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Isolation and characterization of polyvalent bacteriophages infecting multi drug resistant *Salmonella* serovars isolated from broilers in Egypt

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ABSTRACT

In this study, we isolated and characterized three phages named as Salmacey1, Salmacey2 and Salmacey3, infecting multi drug resistant *Salmonella* serovars isolated from broilers in Egypt. The most prevalent *Salmonella* serovars were *S. typhimurium*, *S. enteritidis*, and *S. kentucky*. All these *Salmonella* serovars were found to be resistant to more than two of the ten antimicrobial agents tested. Only *S. kentucky* was found to be resistant to seven antimicrobial agents. Examination of these phage particles by transmission electron microscopy (TEM), demonstrated that two phages (Salmacey1, Salmacey2) were found to belong to family Siphoviridae, and Salmacey3 was assigned to the family Myoviridae. The results of host range assay revealed that these bacteriophages were polyvalent and thus capable of infecting four strains of *Salmonella* serovars and *Citrobacter freundii*. Moreover, the two phages (Salmacey1, Salmacey2) had a lytic effect on *Enterobacter cloacae* and Salmacey3 was able to infect *E. coli*. All phages could not infect *S. para* Typhi, *Staphylococus aureus* and *Bacillus cereus*. One-step growth curves of bacteriophages revealed that siphovirus phages (Salmacey1, Salmacey2) have burst size (80 and 90 pfu per infected cell with latent period 35 min and 40 min respectively), and for the myovirus Salmacey3 had a burst size 110 pfu per infected cell with latent period 60 min. Molecular analyses indicated that these phages contained double-stranded DNA genomes. The lytic activity of the phages against the most multidrug resistant serovars *S. kentucky* as host strain was evaluated. The result showed that these bacteriophages were able to completely stop the growth of *S. kentucky in vitro*. These results suggest that phages have a high potential for phage application to control *Salmonella* serovars isolated from broilers in Egypt.

1. Introduction

Salmonella are the fundamental source of many cases of foodborne disease in developing and developed countries. Poultry are considered the most important reservoir and source of different *Salmonella* serovars, which may cause foodborne illness in human (Pui et al., 2011). Contamination of poultry carcasses and parts with *Salmonella* organisms is well documented and data are available for many parts of the world (Simmons et al., 2003). The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into six subspecies, and contained > 2600 serovars, with many commonly infecting poultry and humans (Foley et al., 2008). In Egypt, some reports on *Salmonella* serovars distribution on broiler farms were documented by (Ammar et al., 2010; Abd El-Ghany et al., 2012; El-Safey, 2013).The frequency of *Salmonella* serovars recovered from the broiler flocks were *S. enteritidis*, *S. typhimurium*, *S. infantis*, *S. kentucky*. The prevalence of *S. enteritidis* and *S. typhimurium* in broiler flocks of Egypt requires drastic measures for their control, since these serovars are known for their potential to cause severe foodborne illness in humans (El-Sharkawy et al., 2017). There are many variables that need to be controlled for poultry farms to be free of *S. enteritidis*, *S. typhimurium* and other *Salmonella* serotypes along with other pathogenic bacteria. Control of *Salmonella* serovars is a challenge to public health because of their emergence/re-emergence and high mutation rate (Jassim and Limoges, 2017), with antibiotic resistance in developing and developed countries (Jassim and Limoges, 2017). Antimicrobial-resistant strains of *Salmonella* spp. are now widespread all over the world and are causing great concern not least due to the spread of multi-drug-resistant strains (Dewaal and Grooters, 2013). In developed countries it is becoming more and more accepted that a majority of resistant strains are of zoonotic origin and have acquired their resistance in an animal host before being transmitted to humans through the food chain (Mølback

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et al., 2002; Threlfall, 2002; WHO, 2004). In broiler meat the prevalence of resistant isolates of *Salmonella* spp. also showed great variation with a relatively high level of resistance to several antimicrobials reported from some countries (Kim et al., 2012).The percentage of strains resistant to 4 or more of the 11 tested antimicrobials varied between 0 and 36% among reporting countries (EFSA, 2005). In the last 70 years, mankind has greatly benefited from the effectiveness of antibiotics protection against various infectious diseases. However, the overuse or misuse of antimicrobials in medicine, animal husbandry and agriculture combined with the great ability of living systems to adjust and resist selective pressure led to the inexorable increase of pathogenic bacteria as *Salmonella* spp. resistant to most antibiotics in current use (El-Sharkawy et al., 2017; Jassim and Limoges, 2014; Shute, 2013). Multiple drug resistant bacteria constitute now a major threat to human health making of the discovery of novel antibiotics or alternative to antibiotics an urgent necessity (Medina and Pieper, 2016).Many studies and researchers have proposed bacteriophages as potential biocontrol and therapeutic agents to maintain current animal production (Jassim and Limoges, 2014; Mahony et al., 2011). Various kinds of bacteriophages infecting *Salmonella* have been isolated and proposed as alternative biocontrol agents against different *Salmonella* serovars (Akhtar et al., 2014; Bielke et al., 2007; Borie et al., 2008; Parra and Robeson, 2016).Treatment with these bacteriophages has been shown to reduce *S. enteritidis* counts in broiler chickens (Borie et al., 2008; Borie et al., 2009). Very recently, (Parra and Robeson, 2016) have isolated three bacteriophages (FC, FP, and FQ) capable of infecting *S. choleraesuis* with different potential applications. These information on *Salmonella phages* make it possible to isolate and characterize new phages against several *Salmonella* serovars. Therefore, the aim of the present study was to isolate and characterize of different bacteriophages, infecting *Salmonella* serovars isolated from broilers in Egypt and determine their potential for the prevention and control of *Salmonella* in poultry.

2. Materials and methods

2.1. Isolation and identification of Salmonella species from broiler chickens

Two hundred samples were collected from different infected broiler flocks located at three different broiler farms in Sharkia Province, Egypt. Different sections of liver and intestine contents of 300 diseased broiler chickens 6 weeks old were collected aseptically and processed for *Salmonella* detection. Collected swabs and tissue samples were immediately stored on ice and transported to laboratory for further investigation. Swab samples were inoculated in 10 mL peptone and incubated at 37 °C overnight. Each tissue samples (25 g) were inoculated in 100 mL peptone and incubated at 37 °C overnight. Following pre-enrichment in BPW, a portion (0.1 mL) of the pre-enriched culture was transferred to 10 mL Rappaport-Vassiliadis Soy Peptone (RVS) broth followed by incubation at 41.5 °C overnight (18–24 h) for selective enrichment. A loopful of inoculated broth was streaked on selective *Salmonella* Shigella (SS) agar, Xylose lysine deoxycholate (XLD) agar, MacConkey agar and Eiosin-Methylene Blue agar. Plates were then incubated at 37 °C for 24 h. Suspected colonies were collected for further biochemical identification, and serotyped in the Serology Unit, Animal Health Research Institute, Dokki, Giza, Egypt using commercial antisera (Difco, Detroit, MI, USA) according to the manufacturer's instructions. Stock cultures were stored in LB broth containing 20% glycerol at − 20 °C.

2.2. Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed using the disk diffusion susceptibility method (Bauer et al., 1966), at Bacteriology Unit, Animal Health Research Institute, Dokki, Giza, Egypt. ten antibiotics were tested: Amoxicillin/clavulanic acid AMC (20 μg), imipenem IPM (10 μg), gentamicin GEN (10 μg), ciprofloxacin CIP (5 μg), trimethoprim/sulfamethoxazole SXT (1.25 μg), doxycycline (15 μg), chloramphenicol (30 μg), Ceftriaxone (30 μg), Rifamycin (5 μg), and streptomycin (10 μg). The test was performed by applying a bacterial inoculum of approximately 2×10^8 cfu/mL to the surface of Mueller-Hinton agar plate. Antibiotic disks are placed on the inoculated agar surface. Plates are incubated for 16–24 h at 37 °C. The zones of growth inhibition around each of the antibiotic disks are measured to the nearest millimeter. The zone diameters of each drug are interpreted using the criteria published by European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2015).

2.3. Isolation of bacteriophages infecting Salmonella species

Bacteriophages were isolated from different sewage water samples obtained from 10th of Ramadan city, Egypt by the enrichment technique (Adams, 1959). For this, the sewage sample was clarified by centrifugation at 6000 r.p.m. for 20 min and filtered through a 0.45 μm membrane-filter. From these filtrate 50 mL were added to an equal volume of LB broth inoculated by 1 mL of young culture of different isolated pathogenic bacteria (2×10^8 cfu/mL) and incubated at shaker incubator 120 r.p.m. at 37 °C for 24 h. The culture was centrifuged at 6000 r.p.m. for 10 min. The supernatant were filtered through a 0.45 μm membrane filter and was used as a source of phage to be detected on the propagative strain. Bacterial lawns of different *Salmonella* serovars (*S. typhimurium*, *S. enteritidis* and *S. kentucky*) were propagated on LB plates by double agar overlay technique as described by (Adams, 1959). Briefly, 200 μL of a mid-exponential phase of each bacterial culture (optical density at 600 of 0.4) was added to 4 mL semi solid LB agar and poured over solid nutrient agar plates. After drying, 10 μL droplets of the phage source previously prepared were spotted onto the lawns and left to dry. The plates were incubated at 37 °C overnight and checked for presence of lysed zones.

2.4. Bacteriophages purification and propagation

Bacteriophages were propagated and purified from single-plaque isolates according to (Adams, 1959; Abdel-Haliem and Askora, 2013). The isolated phages were purified by three successive single-plaque isolation with sterile pasture pipette until homogenous plaques were obtained. Briefly, a single plaque was picked and put into 5.0 mL nutrient broth containing 100 μL of bacterial host and then incubated at 37 °C under shaking condition with 1200 r.p.m. After incubation, the phage-host mixture was centrifuged at 6.000 r.p.m. 10 min and supernatants were filtered through sterilized Millipore filter (0.45 μm pore size) to remove any bacterial contamination. Purified phages were stored at 4 °C.

2.5. Determination of host ranges and cross infectivity of the isolated phages

The isolated phages were investigated for host range specificity and lysis efficiency (no lysis, clear plaque, and turbid plaque). Bacterial lawns of all different bacterial species were propagated on LB agar plates and 10 μL droplets of phages (1×10^7 PFU/mL) were put on the lawns. The plates were incubated 24 h and checked for presence of plaques. The most efficient phage was selected for further studies. The selection criteria included the lysis profiles, plaque clarity and size. To determine the ability of the isolated phages to infect different species other than *E. coli*, *Salmonella*, and 20 μL of each phage were spotted over a lawn of different bacterial species among the Enterobacteriaceae (Table 3). After incubation at 37 °C for 24 h, the development of plaques in the plates was examined.

2.6. Morphological characteristics (electron microscopy)

Three purified bacteriophage particles were stained with Na-