18	19
MDR3	R	R	R	R	IR
MDR4	R	R	R	R	18
MDR5	R	R	R	R	18
MDR6	R	R	R	R	19
MDR7	R	R	R	R	R

CHL, Chloramphenicol; AMP, ampicillin; CRO, ceftriazone; CIP, ciprofloxacin; AK, amikacin; R, Resistant; IR, Intermediate resistant.

Table 2: Minimum Inhibitory Concentration of *Salmonella enteritidis* against various drugs

Strain #	CHL (µg/ml)	AMP (µg/ml)	CRO (µg/ml)	CIP (µg/ml)	AK (µg/ml)
MDR1	1.0	2	0.25	1	0.125
MDR2	0.5	4	0.25	0.125	0.125
MDR3	1.0	2	0.50	0.50	0.500
MDR4	2.0	1	0.25	0.50	0.125
MDR5	2.0	2	0.25	0.50	0.125
MDR6	1.0	2	0.50	0.50	0.125
MDR7	1.0	2	0.50	0.25	0.500

CHL, Chloramphenicol; AMP, ampicillin; CRO, ceftriazone; CIP, ciprofloxacin; AK, amikacin.

Isolation and efficacy of lytic bacteriophages: For the isolation of lytic bacteriophages against MDR *S. enteritidis*, 10 sewage water samples were collected, out of which 7 (70%) were found positive for lytic bacteriophages as shown in Table 3. All the samples collected from hospitals were found positive for bacteriophages. In the recent study, to isolate and purify agar overlay method was used.

Spot assay: All the sewage samples were checked for the presence of *S. enteritidis* specific bacteriophages after filtration and enrichment over all MDR bacterial strains. Spot assay was used to check the lytic potency and host range of prepared bacteriophage lysate. Eff1, Eff5 and Eff10 were found most effective against MDR isolates while three found negative for bacteriophage isolation. Phages were confirmed by the presence of clear zones on the bacterial bed (Fig. 3a).

Plaques morphology and Cell Imaging: The effective filtrates (Eff1, Eff5 and Eff10) showed clear and turbid zones with sharp boundaries against *S. enteritidis* which were confirmed by using ZOETM cell imager (Bio-Rad, USA) (Fig. 3b).

Effect of salts: $CaCl_2$ had significant effect on plaque morphology as well as Plaque Forming Units (PFU) while $MgSO_4$ had non-significant effect. Similarly, insignificant effect of mitomycin was recorded.

DISCUSSION

The irrational and excessive use of antibiotics has resulted in the development of antibiotic resistance against the *S. enteritidis.* This development of resistance has increased the chances of non-typhoidal salmonellosis (gastroenteritis) from animal origin. Bacteriophages are considered as potential candidate for the treatment of MDR *S. enteritidis* (Khalid *et al*., 2017). The current study was designed to check the lytic potential of bacteriophages against the resistance isolates.

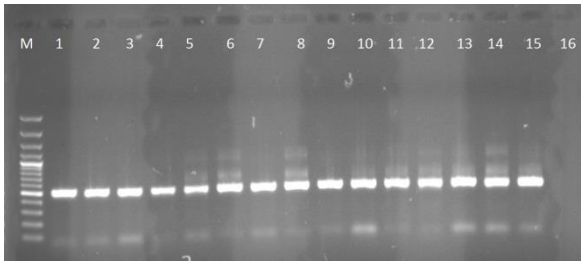

Fig. 1: PCR based detection of *Salmonella enteritidis* using *spv*C (571bp) genes. Lane M: 100bp ladder, Lane 1-15 Positive samples for *Salmonella enteritidis.* Lane 16: negative control.

Table 3: Results of spot assay showing effective filtrate and most susceptible strain

Sewage samples	MDR1	MDR2	MDR3	MDR4	MDR5	MDR6	MDR7
Eff1	+	-	+	+	+	+	+
Eff2	+	-	-	-	-	+	-
Eff3	+	+	-	-	+	+	+
Eff4	+	-	-	-	-	+	-
Eff5	+	+	+	+	+	+	+
Eff7	+	-	+	-	+	+	+
Eff10	+	+	+	+	+	+	+

Eff: Effluent. MDR: Multidrug resistant strain of *Salmonella enteritidis.*

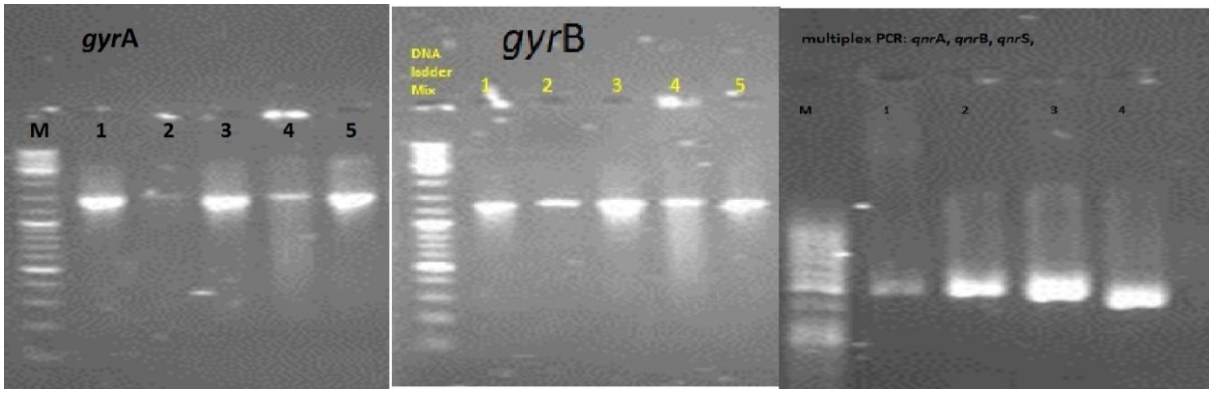

Fig. 2: PCR based detection of fluoroquinolone resistance genes including QRDR (*gyr*A, *gyr*B) and PMQR (*qnr*A, *qnr*B and *qnr*S) showing their respective amplicons.

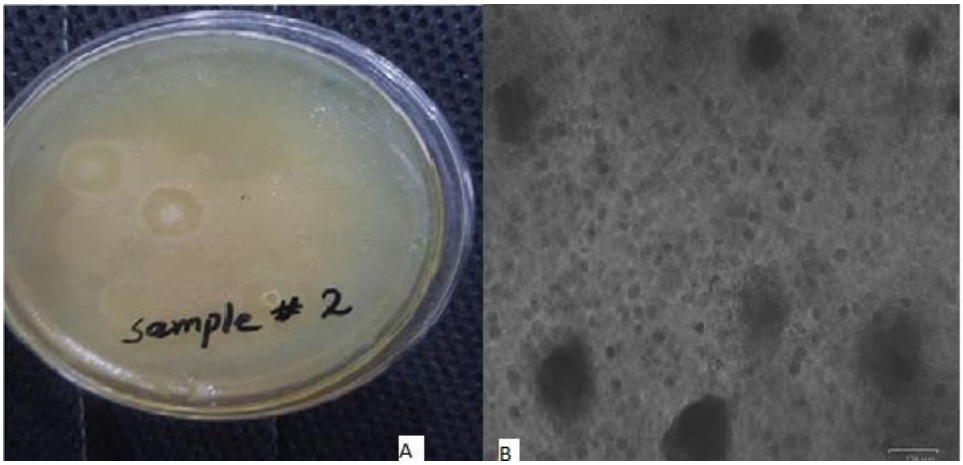

Fig. 3: (A) Plate showing different sized plaques obtained by agar overlay method, (B) Plaques of Different Types and Sizes Viewed through a Cell Imager (Bio Rad).

Table 4: Primer sequence used for the current study

Sr. No.	Gene	Primer sequence	Product size (bp)	Reference
1	*spvC* gene	GTCCTTGCTCGTTTACGACCTGAAT TCTCTTCTGCATTTCGTCA	571	(Amini *et al*., 2010)
2	*gyr*A	GCGATGTCGGTCATTGTTGG CCGAACTGGTCACGGATCAG	1200	(Hou *et al*., 2015)
3	*gyr*B	CTCCGTCTCCGTACAGGATGAC TGTGATAGCGCAGTTTTATCC	1200	(Hou *et al*., 2015)
4	*qnr*A	ATTTCTCACGCCAGGATTTG GATCGGCAAAGGTTAGGTCA	516	(Mak *et al*., 2009)
5	*qnr*B	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC	476	(Mak *et al*., 2009)
6	*qnr*S	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC	416	(Mak *et al*., 2009)

Out of 70 samples from animal products 18 were found positive for the presence of *Salmonella enteritidis*. The identification of isolates were was done by Gram`s staining, motility, citrate utilization test, methyl red test, triple sugar iron and indole test as stated by Akthar *et al*. (2010). Opaque and translucent colony with black center was observed on Salmonella Shigella agar. On MacConkey agar colorless colony with no lactose fermentation was observed. On nutrient agar pale yellow colonies were observed. Almost similar results were described previously which includes pinkish slant and yellow butt with blackening and gas production (Nesa *et al*., 2012; Kaushik *et al*., 2014). In these studies, the incidence of *S. enteritidis* was determined from meat and milk and was found to be 27 and 23.7% respectively. The isolates were confirmed using *spv*C gene of virulent plasmid of *S. enteritidis* (Amini *et al*., 2010). Earlier studies showed that these genes were not present in all isolates. Woodward *et al*. (1999) reported that this gene was detected in 70% of isolates but the present studies showed unexpected and different results. This shows the increase in *spv*C gene in recent years in Pakistan.

These isolates were tested for their susceptibility against most commonly used antibiotics. Zones of inhibition around the discs were measured in mm. Out of 18 strains, 11 (61%) were found to be susceptible to most of the drugs while 7 (39%) strains showed resistance against most of the drugs. These MDR strains were numbered as MDR1, MDR2 and MDR3, MDR4, MDR5, MDR6 and MDR7. Mehdi *et al*. (2009) isolated 51 strains from chicken and beef samples and it was documented that 23.5% isolates were found to be MDR while in current study 18 *S. enteritidis* were isolated from chicken and raw milk samples and 33% were MDR. Khanal *et al*.

(2007) showed 26% isolates of *Salmonella* was MDR and showed decreased susceptibility to different antibiotics. Furthermore, in the present study fluoroquinolones resistance genes (QRDR and PMQR) were investigated through PCR using specific primers (Hu *et al.*, 2014). Reasons behind the choice of fluoroquinolone resistance are the frequent and aberrant use of ciprofloxacin in local clinical settings and prescription of high potency antibiotics by the clinicians.

For the isolation of lytic bacteriophages against MDR *S. enteritidis*, 10 sewage water samples were collected, out of which 7 (70%) were found positive for lytic bacteriophages as shown in Table 3. Similar results were reported by Robeson *et al.* (2008) and Khalid *et al*. (2017). All the samples collected from hospitals were found positive for bacteriophages, mainly because of presence of high bacterial contamination as reported (Rasool *et al*., 2016). In the recent study, to isolate and purify the bacteriophages, agar overlay method was used. Nutrient agar plates were layered with soft agar containing *Salmonella* spp. as a host organism along with enriched filtrate followed by incubation. Clear zones referred to as plaques. To get purified suspension the procedure was repeated multiple times (Debarbieux *et al*., 2010; Henry *et al*., 2013) and same method was employed to isolate and purify bacteriophages.

All the sewage samples were checked for the presence of *S. enteritidis* specific bacteriophages after filtration and enrichment over all MDR bacterial strains. Host range as well as lytic potency of prepared bacteriophage lysate was determined by spot assay. Eff1, Eff5 and Eff10 were found most effective against MDR isolates while three found negative for bacteriophage isolation. Phages were confirmed by the presence of clear zones on the bacterial bed. Clear and turbid zones were observed and confirmed by cell imager (Bio-Rad, USA). Similar plaques have been reported (Rasool *et al*., 2016; Khalid *et al*., 2017).

Phage efficacy can be enhanced by divalent ions as play its role in phage adsorption. There was a significant effect of $CaCl_2$ on improvement of plaque morphology as well as PFU while no significant effect of $MgSO_4$ on plaque morphology and PFU was observed. These findings are in accordance with Chhibber *et al*. (2014). Mitomycin had very little and insignificant effect on induction of bacteriophages from lysogenic phase to lytic phase. Lytic potency of bacteriophage lysate and phage titration was evaluated by bacterial count after infection of lytic phages.

Conclusions: On the basis of present study, it is concluded that high occurrence of *S. enteritidis* from food samples and resistance in these isolates are on the rise. Bacteriophages specific to *S. enteritidis* were isolated from waste water and found effective MDR isolates and can be good substitute for treatment against resistant strains. Further studies will help in control of *S. enteritidis* from food samples.

Authors contribution: ABS and ZN assisted in study concept and design. MAZ and AR wrote the manuscript. BA, NN and RH helped in execution of the project.

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