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# Investigation of Antibacterial Efficiency of Various Lytic Bacteriophages Isolated from Chickens Against Characterized Multidrug-resistant Pathogenic Bacterial Strains

Marwa Fathy[1,2], Afaf Ahmed[2], Mohamed Wael Abd El-Azeem[1], Sabry Hassan[1], Serageldeen Sultan[3*]

[1]Department of Microbiology, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt.
[2]Reference Laboratory for Quality Control on Poultry Production (RLQP), Agriculture Research Center (ARC) / Animal Health Research Institute (AHRI), Luxor, Egypt.
[3]Department of Microbiology, Virology Division, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt.

***Correspondence**
Serageldeen Sultan
Department of Microbiology, Virology Division, Faculty of Veterinary Medicine, South Valley University, Qena 83523, Egypt.
E-mail: s.sultan@vet.svu.edu.eg
ORCID: 0000-0003-3527-7803

## Abstract

This study targeted isolation and characterization of potential bacteriophages (phages) infecting (MDR) pathogenic bacteria recovered from chickens and analyzed their efficacy as bio-control agents. A total of 45 different bacterial isolates (18 *E. coli*, 16 *Salmonella*e spp., 5 *Staphylococcus* spp., 2 *Pseudomonas* spp., 1 *Proteus mirbalis*, 1 *Citrobacter* spp., 1 *Enterobacter aerogenes* and 1 *Klebsiella pneumonia*) were obtained from chickens in the current study and previous studies. The identified isolates were investigated for the presence of virulence genes and MDR using PCR and disc diffusion method, respectively. Nine purified phages classified morphologically into 3 families (*Myoviridae*, *Siphoviridae and Podoviridae*) using Transmission Electron Microscope were recovered from chicken intestinal contents and showed viability at wide pH range, resistance to organic solvents and thermostability at high temperatures (up to 80ºC). The potential phages exhibited various bacterial host ranges using the spot test and the efficiency of plating (EOP) assay. The results revealed the prevalent of pathogenic *E. coli* and *Salmonella* serovars among the recovered isolates with different virulence and genotypic patterns. The lytic phages were highly stable and have the capacity to infect different pathogenic MDR bacterial strains. This study demonstrated that these promising phages of avian origin could be used to control the pathogenic MDR *E. coli* and *Salmonella* serovars which possess public concerns on human health and poultry industry.

KEYWORDS
Bacteriophages, Multidrug-resistant bacteria, Chicken, EOP, TEM, PCR

## INTRODUCTION

Bacteriophages (phages) are viruses infecting bacteria and consider natural killers. Phages have the ability to parasitize the bacterial cell and proliferate inside it producing more progenies and lysis the host cell (Hyman and Abedon, 2012). Phages are numerous and wide distributed in soil, deep sea and water with global population numbers estimated as $10^{30}$ to $10^{32}$ (Kazi and Annapure, 2016). Phages have been used to treat bacterial infections in human, different agricultural settings like treating plant bacterial diseases and showed potentiality in livestock and aquaculture (Kutter and Sulakvelidze, 2004). The efficacy of phages in reducing *Salmonella* Typhimurium and *Salmonella Enteritidis* colonization in broiler chickens has been reported (Nabil *et al*., 2018; Tawakol *et al*., 2019; Sorour *et al*., 2020) Also, the high efficacy of phage cocktails in controlling the infection of ducklings with multidrug resistant *Escherichia coli* (*E. coli*) O168 has been stated (El-daly *et al*., 2018) .The phage cocktail can be potentially used as a biological control agent against *Salmonella* isolated from food products and can reduce biofilms producing *Salmonella* serovars (Islam *et al*., 2019).

The poultry industry is exposed to numerous threats of viral or bacterial origin. The *Salmonella* species (*Sal.* spp.) and *E. coli* have been considered the greatest serious bacterial pathogens responsible for a variety of acute and chronic diseases, associated with economic losses, and foodborne diseases (Ahmed and Shimamoto, 2014).

Suberbugs or multidrug resistant bacteria become a serious problem threatens public health and multiple sectors due to misuse of antimicrobial agents in livestock production (WHO 2014). Many studies have suggested that phages have considered a potential therapeutic agent for the biocontrol of multidrug resistant bacteria in poultry (Jassim and Limoges, 2014; Nabil *et al*., 2018).

The distribution of multidrug resistant bacteria among poultry farms and poultry products have been reported in Egypt. From these bacteria many *E. coli* and *Salmonella* isolates have carried virulence genes and exhibited multidrug resistance (El-daly *et al*., 2018; Merwad and Abdel-Haliem, 2018; Sorour *et al*., 2020).

The infectivity of pathogenic *E. coli* is related to several virulence factors, such as intimin (*eae*A), Shiga toxin 1 (*STX1*), and


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Shiga toxin 2 (*STX2*) genes. The *eae*A gene is responsible for the bacterium adherence to the intestinal mucosa while *STX1* and *STX2* genes are increasing the intestinal motility and solution accumulations (Paton and Paton, 1998). In the other hand several virulence factors are responsible for the infectivity of *Salmonella* serovars such as *inv*E/A for *Salmonella* pathogenicity island 1 (SPI-1) which indicated that all serovars have the ability to invade and to cause gastroenteritis (Odjadjare and Olaniran, 2015; Ekwanzala *et al*., 2017; Lan *et al*., 2018). The *ssa*Q gene has been implicated in the type three secretion system (T3SS) apparatus protein encoded with SPI-2, this genetic element has a central role in systemic infection by *Sal.* spp. and intracellular pathogenesis (Bugarel *et al*., 2011). The *mgt*C gene for SPI-3 is required by the organisms for growth in a magnesium limited environment such as in phagosomes and also nece*ssar*y for intra-macrophage survival (van Asten and van Dijk, 2005). The SPI-4 encoded by *Spi4*R is required for intra-macrophage survival and is suspected to carry a type 1 secretion system (T1SS) which involved in the toxin secretion (van Asten and van Dijk, 2005). The *sop*B gene responsible for SPI-5 plays a significant role in the occurrence of diarrhea through activating secretory pathways or facilitating inflammation and altering ion balances within cells (Ahmed *et al*., 2016). Therefore, this study was conducted to isolate and characterize different phages of chicken origin and assess their efficacy against these multidrug resistant pathogenic strains of *E. coli* and *Salmonella* as well as other pathogens recovered from broiler chicken farms in the South of Egypt.

## MATERIALS AND METHODS

*Collection and preparation of chicken samples*

A total of 80 samples were collected from diseased broiler chickens of varying ages (one-day old to 45 days old) from 23 broiler chicken farms in Luxor governorate, in the South of Egypt. A total of 3-5 diseased birds were taken from each farm for isolation and identification of *Sal.* spp. and *E. coli*. A pooled sample from liver, heart, and lung was collected from each bird in a sterile container to be examined in the laboratory (El Sayed *et al*., 2016; Sorour *et al*., 2020) .

*Bacterial isolates*

Other bacterial isolates include *Sal.* spp., *E. coli*, *Klebsiella* spp., *Staphylococcus* aureus (*Staph. aureus*) *Pseudomonas* spp., *Proteus* spp., *Citrobacter* spp., *Enterobacter aerogenes*, used in this study were obtained from Microbiology Department, Faculty of Veterinary Medicine, South Valley University, Qena, Egypt (Table 1).

The collected samples were weighed, homogenized, and suspended in buffered peptone water (1:10 dilution) then incubated at 37ºC for 18 h. A loopful from incubated buffered peptone water was streaked on MacConkey's agar and Eosin methylene blue (EMB) plates and incubated for 24 h at 37ºC. Suspected lactose fermented colonies and colonies with metallic green sheen on EMB were picked up and kept in semi-solid nutrient agar. The purified isolates of *E. coli* were morphologically identified by Gram stain; biochemical tests according to Quinn *et al*., (2002) and serologically by slide agglutination test using *E. coli* polyvalent antisera (SIFIN, Berlin, Germany) according to Edwards and Ewing (1986).

The pre-enrichment broth containing homogenate was mixed and 0.1 ml of the broth was transferred into a tube containing 10 ml of Rappaport-Vassiliadis (RVS) medium (Oxoid, UK). Another 1 ml of the pre-enrichment broth was transferred into a tube containing 10 ml of Muller-Kauffmann tetrathionate novobiocin broth (MKTTn broth). The inoculated RVS broth was incubated at 41.5°C for 24 h and the inoculated MKTTn broth was incubated at 37°C for 24 h. After that, a loopful from the RVS and MKTTn broth was transferred and streaked separately onto the surface of Xylose Lysine Deoxycholate (XLD) agar (Oxoid, UK), brilliant green (BG) agar (Oxoid, UK). The plates were incubated at 37°C for 24 h then checked for the growth of typical *Salmonella* colonies. Purified isolates were biochemically confirmed by oxidase, urease, and triple sugar iron (TSI) tests. The *Salmonella* isolate gave negative for oxidase, urease, and produced K/A with $H_2S$ gas production on TSI according to ISO, 6579-1 (2017). Serotyping of *Sal.* spp. was performed using polyvalent sera (SIFIN, Berlin, Germany) according to ISO, 6579-1 (2017).

Table 1. List of the source and number of identified bacterial isolates used in the present study

| Isolate name | No. of isolates | Source of isolates |
|---|---|---|
| *E. coli O142* | 2 | This study |
| *E. coli O27* | 2 | This study |
| *E. coli O114* | 1 | This study |
| *E. coli O26* | 1 | This study |
| *E. coli O125* | 4 | This study |
| *E. coli O126* | 3 | This study |
| *E. coli O6* | 1 | This study |
| *E. coli O78* | 1 | This study |
| *E. coli O86a* | 1 | This study |
| *E. coli O146* | 1 | This study |
| *S. Entertidis* | 2 | This study |
| *Sal. Kentucky* | 2 | This study |
| *Sal. Typhimurium* | 1 | This study |
| *Sal. Blegdam* | 1 | This study |
| *Sal. Montevideo* | 1 | This study |
| *Sal. Gueuletape* | 2 | This study |
| *Staph. hominis* | 1 (mec*A*+) * | |
| *Staph. aureus* | 1 | El-nagar *et al*. (2017) |
| *Staph. aureus* | 1(mec*A*+) | |
| *Sal. Anatum* | 1 | |
| *Sal. Enteritidis* | 1 | |
| *Sal. Infantis* | 1 | |
| *Sal. Newport* | 1 | |
| *Sal. Verchio* | 1 | |
| *Sal. Kentucky* | 1 | |
| *Sal. Chester* | 1 | |
| *Proteus mirbalis* | 1 | El Sayed *et al*. (2016) |
| *E. coli O26* | 1 | |
| *Citrobacter spp.* | 1 | |
| *Enterobacter aerogenes* | 1 | |
| *Klebsiella pneumonia* | 1 | |
| *Pseudomonas aeruginosa* | 1 | |
| *Pseudomonas lutca* | 1 | |
| *Staph. aureus* | 1 | |
| *Staph. aureus* | 1 | |

* mecA+; methicillin resistant *Staphylococcus*

*Antimicrobial susceptibility testing*

Antimicrobial susceptibility testing was carried out by the Kir-

by-Bauer Disk Diffusion susceptibility test (Hudzicki, 2009), and the interpretation was done as recommended by the Clinical and Laboratory Standards Institute (CLSI) (CLSI, 2018).

Forty-five different bacterial isolates were tested for their antimicrobial susceptibility against 12 antimicrobial agents namely, Amoxicillin (AM) 10 µg, Nitrofurantoin (F) 30 µg, Gentamycin (CN) 10 µg, Neomycin (N) 30 µg, Tetracycline (TE) 30 µg, Ofloxacin (OFX) 10 µg, Chloramphenicol (C) 30 µg, Streptomycin (S) 10µg, Florfenicol (FFC) 30 µg, Norfloxacin (NOR) 10 µg, Enrofloxacin (ENR) 5 µg, and Cefotaxime (CTX) 30 µg (Oxoid, UK). The bacterial isolates were enriched in brain heart infusion broth (Oxoid, UK) at 37ºC for 24 h. A loopful of bacterial growth was mixed with 5 ml of tryptone soya broth (Oxoid, UK), followed by incubation at 37ºC for 24 h. till reaching the turbidity of 0.5 McFarland standard as previously described (Hudzicki, 2009). The bacterial suspensions were inoculated on Mueller Hinton agar (Oxoid, UK) plates, antimicrobial discs were placed on the inoculated plates, and were incubated at 37ºC for 18 h. The growth-inhibition zones were measured and interpreted as susceptible (S), intermediate (I), or resistant (R) according to the CLSI manual (CLSI, 2018).

*Detection of virulence genes by polymerase chain reaction (PCR)*

Molecular detection of virulence-associated genes was performed using oligonucleotides targeting virulence genes for *Salmonella* and *E. coli* isolates. The DNA was extracted from 200 µl bacterial sample using a QIAamp DNA Mini kit (Qiagen, Germany) following the manufacturer's instructions. Primer sequences and the expected size of the PCR product are shown in Table 2. All PCR reactions were performed using DreamTaq Green master mix kit (Takara, Japan) using the thermal cycler (Biometra, Analytik Jena, Germany) and thermal profiles were described (Dipineto *et al*., 2006; Sánchez-Jiménez *et al*., 2010; Bisi-Johnson *et al*., 2011; El Sayed *et al*., 2016). PCR products were separated in 1.5% agarose gel electrophoresis (Applichem, Germany) stained with ethidium bromide (Sigma-Aldrich, USA) 0.5 ug/ml, and visualized under ultraviolet light using a gel documentation system (Alpha Innotech, Biometra, Germany) for the presence of specific target genes product.

*Phages isolation and enrichment*

The broiler chicken intestine was collected from several birds at private slaughterhouses. The cloaca and cecum were homogenized in an equal amount of buffered peptone water (BPW). The homogenate was shacked at 250 rpm/2 h, and centrifuged at 6000 rpm/20 min. The obtained supernatant was further centrifuged at 13000 rpm/5 min and filtered through 0.45µm pore size filter (Corning, NY, Germany). Each bacterial strain was grown to mid-log phase at 37°C in BPW. The filtrate was then examined for the presence of phages using double layer agar plaque assay (Cormier and Janes 2014). A 250 µl of the host bacterial culture was mixed with 750 µl of the filtrate and incubated for 10 - 15 min at room temperature. Then it was mixed with 3 - 5 ml molten soft (45 - 50 °C) TSB (0.7% w/v agar) and poured on the surface of dry tryptone soya agar (TSA). The plates were invertedly incubated after solidification at 37°C/24 h and subsequently, plates were examined for plaques formation.

*Phage purification, propagation, and titration*

Phages were purified from a single plaque using the soft agar overlay method. Where plaques from each plate were picked based on their size and clarity using a sterile pipette tip and were resuspended in a tube containing 5 ml of saline magnesium (SM) buffer [5.8 g NaCl, 2 g $MgSO4{\cdot}7H_2O$, 50 ml Tris-Cl (1M, pH 7.4), and 5 ml Gelatin (2 % w/v)]. The tubes were left at 4°C/24 h. to allow the phage to diffuse into the buffer. Thereafter tubes were centrifuged at 13000 rpm/5 min and the supernatant were filtered through a 0.45µm filter. The purification process was repeated 3 consecutive times until homogeneous plaques were obtained for each phage isolate. The purified phages were propagated by adding 100 µl of an overnight cultured bacterial host. The phage was enriched overnight at 37°C, then centrifuged at 13000 rpm/5 min and filtered via a 0.45 µm filter. Thence tenfold serial dilution in SM buffer was prepared to determine the phage titer by plaque assay. The visible plaques between 20 and 300 plaques were counted and expressed as plaque forming unit (pfu) ml – 1. The stock phages were stored at 4°C for further analysis.

Table 2. Oligonucleotide sequences used for determination of some virulence factors of *E. coli* and *Salmonella* species.

| Target gene | Primer name | Oligonucleotide sequence (5'-3') | Product (bp) | Reference |
|---|---|---|---|---|
| *STX1* | *STX1* (F) | ACACTGGATGATCTCAGTGG | 614 | Dipineto *et al*. (2006) |
| | *STX1* (R) | CTGAATCCCCCTCCATTATG | | |
| *STX2* | *STX2* (F) | CCATGACAACGGACAGCAGTT | 779 | |
| | *STX2* (R) | CCTGTCAACTGAGCAGCACTTTG | | |
| *intimin (eae*A) | *eaeA* (F) | ATGCTTAGTGCTGGTTTAGG | 248 | Bisi-Johnson *et al*. (2011) |
| | *eaeA* (R) | GCCTTCATCATTTCGCTTTC | | |
| *inv*E/A (SPI-1) | *Inv*E/A (F) | TGCCTACAAGCATGAAATGG | 450 | Sánchez-Jiménez *et al*. (2010) |
| | *Inv*E/A (R) | AAACTGGACCACGGTGACAA | | |
| *ssa*Q (SPI-2) | *ssa*Q (F) | GAATAGCGAATGAAGAGCGTCC | 677 | Soto *et al*. (2006) |
| | *ssa*Q (R) | CATCGTGTTATCCTCTGTCAGC | | |
| *mgt*C (SPI-3) | *mgt*C(F) | TGACTATCAATGCTCCAGTGAAT′ | 655 | Sánchez-Jiménez *et al*. (2010) |
| | *mgt*C (R) | ATTTACTGGCCGCTATGCTGTTG | | |
| *Spi4*R (SPI-4) | *Spi4*R (F) | GATATTTATCAGTCTATAACAGC | 1269 | |
| | *Spi4*R (R) | ATTCTCATCCAGATTTGATGTTG | | |
| *sop*B (SPI-5) | *Sop*B (F) | GATGTGATTAATGAAGAAATGCC | 1170 | Soto *et al*. (2006) |
| | *SopB (R)* | GCAAACCATAAAAACTACACTCA | | |

*Phage characterization*

Transmission Electron Microscope (TEM) analysis

Nine phage isolates were subjected to transmission electron microscopy (TEM), and the phage morphotype was determined using the negative staining technique as previously described (Vinner *et al*., 2017). Briefly, one drop of phage lysate ($10^{10}$ pfu/ml) was fixed on carbon-coated grids (Emsdiasum, Hatfield, PA) and stained with 2% phosphotungstic acid (Emsdiasum, Hatfield, PA). Grids were allowed to dry and examined with the TEM (TEM; JEM-1200TEM, JEOL) provided with a Gatanorius 2kx2k CCD digital camera at 80 kV (Central laboratory in Faculty of Agriculture, Cairo University). The images were taken, and morphological characters were used to classify phage isolates as previously described (Ackermann, 2009).

Host range and cross infectivity of the phage isolates

The lytic activity of isolated phages was evaluated against 45 bacterial hosts (18 *E. coli*, 16 *Sal.* spp., 5 *Staph. aureus*, 2 *Pseudomonas* spp., and one each of *Klebsiella* spp., *Citrobacter* spp., *Enterobacter aerogenes* and *Proteus mirabilis*). The spot test was carried out to assess the host-range specificity of each phage isolate as previously described (Islam *et al*., 2019). A 100 µl of cultured bacteria which is in an exponential phase was transferred to 4 ml of soft molten (45-50°C) TSB (0.7% w/v agar). The mixture was poured onto the surface of TSA plates and allowed to dry for 5 min. When the overlay agar settled, 5 µl of each phage solution was spotted onto bacterial lawns and allowed to dry, and then plates were incubated at 37°C /20-24 h. Any bacterial lawn showing clear spots/plaques were considered a phage susceptible bacterium.

Efficiency of plating of phages

Efficiency of plating (EOP) was performed to determine lytic efficiency of the phage in comparison with their suitable host bacteria as previously described (Islam *et al*., 2019). Each phage was serially diluted and tested in a triplicate on the bacterial host. Bacterial strains were grown overnight at 37°C. A 100 µl of overnight bacterial culture together with 100 µl of diluted phage lysate, dilution factors between $10^{2}$–$10^{10}$ pfu/ml, was applied in double layer agar plaque assay. The plates were incubated overnight at 37°C and the number of plaques was counted. The average EOP value was classified as high efficiency (EOP 0.5 to 1.0), moderate efficiency (EOP 0.2 to <0.5), low efficiency (EOP 0.001 to <0.2), and inefficient (EOP <0.001) based on the reproducible infection on the targeted bacteria (Manohar *et al*., 2019). Relative EOP values were calculated (average pfu on tested bacteria / average pfu on reference bacteria).

Viability and stability of phages at different pH degrees and temperatures

Phages stability and viability were evaluated at different thermal and pH conditions (Lu *et al*., 2019). One milliliter of phage in SM buffer was incubated at 40°C, 50°C, 60°C, 70°C and 80°C for 1 h. and the titer was determined by double layer agar plaque assay. Also, a 100 µl of phage suspension was added to 900 µl of 1% peptone solution with different pH values (2, 4, 6, 8, 10 and 12) using pH meter (Jenway, UK) adjusted by the addition of either mol-1 HCl (AppliChem, Darmstadt, Germany) or mol-l NaOH (AppliChem, Darmstadt, Germany), and subsequently incubated at 25°C for 1 h. The phage viability was determined by the double layer agar plaque method.

Stability and viability of phages against organic solvents

The isolated phage stability in organic solvents as chloroform and ethanol 70% was tested (Jurczak-Kurek *et al*., 2016). Where 1 ml of the phage suspension was mixed with equal volume of each organic solvent separately and incubated at room temperature /1 h. with shaking by hand from time to time. After that, the mixtures were centrifuged at 10000 rpm/ 10 min and the phage titer in the aqueous phase was determined by double layer agar plaque method.

## RESULTS

*Isolation and serotyping of Salmonella serovars and E. coli isolated from broiler chickens*

In this study, 26 bacterial isolates were isolated and serologically identified out of 80 samples collected from diseased broiler chickens. Total of 9 *Sal.* spp. isolates were serotyped into *Sal.* Typhimurium (n=1), *Sal. Enteritidis* (n=2), *Sal. Kentucky* (n=2), *Sal. Blegdam* (n=1), *Sal. Montevideo* (n=1), and *Sal. Gueuletape* (n=2). While the remaining 17 isolates were identified as *E. coli* O27 (n=2), *E. coli* O26 (n=1), *E. coli* O126 (n=3), *E. coli* O125 (n=4), *E. coli* O6 (n=1), *E. coli* O114 (n=1), *E. coli* O78 (n=1), *E. coli* O142 (n=2), *E. coli* O146 (n=1), and *E. coli* O86a (n=1) as shown in Table 1.

*Antimicrobial susceptibility testing*

Variable rates of antimicrobial resistance for *Salmonella* and *E. coli* serotypes were observed using 12 different types of antimicrobial agents (Table 3). The 18 *E. coli* isolates showed a high resistance to Amoxicillin (100%), Tetracycline (94.4%), each of Streptomycin and Enrofloxacin (83.3%), Neomycin (77.7%), Chloramphenicol (61.1%), each of Florfenicol and Ofloxacin (55.5%), and Norfloxacin (50%). On the other hand, lower rates of resistance were observed for Gentamycin (22.2%), and Cefotaxime (16.7%), while all isolates were sensitive to Nitrofurantoin (100%). Regarding the 16 *Salmonella* isolates, the high antimicrobial resistance rates were exhibited to Amoxycillin (87.5%), Neomycin (75%), Streptomycin (62,5%), and Tetracycline (56.25%), but the low antimicrobial resistance rates were observed for Nitrofurantoin (37.5%), each of Florfenicol, Enrofloxacin and Gentamycin (31.25%), Ofloxacin (25%), and each of Chloramphenicol, Norfloxacin, and Cefotaxime (18.75%). Several multidrug resistance (MDR) profiles to three or more antimicrobial classes were detected in 76.7% of *Salmonella* serovars and in 94.4% of *E. coli* serotypes and 14 different MDR patterns were recorded, reflecting the high prevalence of MDR among *Sal.* spp. and *E. coli* isolates (Table 4). Interesting, even the same serovar or serotype of bacterial isolates was present, different MDR pattern was observed.

*Detection and frequency of virulence genes among Salmonella and E. coli isolates*

Tables 5 and 6 presented the detection and frequency of 8 virulence genes, that are *inv*E/A, *ssa*O, *mgt*C, *Spi4*R, *sop*B genes for *Sal.* spp., and *eae*A, *STX1*, and *STX2* genes for *E. coli*. Eight isolates of *E. coli* and *Salmonella* showed at least one virulence-associated gene. The *Salmonella* isolates showed 7 genotypic patterns based on the frequency of the pathogenicity island genes

Table 3. The susceptibility of different *Salmonella* and *E. coli* isolates to different antimicrobial agents

| Antimicrobials | *Salmonella* isolates | | | *E. coli* isolates | | |
|---|---|---|---|---|---|---|
| | R | I | S | R | I | S |
| AM[a] | 14/16 (87.5)[b] | 1/16 (6.25) | 1/16 (6.25) | 18/18 (100) | - | - |
| F | 6/16 (37.5) | - | 10/16 (62.5) | - | - | 18/18 (100) |
| C | 3/16 (18.75) | - | 13/16 (81.25) | 11/18 (61.11) | - | 7/18 (38.88) |
| FFC | 5/16 (31.25) | 1/16 (6.25) | 10/16 (62.5) | 10/18 (55.5) | 7/18 (38.8) | 1/18 (5.5) |
| S | 10/16 (62.5) | | 5/16 (31.25) | 15/18 (83.3) | 3/18 (16.6) | - |
| CN | 5/16 (31.25) | - | 11/16 (68.75) | 4/18 (22.22) | | 14/18 (77.77) |
| N | 12/16 (75) | - | 4/16 (25) | 14/18 (77.7) | - | 4/18 (22.2) |
| OFX | 4/16 (25) | - | 12/16 (75) | 10/18 (55.5) | - | 8/18 (44.4) |
| NOR | 3/16 (18.75) | | 13/16 (81.25) | 9/18 (50) | - | 9/18 (50) |
| ENR | 5/16 (31.25) | | 11/16 (68.75) | 15/18 (83.3) | - | 3/18 (16.6) |
| TE | 9/16 (56.25) | | 7/16 (43.75) | 17/18 (94.44) | | 1/18 (5.55) |
| CTX | 3/16 (18.75) | - | 13/16 (81.25) | 3/18 (16.66) | - | 15/18 (83.3) |

[a] AML (Amoxycillin), F (Nitrofurantoin), C (Chloramphenicol), FFC (Florfenicol), S (Streptomycin), CN (Gentamycin), N (Neomycin), OFX (Ofloxacin), NOR (Norfloxacin), ENR (Enrofloxacin), Tetracycline (TE), CTX (Cefotaxime).

[b] the percentage of reacted isolates

Table 4. The multidrug resistant profiles of the *Salmonella* and *E. coli* isolates against different antimicrobial groups.

| Bacterial isolates | Antimicrobial categories | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Penicillin | Nitrofuran | Phenicol | | Tetracycline | | Cephem | Aminoglycoside | | Fluoroquinolone | | |
| | AML[a] | F | C | FFC | TE | CTX | CN | S | N | OFX | NOR | ENR |
| *Sal. Kentauky* | R | -[b] | R | R | R | R | R | R | R | R | R | R |
| *E. coli O146* | R | - | R | R | R | R | R | R | R | R | R | R |
| *Sal. Kentauky* | R | - | R | R | R | R | - | R | R | R | R | R |
| *E. coli O125* | R | - | R | R | R | R | - | R | - | R | R | R |
| *E. coli O114* | R | - | R | R | R | - | R | R | R | R | R | R |
| *E. coli O125* | R | - | R | R | R | - | R | R | R | R | R | R |
| *E. coli O126* | R | - | R | R | R | - | R | R | R | R | R | R |
| *E. coliO27* | R | - | R | R | R | - | - | R | R | R | R | R |
| *EcoliO142* | R | - | R | R | R | - | - | R | - | R | R | R |
| *EcoliO142* | R | - | R | R | R | - | - | R | R | - | - | R |
| *E. coliO27* | R | - | - | - | R | R | - | R | R | R | R | R |
| *E. coli O125* | R | - | - | - | R | - | - | R | R | R | R | R |
| *Sal. Enteritidis* | R | R | - | R | R | - | R | R | R | - | - | R |
| *Sal. Verchio* | R | R | - | R | R | - | R | R | R | - | - | R |
| *Sal. Chester* | R | R | - | - | R | - | R | R | R | - | - | R |
| *Sal. Gueuletapee* | R | - | R | R | R | - | R | R | R | - | - | - |
| *E. coli O86* | R | - | R | R | R | - | - | R | R | - | - | - |
| *E. coli O6* | R | - | R | R | R | - | - | R | R | - | - | R |
| *Sal. Newport* | R | - | - | - | R | - | - | R | R | - | - | - |
| *E. coli O26* | R | - | - | - | R | - | - | R | R | - | - | - |
| *Sal. Infantis* | R | - | - | - | R | - | - | R | - | - | - | - |
| *E. coli O26* | R | - | - | - | R | - | - | - | R | - | - | R |
| *E. coliO78* | R | - | - | - | R | - | - | - | R | - | - | R |
| *Sal. Blegdam* | R | R | - | - | R | - | - | - | R | - | - | - |
| *Sal. Enteritidis* | R | R | - | - | - | - | - | - | R | - | R | - |
| *E. coli O126* | R | - | R | - | R | - | - | R | - | - | - | R |
| *E. coli O125* | R | - | - | - | R | - | - | R | - | R | - | R |

[a]AML (Amoxycillin), F (Nitrofurantoin), C (chloramphenicol), FFC (Florfenicol), S (Streptomycin), CN (Gentamycin), N (Neomycin), OFX (Ofloxacin), NOR (Norfloxacin), ENR (Enrofloxacin), TE (Tetracycline), CTX (Cefotaxime)

[b] means sensitive to antimicrobial agent

(Table 5). Also, the *E. coli* serotypes showed 4 genotypic patterns depend on the absence or presence of one or more virulence genes (Table 6). Although the same serovar or serotype of bacterial isolate was identified, the different or same virulence pattern was shown.

Table 5. The genotypic patterns of *Salmonella* serovars according to the presence of different pathogenicity islands genes.

| Genotype | *Salmonella* serovars | *inv*E/A | *ssa*O | *mgt*C | *Spi4*R | *sop*B |
|---|---|---|---|---|---|---|
| I | *Sal. Montevideo* | + | + | + | + | + |
| | *Sal. Enteritidis* | + | + | + | + | + |
| II | *Sal. Enteritidis* | + | + | + | - | + |
| | *Sal. Gueuletapee* | + | + | + | - | + |
| III | *Sal. Kentauky* | + | + | - | - | + |
| IV | *Sal. Typhymurum* | + | + | - | - | - |
| V | *Sal. Blegdam* | + | - | + | - | - |
| VI | *Sal. Gueuletapee* | + | - | - | - | - |
| VII | *Sal. Kentauky* | - | - | - | - | - |

Table 6. The distribution of virulence pattens among different *E. coli* serotypes.

| Virulence pattern | *E. coli* serotypes | Virulence genes | | |
|---|---|---|---|---|
| | | *eae*A | *STX1* | *STX2* |
| 1 | *E. coli O86a* | - | - | - |
| | *E. coli O125* | - | - | - |
| | *E. coli O27* | - | - | - |
| | *E. coli O27* | - | - | - |
| | *E. coli O125* | - | - | - |
| | *E. coli O78* | - | - | - |
| | *E. coli O126* | - | - | - |
| | *E. coli O6* | - | - | - |
| | *E. coli O142* | - | - | - |
| 2 | *E. coli O114* | + | - | - |
| | *E. coli O126* | + | - | - |
| 3 | *E. coli O27* | + | + | - |
| | *E. coli O146* | + | + | - |
| 4 | *E. coli O26* | + | - | + |
| | *E. coli O126* | + | - | + |
| | *E. coli O125* | + | - | + |
| | *E. coli O125* | + | - | + |

*Morphological characterization of phages using TEM*

Phage isolates were subjected to TEM analysis to determine their morphotypes. The phage structural dimensions and its family was shown in Fig. 1 (A-I) and table 7, respectively. Phage isolates were classified as per the International Committee for Taxonomy of Virus (ICTV) classification based on the three-dimensional structure was observed. Structurally, the phage had an icosahedral head and a neck attached to a tail with tail fibers, and it was classified under the order Caudovirales and *Siphoviridae*, *Myoviridae* and Podoviridae families.

The nomenclature of phages was designed depending upon three main criteria preceded by vB (bacterial virus), followed by abbreviation for the host bacteria name, the viral family, and a simple abbreviation of specific laboratory designation (laboratory code). The tail diameter (td) value was used to classify viruses with long tails as either *Siphoviridae* (td < 16 nm) or *Myoviridae* (td ≥ 16 nm). Phages *vB_salk1S*, *vB_salk3S*, *vB_salNS* and *vB_EnaS* (Fig. 1A-D) belong to the family *Siphoviridae*. While phages *vB_Salk2M*, *vB_SauM*, *vB_EO26M* and *vB_EO27M* (Fig. 1E -G) belong to the *Myoviridae* family and the phage *vB_EO114P* (Fig. 1-I) belongs to Podoviridae family (Table 7).

*Phage isolates identification*

A total of 18 phage isolates were obtained but only 9 were successfully purified. The 18 phage isolates have different bacterial hosts: 7 different phages for *E. coli*, 9 different phages for *Salmonella*, and one phage each for *Staph. aureus*, and *Enterobacter aerogenes*, that were assigned based on plaque morphology and size (Fig. 2A-F). The purified phages were named as *vB_salk1S*, *vB_Salk2M*, *vB_salNS* and *vB_salk3S* for *Salmonella* specific phages, *vB_EO27M*, *vB_EO114P* and *vB_EO26M* for *E. coli* specific phages, one phage *vB_SauM* specific for *Staph. aureus*, and one phage *vB_EnaS* specific for *Enterobacter aerogenes*. The phages which produced round clear plaques with their respective host bacterial isolates after overnight incubation at 37˚C had been confirmed as lytic phages (Fig. 2A-F).

*Phages propagation*

The phages titers after propagation were ranged from 109 to 1014 pfu/ml. The *vB_SauM* and *vB_EO27M* phages had the lowest titer, while the phage *vB_salk3S* had the highest titer in comparison to other phages.

*Phage host range and efficiency of plating (EOP) analysis*

The spot test was performed to determine the host range of 9 selected lytic phages against different bacterial host species (Table 8). Phages from this study showed narrow host range. The staphylococcal phage was able to lyse the strain in which it was isolated and another *Staph. aureus* isolate, and 2 phages each for *E. coli* and *Salmonella*, and a phage for *Enterobacter aerogenes* were able to lyse the strain in which they were isolated. On the other hand, 2 *Salmonella* and one *E. coli* phages were able to lyse 1 - 4 bacterial strains (Table 8).

The results of spot test indicated that the phage *vB_SauM* had the capacity to form completely clear zones on 3 out of 5 strains, while the phage *vB_salk1S* formed turbid zones on 11 out of 45 isolates. The phage *vB_Salk2M* formed turbid zones on 9 out of 45 bacterial isolates The phage *vB_EO27M* formed turbid zones on 4 out of 45 isolates. The phages *vB_salNS* and *vB_salk3S* formed clear lysis zones on 4 *Salmonella* isolates lawns, and the *vB_EO26M* phage formed clear lysis zones on 5 *E. coli* isolates and an *Enterobacter aerogenes* isolate.

However, EOP results indicated that the *vB_SauM* phage had plaques against one *Staph. aureus* isolate with high efficiency (EOP = 1), the *vB_EO26M* phage had plaques against 3 *E. coli* isolates with low efficiency (EOP = 0.012, 0.01 and 0.02) and one *Enterobacter aerogenes* isolate with a low efficiency (EOP = 0.006). The *vB_salNS* phage had plaques against one *Salmonella* isolate with a high efficiency (EOP = 0.6) and a low efficiency (EOP = 0.06) against another *Salmonella* isolate. The *vB_salk3S* phage had plaques against 3 *Salmonella* isolates of low efficiencies (EOP = 0.01, 0.025 and 0.021), while the *vB_Salk2M*, *vB_salk1S*, *vB_EO27M*, and *vB_EnaS* phages had no effect on tested isolates (Table 9).

*Phage stability and viability at different temperatures*

The results in Fig. 3A showed the stability and viability of phages at different temperatures. The phages maintained their maximum infectivity at 40°C/1 h. Also, all phages were active at 50°C and 60°C with reduced titer from 1-5 $log_{10}$, but the staphylococcal phage was completely lost its infectivity. Five phages were active with reduced titer at 70°C, while four phages were completely inactivated at 70°C. All phages were com¬pletely inactivated when they were incubated at 80°C /1 h. except for the phage *vB_salNS* which remained infective.

*Phage stability and viability against different hydrogen ion levels*

The stability of phages at different pH degrees from 2-12 was presented in Fig. 3B. The results showed that most phages found infective in all pH values from 4-12 with different proportions. At pH 2 all phages were completely inactivated except the *vB_salNS*, *vB_salk3S*, and *vB_EO26M* phages. While all phages were active at pH 4 except for the phage *vB_Salk2M* was completely inactivated. All phage isolates were able to propagate at pH 6-12 with reduced phage particles titer from 1-2 $log_{10}$ except the *vB_SauM* phage was completely inhibited at pH 10 and 12.

Table 7. The physical properties (size in nm) and belonging family of obtained phages.

| Phage name | Physical properties [a] | | | | | Family |
|---|---|---|---|---|---|---|
| | hd | hl | tl | td | Tail character | |
| *vB_salk3S* | 52±5 [b] | 51±3 | 120±5 | 9±1 | non-contractile | *Siphoviridae* |
| *vB_EnaS* | 67±4 | 66±2 | 124±3 | 12±0.6 | non-contractile | *Siphoviridae* |
| *vB_salk1S* | 55±1 | 60±3 | 121±3 | 8±2 | non-contractile | *Siphoviridae* |
| *vB_salNS* | 50±3 | 47±3 | 161±8 | 8±0.6 | non-contractile | *Siphoviridae* |
| *vB_SauM* | 136±5 | 124±3 | 161±3 | 34±2 | contractile | *Myoviridae* |
| *vB_Salk2M* | 61±2 | 62±3 | 126±3 | 18±3 | contractile | *Myoviridae* |
| *vB_EO27M* | 72±4 | 80±1 | 115±3 | 20±5 | contractile | *Myoviridae* |
| *vB_EO26M* | 64±8 | 61±3 | 106±7 | 19±2 | contractile | *Myoviridae* |
| vB_ EO114P | 113±8 | 110±6 | 16±3 | - | non-contractile | *Podoviridae* |

[a]Head diameter (hd); width perpendicular to the tail, head length (hl); along the tail axis, tail diameter (td), and tail length (tl).
[b]The mean values and standard deviations of measurements.

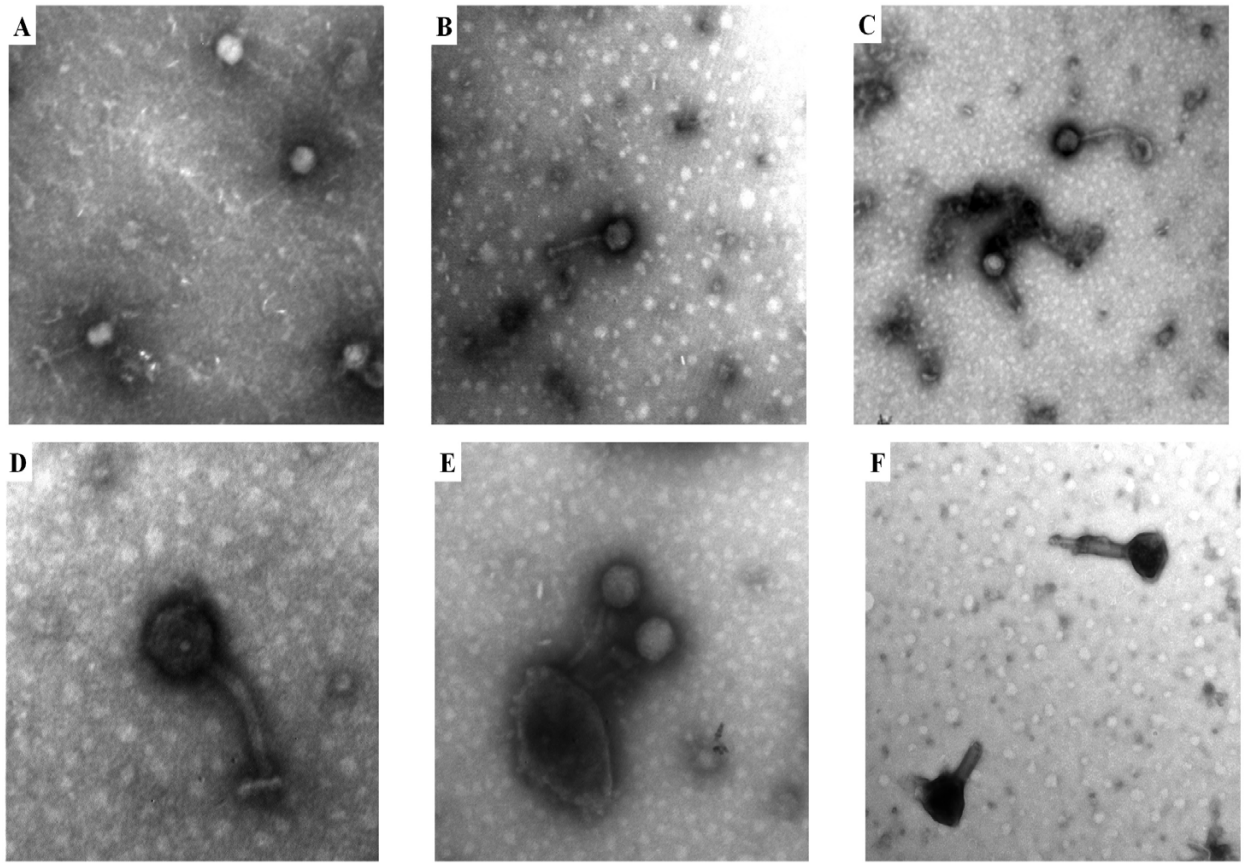


Fig. 1. Morphological characterization of phages stained with 2% phosphotungstic acid and examined by TEM (magnification 100000x – 150000x/100 nm). The tail diameter (td) value was used to classify viruses into *Siphoviridae* with long non-contractile tails (td < 16 nm) such as *vB_salk3S* (A), *vB_salk1S* (B), *vB_EnaS* (C, D) and *vB_salNS* (E). The *Myoviridae* has thick contractile tail (td ≥ 16 nm) as *vB_SauM* (F).

*Effect of organic solvents on the phage stability*

The effect of organic solvents (ethanol 70% and chloroform) on the phage virions stability was also studied. The results revealed that all phages were quite resistant to chloroform with titer reduction from 1-2 $log_{10}$ Conversely, 3 phages were completely inactivated after exposure to ethanol 70% and 6 phages showed a low titer from 1-7 $log_{10}$ (Fig.3C).

Table 8. The spot test to determine the efficacy of the isolated phages against different bacterial isolates

| Isolates | Spot test of bacteriophages | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | vB_EO27S | *vB_salk1S* | *vB_Salk2M* | *vB_SauM* | *vB_EnaS* | vB_E. O114p | *vB_salNS* | *vB_salk3S* | *vB_EO26M* |
| *E coli O27(H)* | +[a] | - | - | - | - | - | - | - | + |
| *E coli O114(H)* | -[b] | - | - | - | - | + | - | - | - |
| *E. coli O27* | +/-[c] | - | - | - | - | - | - | - | - |
| *E. coli O6* | - | +/- | - | - | - | - | - | - | - |
| *E coli O142* | +/- | - | - | - | - | - | - | - | - |
| *E coli O142* | - | - | - | - | - | - | - | - | - |
| *E coli O26* | +/- | - | - | - | +/- | - | - | - | + |
| *E coli O146* | - | - | - | - | - | - | - | - | + |
| *E coli O125* | - | - | - | - | - | - | - | - | + |
| *E coli O125* | - | - | - | - | - | - | - | - | + |
| *E coli O125* | - | - | - | - | - | - | - | - | - |
| *E coli O86a* | +/- | - | - | - | - | - | - | - | - |
| *E coli O78* | - | - | - | - | - | - | - | - | - |
| *E coli O126* | - | - | - | - | - | - | - | - | + |
| *E coli O126* | - | - | - | - | - | - | - | - | - |
| *E coli O126* | - | - | - | - | - | - | - | - | - |
| *E coli O125* | - | - | - | - | - | - | - | - | - |
| *E coli O26* | - | - | - | - | - | - | - | - | - |
| *Sal. Enteritidis* | - | +/- | +/- | - | - | - | + | + | - |
| *Sal. Enteritidis* | - | +/- | +/- | - | - | - | - | - | - |
| *Sal. Enteritidis* | - | - | - | - | - | - | - | - | - |
| *Sal. Kentucky* | - | + | +/- | - | - | - | + | + | - |
| *Sal. Kentucky* | - | +/- | + | - | - | - | - | + | - |
| *Sal. Kentucky* | - | - | - | - | - | - | - | - | - |
| *Sal. Anatum* | - | - | - | - | - | - | - | - | - |
| *Sal. Infantis* | - | +/- | +/- | - | - | - | + | + | - |
| *Sal. Newport* | - | +/- | +/- | - | - | - | + | + | - |
| *Sal. Verchio* | - | +/- | +/- | - | - | - | - | - | - |
| *Sal. Chester* | - | - | - | - | - | - | - | - | - |
| *Sal. Blegdam* | - | +/- | +/- | - | - | - | - | - | - |
| *Sal. Montevidea* | - | - | - | - | - | - | - | - | - |
| *Sal. Gueuletapee* | - | +/- | +/- | - | - | - | - | - | - |
| *Sal. Gueuletapee* | - | +/- | +/- | - | - | - | - | - | - |
| *Sal. Typhymurum* | - | - | - | - | - | - | + | - | - |
| *Enterobacter.aerogenes* | - | - | - | - | + | - | - | - | + |
| *Proteus mirbalis* | - | - | - | - | - | - | - | - | - |
| *Citrobacter spp.* | - | - | - | - | - | - | - | - | - |
| *Klebsiella pneumonia* | - | - | - | - | - | - | - | - | - |
| *Pesudomonas aeruginosa* | - | - | - | - | - | - | - | - | - |
| *Pseudomans lutca* | - | - | - | - | - | - | - | - | - |
| *Staph. aureus* | - | - | - | - | - | - | - | - | - |
| *Staph. aureus* | - | - | - | + | - | - | - | - | - |
| *Staph. hominis* | - | - | - | + | - | - | - | - | - |
| *Staph. aureus* | - | - | - | + | - | - | - | - | - |
| *Staph. aureus* | - | - | - | + | - | - | - | - | - |

[a] means production of clear plaques, [b] means no plaques, [c] means production of turbid plaques

Table 9. Efficiency of plating (EOP) to determine the phages efficiency against the different bacterial isolates showing positive spot test

| Isolates | EOP score of the phages | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | vB_EO27S | *vB_salk1S* | *vB_Salk2M* | *vB_SauM* | *vB_EnaS* | *vB_salNS* | *vB_salk3S* | *vB_EO26M* |
| *E. coli* O27(H) | 1 | ND[b] | ND | ND | ND | ND | ND | <0.001 |
| *E. coli* O27 | <0.001 | ND | ND | ND | ND | ND | ND | ND |
| *E. coli* O6 | ND | <0.001 | ND | ND | ND | ND | ND | ND |
| *E. coli* O142 | <0.001 | ND | ND | ND | ND | ND | ND | ND |
| *E. coli* O26 | 0.001 | ND | ND | ND | <0.001 | ND | ND | 1 |
| *E. coli* O146 | ND | ND | ND | ND | ND | ND | ND | 0.012 |
| *E. coli* O125 | ND | ND | ND | ND | ND | ND | ND | 0.01 |
| *E. coli* O125 | ND | ND | ND | ND | ND | ND | ND | <0.001 |
| *E. coli* O86a | <0.001 | ND | ND | ND | ND | ND | ND | ND |
| *E. coli* O126 | ND | ND | ND | ND | ND | ND | ND | 0.02 |
| *S. Enteritidis* | ND | <0.001 | <0.001 | ND | ND | 0.06 | <0.001 | ND |
| *S. Enteritidis* | ND | <0.001 | <0.001 | ND | ND | ND | ND | ND |
| *S. Kentucky* | ND | 1 | <0.001 | ND | ND | <0.001 | 0.01 | ND |
| *S. Kentucky* | ND | <0.001 | 1 | ND | ND | ND | 1 | ND |
| *S. Infantis* | ND | <0.001 | <0.001 | ND | ND | 0.6 | 0.025 | ND |
| *S. Newport* | ND | <0.001 | <0.001 | ND | ND | 1 | 0.021 | ND |
| *S. Verchio* | ND | <0.001 | <0.001 | ND | ND | ND | ND | ND |
| *S. Blegdam* | ND | <0.001 | <0.001 | ND | ND | ND | ND | ND |
| *S. Gueuletapee* | ND | <0.001 | <0.001 | ND | ND | ND | ND | ND |
| *S. Gueuletapee* | ND | <0.001 | <0.001 | ND | ND | ND | ND | ND |
| *S.* Typhymurum | ND | ND | ND | ND | ND | <0.001 | ND | ND |
| *E. aerogenes* | ND | ND | ND | ND | 1 | 0.000 | ND | 0.006 |
| *Staph. aureus* | ND | ND | ND | 1 | ND | ND | ND | ND |
| *Staph. hominis* | ND | ND | ND | <0.001 | ND | ND | ND | ND |
| *Staph. aureus* | ND | ND | ND | 1 | ND | ND | ND | ND |
| *Staph. aureus* | ND | ND | ND | <0.001 | ND | ND | ND | ND |

[a] EOP score; 0.5 to 1.0 (high efficiency); 0.2 to <0.5 (moderate efficiency); 0.001 to <0.2 (low efficiency) and <0.001 (inefficient).
[b] ND not done (Spot test negative).

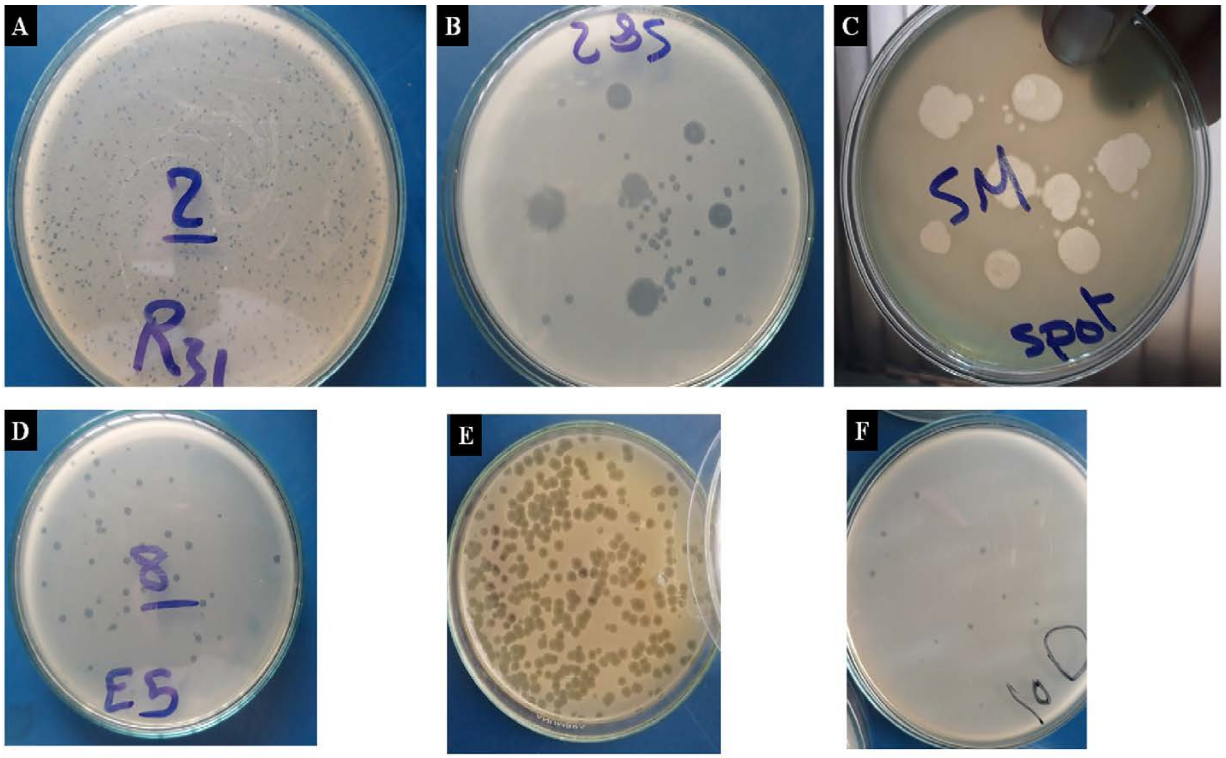


Fig. 2. The spot test determining the host range of the 9 lytic phages against different bacteria species. The clear plaques are typical for lytic (virulent) phages; *vB_SauM* (A), *vB_Salk2M* (B), *vB_EO27M* (C), *vB_EO114P* (D), *vB_salk3S* (E) and *vB_salk1S* (F). The plaque size of *vB_SauM* is 1 mm. The phages *vB_EO27M* and *vB_salk1S* showed plaque size 1.5 – 2 mm. The phages *vB_Salk2M*, *vB_EO114P* and *vB_salk3S* showed clear plaque size 3 mm.

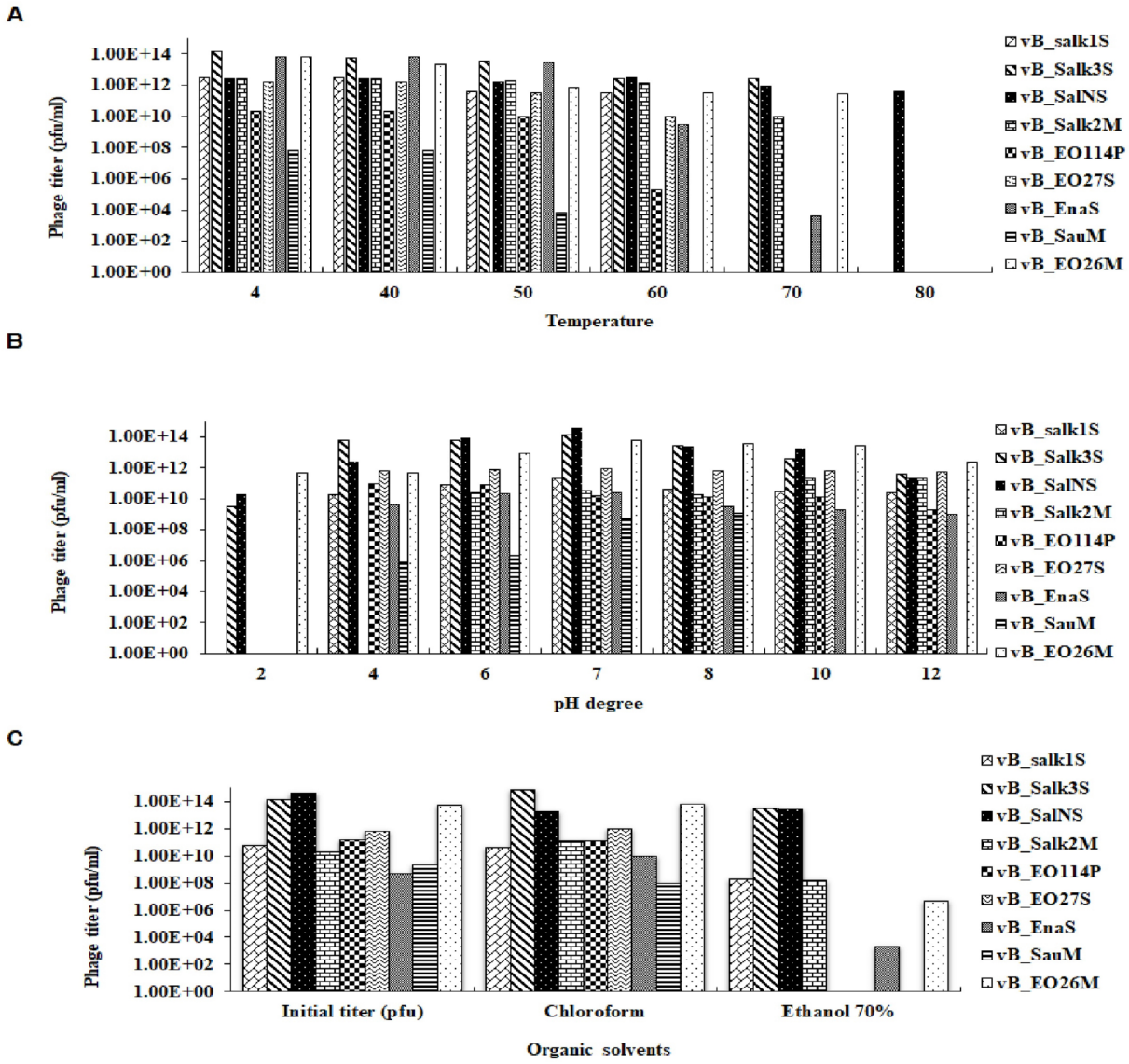


Fig. 3. The biological characterization of the 9 lytic phages stability and viability after exposing to different factors including: A) different temperatures, B) different pH degrees and C) different organic solvents.

## DISCUSSION

The emergence of antibiotic resistance among pathogenic bacteria from food animals and birds has a great potential for the possible use of lytic phages as an alternative biocontrol strategy (Rios *et al*., 2016). Because of their ability to lyse MDR pathogens, lytic phages are considered as a natural and green technology for food safety (Moye *et al*., 2018).

The isolation, identification, and characterization of the bacterial host is a prerequisite for the successful isolation of suitable lytic phages intended for biocontrol of MDR pathogens. Out of 80 examined clinical samples from 23 broiler chicken farms, 17 (73%) *E. coli* and 9 (39%) *Salmonella* spp. were isolated. Results in this study showed a higher prevalence of *Salmonella* spp. and *E. coli* infection than those reported by (Merwad and Abdel-Haliem, 2018; Tawakol *et al*., 2019; Sorour *et al*., 2020). The antimicrobial susceptibility test indicated that MDR bacteria to three or more antimicrobial classes was detected in 76.7% of *Salmonella* serovars and 94.4% of *E. coli* isolates. While previous reports indicated that 86% of *Salmonella* isolates and all *E. coli* strains recovered from broiler chickens were multidrug resistant (Tawakol *et al*., 2019; Rady *et al*., 2020).

The molecular typing of virulence associated genes has determined the genetic background or pathogenicity of bacteria. Overall, the analysis of virulence determinant genes in the isolated *Salmonella* serovars revealed that 8 *Salmonella* isolates carry at least one SPIs gene. Although another study recorded that most *Salmonella* isolates obtained from broiler chickens in distant geographical area have five pathogenicity islands genes (El Sayed *et al*., 2016). In the present study, the *inv*E/A gene was harbored by 88.9% of *Salmonella* serovars which are nearly in agreement with (Osman *et al*., 2014; Ahmed*et al*., 2016) indicating that these isolates have the ability to invade and to cause gastroenteritis (Odjadjare and Olaniran, 2015; Ekwanzala *et al*., 2017; Lan *et al*., 2018).

The *ssa*Q gene has a central role in systemic infections by *Salmonella* spp., and intracellular pathogenesis (Bugarel *et al*.,2011). The *mgt*C gene enables the organisms for growth in phagosomes and essential for intra-macrophage survival (van Asten and van Dijk, 2005). The *Spi4*R gene is responsible for intra-macrophage survival and involves in the toxin secretion (van Asten and van Dijk, 2005). The *sop*B gene plays a signifi¬cant role in the occurrence of diarrhea (Ahmed *et al*., 2016).

Pathogenic *E. coli* infectivity is related to several virulence factors, such as the *eae*A gene which is responsible for the bacterium adherence to the intestinal mucosa. While *STX1* and *STX2* genes increase the intestinal motility and solution accumulations (Paton and Paton, 1998). In this study 8 *E. coli* isolates have the *eae*A gene, 2 have the *STX1* gene and 4 have the *STX2* gene. The prevalence of *eae*A gene among examined *E. coli* in the present study was 47%, which was higher than the prevalence of *eae*A gene reported in the previous study 33.3% (Eid *et al*., 2016).

Phages are numerous and spread in the environment, where their host present in rivers, soil, sewage, poultry or animal feces,

water ponds, and sea water (Mulani *et al*., 2015). Several studies have reported that phages were successfully isolated from fresh water ponds, soil, and animal waste collected from different livestock farms (Yordpratum *et al*., 2011; Shukla *et al*., 2014) as well as from chicken cloacae, pig rectal swabs, and urban sewage (Cortés *et al*., 2015; Jurczak-Kurek *et al*., 2016). Furthermore, others reported the isolation of 15 phages from domestic sewage, 10 phages from poultry sources, and 6 phages from chicken and beef offal samples (Huang *et al*., 2018; Lukman *et al*., 2020).

In this study, total of 18 specific phages were isolated from the intestine of broiler chickens using different MDR bacteria as a host, but nine purified phages were successfully exhibited clear and discrete plaques, while the rest of phages produced turbid, very small or hardly visible plaques typical for lysogenic phages (Yoon *et al*., 2007). Three phages were effective against 3 MDR *E. coli* isolates, 2 of them were Shiga toxin-producing *E. coli*. In addition to that, 4 phages were effective against 3 MDR *Salmonella* serovars whereas 2 serovars harboring virulence genes. Also, one phage was effective against antimicrobial resistant *Enterobacter aerogenes*, and another was effective against antimicrobial susceptible *Staph. aureus*.

The plaque sizes revealed by double layer plaques assay were 1 – 3 mm. The previous study reported that plaque sizes ranged from 1 - 7 mm (Jurczak-Kurek *et al*. 2016). The size and appearance of plaque affected by the volume and density of agar, the concentration and the stage of the host bacterium growth, and the constancy of top agar (Cormier and Janes, 2014). The plaque size of the *vB_SauM* phage was 1 mm, 3 mm for *vB_salk1S*, *vB_Salk2M vB_salNS*, and *vB_salk3S*, 2 mm for *vB_EO26M*, 1.5 – 2 mm for *vB_EO27M* and *vB_EO114P*, and 2 - 2.5 mm for *vB_EnaS*.

Transmission electron microscopy (TEM) of the phage isolates allows morphological and particle stability assessment (Casey *et al*., 2018). The purified phages were classified into order Caudovirales. This order was divided into three families based on the tail morphology, whereas the phage with a long contractile tail was classified as *Myoviridae* family, while *Siphoviridae* family had a long non-contractile tail but Podoviridae family had a short non-contractile tail (Ackermann, 1998). In this study phages *vB_Salk2M*, *vB_EO26M*, *vB_EO27M*, and *vB_SauM* had an icosahedral head and a long contractile tail which is a characteristic to the family *Myoviridae*. While phages *vB_EnaS*, *vB_salk1S*, *vB_salk3S*, and *vB_salNS*, had an icosahedral head and a long, thin, non-contractile flexible tail which characterizes the family *Siphoviridae*. The *vB_EO114P* phage had an icosahedral head with a short non contractile tail which characterizes the Podoviridae family.

The host range is one of the most important criteria when selecting phages intended for biocontrol of antimicrobial foodborne pathogens (Duc *et al*., 2018). From the findings regarding host range, the spot test revealed that the *vB_EO27M* phage infects 4 different *E. coli* serotypes, the *vB_EO26M* phage is lysis 5 *E. coli* isolates and an *Enterobacter aerogenes* isolate, the *vB_salk1S* phage infects 10 *Salmonella* serovars and one *E. coli* isolate, the *vB_Salk2M* phage infects 9 serotypes of *Salmonella*, the *vB_SauM* phage destroys 3 isolates of *Staphylococcus*, and the *vB_EnaS* phage exterminate only one *E. coli* isolate. Whereas the vB_E O114P phage was a highly specific and infected its host bacteria only. The previous studies on *E. coli* phages showed that many of the isolated phages are specific to a single *E. coli* strain or had a narrow host range activity (Baig *et al*., 2017). The narrow host range specificity of phages mainly attributed to the use of standard isolation procedure whereas the single host strain of bacteria is used (Ross *et al*., 2016; Hamdi *et al*., 2017). The isolation of 10 lytic phages from cattle feces against Shiga toxin-producing *E. coli* isolates was reported (Bumunang *et al*., 2019). Also, phages specific for *E. coli* were isolated from poultry sewage and feces (Bhensdadia *et al*., 2014) . Another study stated the isolation of phages against MDR *Salmonella*e (Merwad and Abdel-Haliem 2018). Furthermore, 60 phages infecting *E. coli*, 10 phages infecting *Pseudomonas aeruginosa*, 4 phage infecting *Salmonella Enterica*, 3 phages infecting *Staphylococcus sciuri*, and 6 phages infecting *Enterococcus faecalis* were isolated from the urban sewage (Jurczak-Kurek *et al*., 2016). The isolation of 3 phages effective against *Staph. aureus* and one effective against *E. coli* from chicken, beef and vegetables samples as well as 17 phages isolated from feces, feed, soil and drinking water from poultry farms was documented (Petsong *et al*., 2019). A lytic phage specific for *Enterobacter aerogenes* was isolated from a hospital sewage (Zhao *et al*., 2019).

The obtained results from the spot test and double layer plaques assay for host range activity determination were different, so the double layer plaques assay is recommended in order to obtain productive infection for the determination of phage host range activity (Mirzaei and Nilsson, 2015).

Based on the results of EOP, the *vB_EO27M* phage was negative with 2 isolates and inefficient with 2 isolates. The *vB_salk1S* phage was negative with 4 isolates and inefficient with 6 isolates. The *vB_Salk2M* phage was negative with 3 isolates and inefficient with 5 isolates. The *vB_EnaS* phage was negative with one *E. coli* strain.

The *vB_EO26M* phage had plaques against 3 *E. coli* isolates and one *Enterobacter aerogenes* isolate with a low efficiency. The *vB_salNS* phage had plaques against 2 *Salmonella* serovars one with a high efficiency and the other with a low efficiency, while the *vB_salk3S* phage had plaques against 3 *Salmonella* serovars with a low efficiency. The *vB_SauM* phage had plaques against an isolate of *Staph. aureus* with a high efficiency.

In addition to biological characterization, the newly isolated phages should be assessed for their stability and viability when exposed to different external environmental conditions to confirm their biocontrol potential (Hagens and Loessner, 2010). External environmental factors such as pH and temperature may influence the stability and infectivity of the phages (Yin *et al*., 2019).

Temperature is an important factor for the phage replication process including attachment, penetration, and multiplication (Olson *et al*., 2004). In this study, *vB_EO114P*, *vB_EnaS*, *vB_salk1S*, *vB_Salk2M*, *vB_salNS*, *vB_salk3S*, *vB_SauM*, *vB_EO27M*, *vB_EO26M*, and *vB_salk3S* phages were found to be stable at up to 50ºC without any significant change in their titers. Meanwhile, 1-5 $\log_{10}$ titers reduction in *vB_salk1S*, *vB_salk3S*, *vB_EO26M*, *vB_EO27M*, *vB_EnaS*, and *vB_EO114P* were observed at 60ºC. While slight decrease in the titers of *vB_salNS*, *vB_salk1S*, and *vB_Salk2M* phages and complete reduction in the phage *vB_SauM* titer was recorded at 60ºC. The titer of *vB_EnaS* phage was sharply decreased while the titers of *vB_EO26M*, *vB_salNS*, *vB_salk3S*, and *vB_Salk2M* phages was dropped 1-2 $\log_{10}$ at 70ºC. Phages *vB_EO114P*, *vB_salk1S vB_SauM*, and *vB_EO27M* titers were diminished at 70ºC. All phages except the *vB_salNS* phage showed no growth at 80ºC suggesting that the phage is not resistant to an extremely high temperature over 70 ºC, this attributed to the effect of high temperatures on phage proteins (Ackermann *et al*., 2004).

Acidity or alkalinity of the environment is an important factor for phage survivability which results in denaturation of phage proteins and consequently loss of phage viability (Krasowska *et al*., 2015). The growth of phages *vB_EO26M*, *vB_salNS*, *vB_salk3S vB_salk1S*, *vB_EO27M*, *vB_EO114P*, and *vB_EnaS* exhibiting stable titers at pH degrees 4 – 12 was observed, while the phage *vB_Salk2M* exhibited tolerance at pH values 6 -12, indicating that these phages are active at a wide pH range. The *vB_SauM* phage exhibited tolerance at the pH value 8 and decreased 101 at pH 6 and 102 at pH 4 and it was completely inactivated at pH 2, 10, and 12. Interestingly, *vB_salNS*, *vB_salk3S*, and *vB_EO26M* phages were able to grow at a high acidity degree (pH=2) and all phages except the *vB_Salk2M* phage showed high titers at pH 4. Researchers have reported that most tailed phages are stable at pH 5.0 - 9.0 (Fan *et al*., 2017).

Regarding the stability of phage against organic solvent (chloroform and ethanol 70%) the phages showed no significant titer changes in comparison to the initial titer after exposure to chloroform. These results are accepted because the chloroform is used as antimicrobial agent and prevent the bacterial contamination of the phage solution, also the chloroform has been used in the phage stock preparation during the isolation, purification

as well as incorporated into the growth medium to enhance the phage lytic cycle (Cotton and Lockingen, 1963). Meanwhile, the incubation with 70% ethanol/1 h. resulted in the complete inactivation of *vB_EO27M*, vB_EO114, and *vB_SauM* phages while *vB_salk1S*, *vB_Salk2M*, *vB_EnaS*, and *vB_EO26M* phages showed 2, 3, 5 and 7 $\log_{10}$ lower titers, respectively, than the initial titers. Interesting, *vB_salNS* and *vB_salk3S* phages showed similar titer as the initial titer.

## CONCLUSION

The multi-drug resistant bacteria consider a public health, animal, and poultry hazard. The isolated bacteria from chickens showed different virulence patterns and multi-drug resistant profiles which indicated their pathogenicity. The obtained phages showed viability and thermostability against high temperatures, wide range of hydrogen-ion levels (acidity and alkalinity) and organic solvents. Also, some phages could infect several bacterial hosts. This study demonstrated the ability to use these promising phages of avian origin to control the prevalent pathogenic multi-drug resistant bacteria mainly *E. coli* and *Salmonella* serovars, which damage the poultry industry and affect the human health.

## CONFLICT OF INTEREST

Authors declares that they have no competing interests to disclose.

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