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Isolation and characterization of lytic bacteriophages against *Escherichia coli* serogroups O1, O2, and O78

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Summary

The goal of this study was to isolate and characterize a complete set of phages that are active against *Escherichia coli*serogroups O1, O2, and O78, the main causative agents of avian colibacillosis. A mixture of *E.coli* (O1:K1), (O2:K1), and (O78:K80) used as host to isolate phages from wastewater and fecal samples from poultry processing plants. Eleven phages were isolated, only two of them EC-NJ4 and EC-NJ7 were selected for further characterization. EC-NJ4 and EC-NJ7 had icosaheadral heads, necks and contractile tails, with tail fibers and therefore belonged to *Myoviridae*, with genome sizes of 67.06 – 68.04 kb and they lysed 100% of serotype O1, O2, and O78. The two phages were resistant to pH 5-9, and phage EC-NJ7 was slightly more resistant to acid and alkali environments. It was concluded that Phage EC-NJ4 and EC-NJ7 are highly active against O1, O2, and O78 colibacillosis strains and it might be suitable candidate for phage therapy.

Running title: APEC, Phages, E. coli O1, O2, O78

عزل وتشخيص العاثيات المحلله والمضاده لعترات بكتريا الاشريكيا القولونيه O1, O2, O78

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الخلاصة

الهدف من هذة الدراسه هو عزل وتشخيص مجموعه متكامله من العاثيات النشطه والمضاده لعترات بكتريا الاشريكيا القولونيه المسبب الرئيسي لمرض عصويات القولون في الدواجن. تم استخدام خليط من هذة البكتريا كمضيف لعزل العاثيات من نماذج ماخوذه من مياة المجاري وخروج الدواجن ضمن الوحدات الخاصه بتربيتها.تم اختيار عاثيتين فقط من مجموع احد عشر عاثيه عزلت خلال هذه الدراسه للتعرف عليها اكثر وسميت (EC-NJ4 and EC-NJ) تتميز هذه العاثيات بانها تملك راس اهليليجي, عنق, وعلى ذيل متقلص مع الياف ذيليه وعليه تصنف ضمن عائلة العاثيات المعويه, وتتروح احجام الحمض النووي بين 67.06-68.04 كيلوبيز. هذة العاثيات قادرة على قتل وتحليل العتر الجرثوميه بنسبه مئويه قدرها 100%. تتصف هذة العاثيات بمقاومتها للاس الهايدروجيني بين 5-9 وان العاثية O1, O2, O78اكثر تحملا للاجواء الحامضيه والقاعديه EC-NJ7ومن خلال هذة النتائج تم الاستنتاج ان هاتين العاثيتين نشطتين جدا وقادره على قتل وتحليل العترات الجرثوميه المسببه لمرض العصيات القولونيه في الدواجن وقد تكون المرشح الفعال للعلاج بالعاثيات.

Introduction

Avian pathogenic *Escherichia coli* (APEC) are a group of bacteria cause avian colibacillosis in chickens, turkeys, and other avian species. This infectious disease is often causes severe mortality and subsequently results in economic losses to the poultry industry (1, 2). The disease is characterized by septicemia in its acute form resulting in death;and alsoassociated with a complete set of syndromes including airsaculties, pericarditis, perihepatitis, and Swollen Head Syndrome(3, 4).Many *E. coli* isolates are commonly associated with colibacillosis in poultry, of these, serogroups O1, O2 and O78 have been recognized as the predominant sources involved in this disease (5, 6).

A high rate of antibiotic resistance was observed while testing these serogroups, which probably originates from the extensive use of antibiotics in the poultry industry (7). Acquisition of R plasmids is an addition reason to make these organism resistances for

multiple antibiotics as well (8, 9). Numerous concerns of using antibiotics in poultry have been raised in due to further selection of drug resistant strains (10, 11). There are also issues involved human health and potential transfer of *E. coli* from animal via the food (11, 12) which attracted a considerable attention to researchers in developing alternatives for controlling and treatment of coibacillosis in animals.

One promising alternative to antibiotics is the use of lytic bacteriophage against *E. coli* serogroups O1, O2, and O78, a well-established approach that phages for these serogroups are able to be isolated and used as a phage therapy to attack bacterial cells. Bacteriophages are a class of viruses that live and replicate in bacteria (13), and have the ability toattack a single species or subset of a species of bacterium making them potential antibacterial agents.
Previous studies on phage isolation and phage therapy have been reported in animals against *E. coli* infection (14, 15, 16, 17). Huff and colleagues have conducted several studies use of bacteriophage to prevent and treat colibacillosis in broiler chickens (18, 19, 20, 21), however, the research work was restricted only to the isolation of the phages against serotype O2. Our ultimate goals of this study were therefore to isolate a more complete set of phages that are active against serotypes O1, O2, and O78 and to characterize these species with respect to morphology, genome size and restriction endonuclease digestion, bacterial host range, activity assay, and pulse field gel electrophoresis.

Materials and Methods

Bacterial strains, Culture media and chemicals

Avian pathogenic *Escherichia coli* (APEC) strains (O1:K1), (O2:K1) and (O78:K80) were isolated from colisepticemic chickens (Maysan Veterinary Hospital).MacConkeyagar. Brain Heart Infusion (BHI) Broth, BHI agar, and BHI top agar (soft agar) were prepared as described by Sambrook et al. (22). Bacteriophage broth was used to isolate the phage (23). The ingredients were dissolved in 1 litre of distilled water containing peptone (100g), beef extract (30g, Difco), yeast extract (50 g, Fisher), NaCl (25 g, Fisher), and potassiumdihydrogen phosphate (80 g). The other reagents were also used including $MgSO_4$, agarose, RNaseI ,DNase I, proteinase K (Qiagen, Canada), ethanol (Commercial Alcohols), and ethidium bromide.

Bacteriophage Isolation and purification

Bacteriophages were isolated from wastewater and fecal samples collected from poultry processing plants in Maysancity during the period of July and September 2011 according to the procedure described by Jamalludeen et al., (24). BHI broth was inoculated with a mixture of O1, O2, and O78 *E. coli* strains and incubated for 24 h at 37°C. A volume of 200 ml wastewater or 200 g of fecal samples were homogenized and were aseptically poured into a sterile 1-L flask. Twenty milliliters of bacteriophage broth, 20 ml of BHIB with $MgSO_4$, and 20 ml of a suspension of *E. coli* strains in broth culture (OD_{600}=1.4) were aseptically added to the flask, and the mixture was subsequently incubated at 37°C for 24 h. After incubation, the total mixture was centrifuged at 4,000 × g for 15 min and the supernatant was collected into a clean flask, then filtered through a sterile 0.45 μm membrane filter (Fisher).

The filtrate was sequentially diluted (10^{-1} to 10^{-9}) in SM buffer, which is a buffer used for storage and dilution of bacteriophage stocks (5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 ml/L of 1 M Tris pH 7.5, 5 ml/L gelatin in distilled water). One hundred microliters of diluted filtrate were added to 100 μl of the *E. coli* strains (OD_{600}=1.4) in a test tube and incubated at 37°C for 20 minutes. Then, 3 ml of top agar (7.0 g/l) prepared according to Sambrook et al., (22) was added and the tube contents were mixed and poured onto the surface of an BHI agar plate and allowed to solidify. The plates were incubated overnight at 37°C and examined for the presence of plaques. A previously described protocol (22, 24) was used to isolate a single plaque. Only two phages, named EC-NJ4 and EC-NJ7, out of a total of 11phages isolated

were considered for further characterization. Phages EC-NJ4 and EC-NJ7 were propagated on *E. coli* (O2:K1) and the titre of each phage was determined by plaquing 10-fold dilutions by the soft agar overlay method, this procedure was repeated three times to obtain purified phages. The phage preparations were stored at 4°C. The phage suspension was purified using CsCl gradient according to the protocol of Sambrook et al. (22).

Electron Microscopy

Phages EC-NJ4 and EC- NJ7 were examined by electron microscopy of negatively stained preparations. A drop of pure phage preparations suspended in 0.5 ml sterile 0.1 M HEPES buffer (Boehringer Mannheim) was applied to the surface of a formvar-coated grid (200 mesh copper grids). The samples were negatively stained with 2% uranyl acetate, and then examined in a LEO 912AB energy filtered transmission electron microscope operated at 100 kV (Guelph Reginal STEM Facility, University of Guelph, ON, Canada). The phages (EC-NJ4 and EC-NJ7) were classified according to the guidelines of International Committee on Taxonomy of Viruses (25) based on their morphological features.

Extraction of phage DNA

Phage DNA was extracted using the Lambda Maxi Kit (Qiagen) according to the manufacturer's instructions. Briefly, the bacteriophage pellets were resuspended with 10 ml buffer P1 and then 10 ml of buffer P2 were added to lyse the pellet. The mixture appeared viscous until 10 ml of chilled buffer P3 were added and incubated for 20 min on ice. Then, the mixture was centrifuged at 20,000 x g for 30 min at 4°C and the supernatant wascollected. A 500 Qiagen-tip was equilibrated with 10 ml buffer QBT, and the column was allowed to empty by gravity flow. Then, the supernatant from the previous step was applied to the tip and allowed to enter by gravity flow as well. The Qiagen-tip was washed two times with 30 ml of buffer QC provided with kits and then the DNA eluted with 15 ml of buffer QF. The eluted DNA was precipitated with 10.5 ml of isopropanol and centrifuged immediately. DNA pellets from this step were washed with 5 ml of room-temperature 70% ethanol and centrifuged at 15, 000 x g for 10 min. The supernatant was decanted and the pellets were air-dried and redissolved with a suitable volume of buffer TE, pH 8.0 and kept in deep freeze until used (Qiagen).

Host range determination

The isolates (O1:K1), (O2:K1), (O78:K80) and 72 strains that comprise the *E. coli* reference (ECOR) collection (26) were used to test the spectrum of virulence `of phage EC-NJ4 and EC-NJ7 according to the Spot test procedure described by Sambrook et al., (22). A log phase culture of (O2:K1) bacteria [OD_{600}=1.4] (5 μL) was spread over each square on a BHI agar plate, which was divided to four squares by marking the surface. The plates were allowed to dry and phage suspension (10^9pfu/ml; 10 μL) was dropped in the center of each square. Following incubation at 37°C overnight, these plates were examined for lysis. A clear zone in the bacterial lawn was recorded as complete lysis.

Acidity and alkalinity resistance

The method of Jamalludeen et al (24) was used to evaluate the activity of phages to survive at different pH levels. Briefly, phage suspension was exposed to a certain pH value adjusted from 1 to 11 using 0.1 M HCl or NaOH over 16 h of incubation at 37°C, and then checked for survival.

Pulsed-field gel electrophoresis

Genome sizes of EC-NJ4 and EC-NJ7were determined by using pulsed-field gel electrophoresis (PFGE). Phages embedded in 1.0% Seakem Gold agarose (Mandel Scientific, Guelph. ON) were electrophoresed in 0.5× TBE buffer at 14°C for 18 h, using a Chef DR-III Mapper electrophoresis system (Bio-Rad, Mississauga, ON), with pulse times of 2.2-54.2 s pulses, at 6 V/cm. The bands were visualized under UV transillumination after staining with

ethidium bromide. PFGE results were analyzed using BioNumerics software (Applied Maths, Inc. Austin, TX).

Restriction enzyme digestion patterns

Phage nucleic acids (2μg) were treated with the restriction enzymes *Acc*I and EcoRI (New England Biolab,ON, Canada) following standard procedures (22). DNAs (3 μL volumes) were digested for 8 h at 37°C and the cleaved nucleic acids were subjected to electrophoresis in a 1% (w/v) agarose gel and stained with ethidium bromide.

Results

1. Isolation of phages

Eleven phages were isolated using a mixture of O1, O2, and O78 avian *E. coli* strains as hosts. The phages were named (EC-NJ1 to EC-NJ11). Phages EC-NJ4 and EC-NJ7 were selected for further characterization according to their morphological features (Fig. 1).

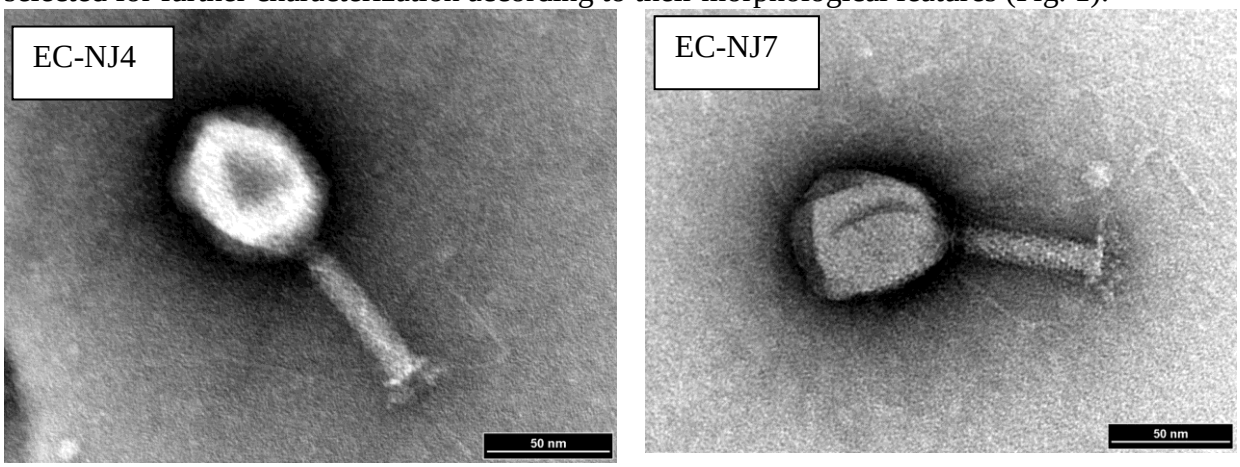

Figure 1.Electron microscope appearance of phages EC-NJ4 and EC-NJ7. The phages have a neck and a contractile tail and icosahedral head. Bar = 50 nm

2**. Morphology of the phages EC-NJ4 and EC-NJ7 and genome sizes:**

Phages were morphologically classified into the family *Myoviridae*, The electron microscopy preparations showed that the phages possessed icosahedral heads, necks and contractile tails, with tail fibers (Fig. 1). The head dimensions for EC-NJ4 and EC-NJ7 were 71nm x 57nm, 70nm x 56nm and tail dimensions were 72nm x 14nm, 57nm x 13nm, respectively (Table 1). The data were acquired based on unbiased experiments, six images were measured and the mean values were recorded. Based on PFGE the entire genome of these two phages (Fig. 2), phage EC-NJ4 had a genome size of 67.06 kb and phage EC-NJ7 had a size of 68.04 kb.

Table 1. Estimated dimensions and genome sizes of bacteriophages EC-NJ4 and EC-NJ7

Phage	Genome size (kb)	Head dimensions (nm)		Tail dimensions (nm)	
		Length	Width	Length	Width
EC-NJ4	67.06	71	57	72	14
EC-NJ7	68.04	70	56	57	13

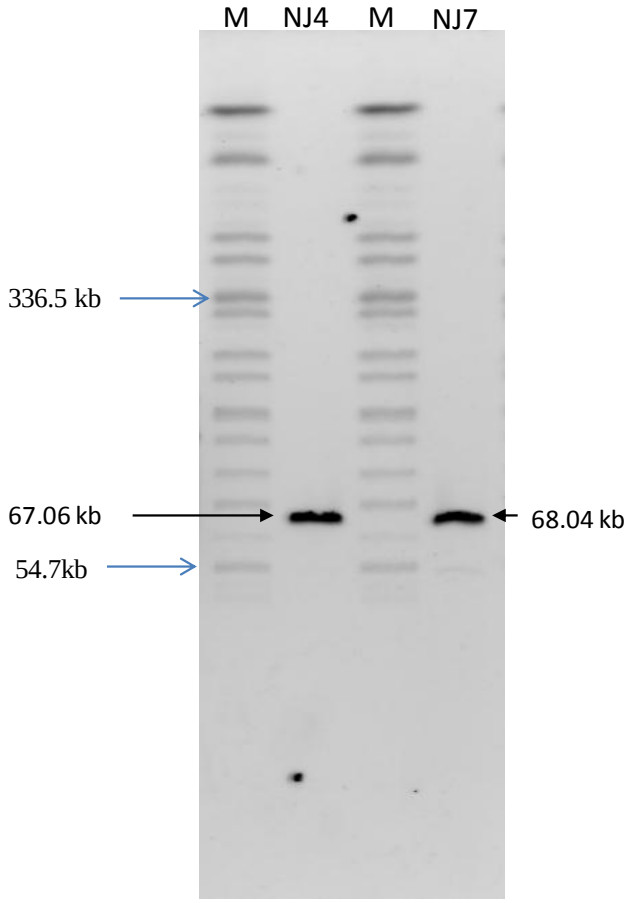

Figure 2.Pulsed-field gel electrophotogram of phages EC-NJ4 and EC-NJ7 genome. M = Marker: *Xba*1 digested *Salmonella*Braenderup, 1% Seakem Gold agarose (Mandel Scientific, Guelph. Ontario,Canada); NJ4 = DNA from phage EC-NJ4; NJ7 = DNA from phage EC-NJ7. EC-NJ4 = 67.06 kb and EC-NJ7 = 68.04 kb.

3. Host range

Phage EC-NJ4 and EC-NJ7 lysed 100% of O1, O2, O78 and 35.6%, 42.3% of 72 strains of ECOR collection, respectively. The results are summarized in Table 2.

Table 2. Summary of lytic activity of two phages against colibacillosis strain and ECOR

E. coli strain	Phage activity (% of strains lysed)	
	EC-NJ4	EC-NJ7
(O1:K1)	100	100
(O78:K80)	100	100
(O2:K1)	100	100
ECOR collection (72)	35.6	42.3

4. Resistance to acidity and alkalinity

The two phages were resistant to pH 5-9. In contrast, Phage EC-NJ7 was slightly more resistant to acidic and alkali environments than phage EC-NJ4 (Table 3).

Table 3. Survival of phages EC-NJ4 and EC-NJ7 following exposure to pH 1-11

pH	Titre of surviving, viable phages (pfu/mL)	
	EC-NJ4	EC-NJ7
1 and 2	ND[a]	ND
3	6.5×10^6	2.4×10^8
4	1.2×10^7	5.6×10^8
5-9	$\geq 10^8$	$\geq 10^8$
10	5.1×10^6	2.3×10^8
11	4.3×10^6	2×10^7
control	$\geq 10^8$	$\geq 10^8$

a= Not detected.

5. Restriction enzyme digestion patterns

Phages EC-NJ4 and EC-NJ7 appear to have similar profiles of the nucleic acid fragments generated by digestion of their DNAs with *Acc*I and *Eco*RI. The patterns for those enzyme cleaved products are shown in Fig. 3.

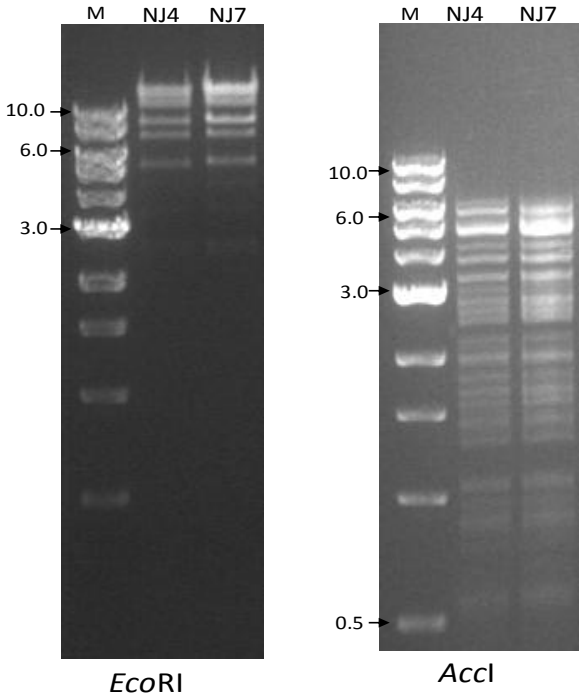

Figure 3.Electrophoresis on 1% agarose of *Acc*I and *Eco*RI restriction enzymes digest of phages EC-NJ4 and EC-NJ7 genome. M = marker; NJ4 = DNA from phage EC-NJ4; NJ7 = DNA from phage EC-NJ7.

Discussion

This study was conducted to isolate and characterize phages that were active against avian colibacillosis strains (O1, O2, O78), as an alternative to antibiotics and as a response for the increasing concerns of the emergence of antibiotic resistance on farms (27). We isolated phages EC-NJ4 and EC-NJ7 active among the predominant strains that cause collibacilosis in poultry from wastewater and poultry processing plants, which are the main sources for these phages. Previous studies by Huff et al. (18) also identified phages, designated SPRO2 and DAF6, which were active only against *E. coli* serotype O2 in broiler chickens. Phages isolated from our study target all the dominant strains from Iraq providencesand are broadly active against *E. coli* serotype O1, O2, and O78.

Phages EC-NJ4 and EC-NJ7 are members of the family *Myoviridae* based on their morphological features and their contractile tails (Fig. 1). *Myoviridae* are characterized by those having icosahedral or elongated head and contractile tails that are more or less rigid,

long and relatively thick (25). DNA structure, protein composition, base sequence similarity, host range and infection characteristics also define the tailed virus species (28, 29).
The phages were tested for their host ranges on the O1, O2, and O78, the predominant avian colibacillosis strains, as well as their host range among the 72 *E. coli* of the ECOR collection which is a widely used set of reference strains isolated between 1973 and 1983 from different hosts and geographical locations that represents the range of genotypic variation in *E. coli* (26).

Both phages were highly susceptible to acidity at pH 1 and 2. EC-NJ4 seems to be more susceptible to pH 3 than EC-NJ7 (Table 3). Phages are often quite sensitive to protein denaturation in an acidic environment (30). On the other hand, both phages were stable and survive at close to neutral pH values between 5 and 9. The results are consistent with the previous observations by Ackermann and DuBow, (31) and Jamalludeen et al., (24) that most phages are able to survive well over a wide range of pH 5-9 at physiological conditions which maintains the native virion structure and stability.

Both phages appear to be closely related based on similar patterns of the DNA fragments obtained from *Acc*I and *Eco*RI restriction enzymes (Fig. 3), which established a close genetic relationship each other. Similar observations have been reported previously (24). Much more information can be obtained by sequencing the complete genome of these two phages, whereupon exact differences may be highlighted.

In conclusion, Phage EC-NJ4 and EC-NJ7 are highly active against O1, O2, and O78 colibacillosis strains. There are slight differences in morphology and in their tolerance of acidic environments, but other characteristics indicate that they are closely related. Those two phages are good candidate for phage therapy and it needs further studies to evaluate their activity at the field trails.

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References

1. Dho-Moulin, M., and Fairbrother, J. M. (1999). Avian Pathogenic *Escherichia coli* (APEC). Vet. Res. 30: 299-316.
2. Gibbs, P.S., Petermann, S.R., and Wooley, R. E. (2004). Comparison of several challenge models for studies in avian colibacillosis. Avian Dis. 48: 751-758.
3. Cheville, N. F., and Arp, L. H. (1978). Comparative pathologic findings of *Escherichia coli* infection in birds. J. Am. Vet. Med. Assoc. 173: 584-587.
4. Rodriguez-Siek, K. E., Giddings, C. W., Doetkott, C., Johnson, T. J., and Nolan, L. K. (2005). Characterizing the APEC pathotype. Vet. Res. 36: 241-256.
5. Whittam, T. S., and Wilson, R. A. (1988). Genetic relationships among pathogenic strains of avian *Escherichia coli*. Infect. Immun. 56: 2458-2466.
6. McPeake, S.J., Smyth, J. A., and Ball, H. J. (2005). Characterization of avian pathogenic *Escherichia coli* (APEC) associated with colisepticaemia compared to faecal isolates from healthy birds. Vet. Microbiol. 110: 245-253.
7. Allan, B. J., van den Hurk, J. V., and Potter, A. A. (1993). Characterization of *Escherichia coli* isolated from cases of avaincolibacillosis. Can. J. Vet. Res. 57: 146-151.
8. Johnson, T. J., Siek, K. E., Johnson, S. J., Nolan, L. K. (2005). DNA sequence and comparative genomics of pAPEC-O2-R, an avian pathogenic *Escherichia coli* transmissible R plasmid. Antimicrob Agents Chemother. 49:4681-4688.
9. Skyberg, J. A., T. J. Johnson, J. R. Johnson, C. Clabots, C. M.Logue, and L. K. Nolan. (2006).Acquisition of avian pathogenic*Escherichia coli* plasmids by a commensal *E. coli* isolate enhancesits abilities to kill chicken embryos, grow in human urine, andcolonize the murine kidney. Infect. Immun. 74:4681–4688.

10. Franklin, A. (1999). Current status of antibiotic resistance in animal production. Acta. Vet. Scand. Suppl. 92: 23-28.
11. Angulo, F. J., Nunnery, J. A., and Bair, H. D.(2004). Antimicrobial resistance in zoonotic enteric pathogens. Rev. Sci. Tech. 23: 485-496.
12. Johnson, J. R., Delavari, P., O'Bryan, T. T., Smith, K. E., and Tatini, S. (2005). Contamination of retail foods, particularly turkey, from community markets (Minnesota, 1999-2000) with antimicrobial-resistant and extraintestinal pathogenic *Escherichia coli* as assessed by multi-locus sequence analysis. Microbes Infect. 8: 1702-1713.
13. Ackermann, H-W. (2000). Bacteriophages. Encyclopedia of Microbiology. 2nd ed. Vol 1 Academic Press. pp. 398-411.
14. Smith, H. W., and Huggins M. B. (1982). Successful treatment of experimental *Escherichia coli* infections in mice using phage: its general superiority over antibiotics. J. Gen. Microbiol. 128: 307-318.
15. Smith, H. W., and Huggins M. B. (1983). Effectiveness of phages in treating experimental *Escherichia coli*diarrhoea in calves, piglets and lambs. J. Gen. Microbiol. 129: 2659-2675.
16. Smith, H. W., Huggins, M. B., and Shaw K. M. (1987). The control of experimental *Escherichia coli* diarrhea in calves by means of bacteriophages. J. Gen. Microbiol. 133: 1111-1126.
17. Jamalludeen, N., Johnson, R. P., Shewen , P. E. and Gyles, C. L. (2009). Evaluation of bacteriophages for prevention and treatment of diarrhea due to experimental enterotoxigenic*Escherichia coli* O149 infection of pigs.Vet. Microbiol. 136:135-141.
18. Huff, W. E., Huff, G. R., Rath, N. C., Balog, J. M., Xie, H., Moore,Jr, P. A., and Donoghue, A. M. (2002). Prevention of *Escherichia coli* respiratory infection in broiler chickens with bacteriophage (SPR02). Poult. Sci. 81: 437-441.
19. Huff, W. E., Huff, G. R., Rath, N. C., Balog, J. M., and Donoghue, A. M. (2003). Bacteriophage treatment of a severe *Escherichia coli* respiratory infection in broiler chickens. Avian Dis. 47: 1399-1405.
20. Huff, W. E., Huff, G. R., Rath, N. C., Balog, J. M., and Donoghue, A. M. (2004). Therapeutic efficacy of bacteriophage and Baytril (enrofloxacin) individually and in combination to treat colibacillosis in broilers. Poult. Sci. 83: 1944-1947.
21. Huff, W. E., Huff, G. R., Rath, N. C., Balog, J. M., and Donoghue, A. M. (2005). Alternatives to antibiotics: utilization of bacteriophage to treat colibacillosis and prevent foodborne pathogens. Poult. Sci. 84: 655-659.
22. Sambrook J., Fritsch E.F., Maniatis T., (1989). Molecular Cloning: A Laboratory Manual, 2nd Ed. Cold Spring Harbour Press, Cold Spring Harbour, NY,
23. Cappuccino, J. G., and Sherman, N. (1992). Microbiology a laboratory manual. 3rded. The Benjamin/Cummings Publishing Company, Inc. PP. 211-225.
24. Jamalludeen, N., Johnson, R. P., Friendship, R., Kropinski, A. M., Lingohr, E. J., and Gyles, C. L. (2007). Isolation and characterization of nine bacteriophages that lyse O149 enterotoxigenic*Escherichia coli*. Vet. Microbiol. 124: 47-57.
25. International Committee on Taxonomy of Viruses (ICTV). (1995). Virus taxonomy, classification and nomenclature of virus. Sixth Report of the International Committee on Taxonomy of Viruses, Springer-Verlag/Wien, Austria. pp. 49-54.
26. Ochman, H., Selander, R. K. (1984). Standard reference strains of *Escherichia coli* from natural populations. J. Bacteriol. 157: 690-693.
27. Kuehn, B. M. (2007). Antibiotic-Resistant "Superbugs" may be transmitted from animals to humans. JAMA. 298: 2125-2126.
28. Ackermann, H-W., DuBow, M. S., Jarvis, A. W., Jones, L. A., Krylov, V. N., Maniloff, J., Rocourt, J., Safferman, R. S., Schneider, J., Seldin, L., Sozzi, T., Stewart, P. R., Werquin, M., Wunsche, L. (1992). The species concept and its application to tailed phages. Arch. Virol. 124: 69-82.
29. Maniloff, J., Ackermann, H-W. (1998). Taxonomy of bacterial viruses: establishment of tailed virus genera and the order *Caudovirales*. Arch. Virol. 143: 2051-2063.
30. Hazem, A. (2002). Effects of temperatures, pH-values, ultra-violet light, ethanol and chloroform on the growth of isolated thermophilic*Bacillus* phages. Microbiologica 25: 469-474.
31. Ackermann, H-W., and DuBow, M. S. (1987). Viruses of Prokaryotes. Vol. 1. CRC Press, Inc., Boca Raton, Fla., pp. 49-85