ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

International Journal of Poultry Science 3 (8): 538-542, 2004

Application of Coliphage Lysate: A Preliminary Trial to Treat an Experimental *Escherichia coli* Infection in Broiler Chicken

M. Sajjad, S.U. Rahman, I. Hussain and M.H. Rasool
Department of Veterinary Microbiology, University of Agriculture, Faisalabad 38040, Pakistan
E-mail: drsajjad@myway.com

Abstract: Pathogenic *E. coli* was isolated from the birds suffering from colibacillosis showing characteristic lesions. Two types of coliphages T_1 and T_4 were isolated from the sewage water collected from different areas of Faisalabad, Pakistan. These phages were separately tested for *in vitro* lysis of their host cell on tryptone agar plates, with equal (1:1)) concentration (10^7 CFU and 10^7 PFU) of *E. coli* and phages, which resulted in complete absence of colonies of bacteria after 10 minutes. When phage concentration was reduced (1:½ and 1:¼), colonies continued to appear even after 10 minutes. Later these phages were mixed and evaluated through *in vivo* trials in broiler chickens of two weeks age. A total of 100 broilers were divided into four groups (A, B, C and D) having 25 birds in each group. First three groups were administered with equal (1:1) concentration of phages as well as *E. coli* (10^7/ml) through oral, intraperitoneal and intramuscular routes. The control group (D) was further subdivided into two groups of D_1 and D_2, both of which were deprived of phages and *E. coli* respectively. The 13 birds of group D_2 that were given only the phages did show neither the signs of disease nor mortality. The results indicated a good protection level using coliphages when compared with the control group. It was concluded that phages may be used effectively as good alternatives of those antibiotics to which bacteria have become resistant.

Key words: Coliphages, *E. coli*, broiler chicken, phage therapy

Introduction

A century ago, Hanbin (1896) reported that water of Ganges and Jamna rivers in India possessed marked antibacterial action, which could pass through a very fine porcelain filter. This activity was lost after boiling. He studied its effects on *Vibrio cholera* and suggested that what so ever the antibacterial agent was, it kept cholera epidemic from being spread through ingestion of water from these rivers. Now this was a magical scenario that invited a great many scientists to come forward and ponder over the situation. As a consequence, a British bacteriologist, Twort (1915) appeared as first person to recognize these agent as viruses which infect bacteria, followed by Healle (1917) who virtually gave them the name, bacteriophages the devourers of bacteria.

Phages which are about one fortieth the size of most bacteria are perhaps the simplest most abundant organisms on earth thriving wherever bacteria grow in raw sewage, in our bodies, in spring and oceans and nearly everywhere else. On account of their cosmopolitan distribution, phages have had a profound positive impact on the reduction of microbial pollution from the environment in that they don't let bacteria grow in heaps, so they are part and parcel of natural check and balance. Total bacterial count from the sewage ranges from 10^6 to 10^7 per ml of which about 10^5 per ml are *E. coli* (Hilton and Stotzky, 1973). Population density of their host cells is the major component, which determines the density and distribution of phages (Ogunseitan *et al*., 1990; Proctor and Fuhrman, 1990). In sewage, enumeration by electron microscopy revealed 10^8 to 10^{10} phages per ml (Ewert and Oaynter, 1980) compared to 10^8 phages per ml in fresh water and open ocean (Bergh and Borsheim, 1989). It is quite evident that in the absence of coliphages, the coliforms are likely to create great pandemic problem (Bell, 1979).

Bacteria undergo many drastic changes to survive the periods of starvation, which increases their resistance to a variety of environmental insults (Kolter, 1992). All over the world, bacterial resistance to antibiotics is becoming a grave medical problem. Now a days, phage therapy is being taken as possible alternative for treating bacterial infections i.e. the harnessing of specific kind of viruses that attack only bacteria (Levine and Bull, 1996; Lederberg, 1996; Radetsky, 1996; Barrow and Soothil, 1997). First known report of phage therapy came from Bruynoge and Maisin (1921), who used phages to treat staphylococcal skin infection. Since then, phage therapy has been tried extensively and many successes have been reported for a variety of diseases including dysentery, typhoid, paratyphoid fever, cholera, pyogenic and urinary tract infections. Phages were poured directly into lesions, given orally, applied through aerosol or enemas. They were also given as injections through intradermal, intravascular, intramuscular, intra duodenal, intraperitoneal routes and even into lungs, carotid artery and pericardium (Kutter, 1997).

The present study will provide first ever effort in Pakistan focusing coliphages in the treatment of coliform related diseases in poultry. The main objective of this study was

Table 1: Biochemical characterization of pathogenic local isolate of *E. coli* from broiler birds

Biochemical test	Results
Indole production test	+
Methyl red test	+
Voges proskauer test	-
Citrate utilization test	-
Catalase test	+
Nitrate reduction test	+
Hydrogen sulphide test	-
Glucose fermentation test	+ (Acid with Gas)
Fructose fermentation test	+ (Acid with Gas)
Lactose fermentation test	+ (Acid with Gas)
Maltose fermentation test	+ (Acid with Gas)
Sucrose fermentation test	+ (Acid with Gas)
Manitol fermentation test	+ (Acid with Gas)

to investigate the strength of lytic action of bacteriophages *in vivo*. Such efforts will also be able to step forward the concept of nature farming in the country.

Materials and Methods

Isolation and identification of host organism: *Escherichia coli* was isolated from diseased cases of poultry suffering from colibocillosis exhibiting the typical postmortem lesions of perihepatitis, pericarditis, enteritis and air-sacculitis. Liver samples were directly streaked on MaConkey's agar, Eosin methylene blue agar and Blood agar plates. Organism was identified by cultural and colony characteristics and through Gram's staining (Harrigen and McCance, 1976). For further identification routine biochemical tests were employed as described by Cruickshank *et al*. (1975); Buxton and Frazer (1977). The organism was further confirmed through sugar fermentation tests using glucose, maltose, fructose, lactose, manitol and sucrose. Pathogenicity of isolated organism was checked on Congo red medium *in vitro* and through ileal loop ligation test *in vivo* (Altwegg and Bockemubi, 1998). Finally, the total bacterial count was determined according to the Breed's smear method described by Awan and Rahman (2002).

Isolation, purification and enumeration of coliphages: Coliphages were isolated from sewage water collected from different areas of Faisalabad, Pakistan. These samples were subjected to phage-assay as described by Caccupccino and Sherman (1999). Tryptone agar in the form of hard and soft agar layers was used for the isolation. Phages were identified on the basis of plaque morphology, purified and plaque forming unit (PFU) contents per ml were calculated. They were processed for long term storage by adding sterile glycerol to the broth lysate to make a 50% v/v solution and kept under refrigeration.

***In vitro* assessment of phage activity:** *In vitro* test was performed on tryptone agar plates by keeping the concentration of *E. coli* constant (10^7/ml) each time and by decreasing the phage concentration to its half twice i.e. *E. coli* to phage ratio was set as 1:1, 1: ½ and 1: ¼. Then the effect of different adsorption times i.e. five, ten and fifteen minutes was studied and results were recorded.

***In vivo* assessment of phage activity:** The mixed culture of coliphage (LP) and coliphage (SP) lysate was used for *in vivo* trials in 14 days old broiler birds. A total of 100 broilers were divided into four groups i.e. A, B, C and D having 25 birds in each group. Group A received an equal dose of phage and *E. coli* (10^7/ml) for three consecutive days through drinking water. Group B was given *E. coli* as intraperitoneal injection for one day and phage treatment through drinking water for three days. Group C was injected with *E. coli* intramuscularly in right gastrocnemius muscle and phages in the left gastrocnemius muscle. The fourth group was further subdivided into two groups i.e. D_1 and D_2. Group D_1 was administered with only *E. coli* and D_2 with phages only for three days. The birds were observed for the appearance of clinical signs and morbidity and mortality was recorded.

Results and Discussion

Isolation of *E. coli*: *Escherichia coli* was taken as host for phages. *E. coli* was isolated from diseased cases of poultry suffering from colibacillosis. The organism formed pink colour colonies on MacConkey's agar and greenish black colonies with metallic sheen on Eosin methylene blue agar. On blood agar organism produced circular, smooth and colour less colonies. These colonies exhibited distinct colour less area all around, indicating hemolysis by the organism. The gram stained smear of organism when observed under the oil immersion lens, exhibited a negative reaction. Small rod shaped organisms were equally distributed in the microscopic field either singly or in pairs. These results are in total agreement with those obtained by Baily and Scott (1966), Kreuger (1953) and Mckinny (1962) who also reported same sort of information about the cultural characteristics of *E. coli*.

The isolated strain of *E. coli* was found to be positive for indole production, methyl red, catalase and nitrate reduction tests. It was unable to show positive results for Voges Proskauer, citrate utilization and hydrogen sulphide production tests. The organism was potentially active to ferment glucose, maltose, lactose, manitol, fructose and sucrose (Table 1). These results are in accordance with those described by Cowan (1974); Davis *et al*. (1973); Duke and Jarvis (1972); Fimlt and Fimlt (1975); Janin (1975); Merckant and Pecker (1966); Robertson and Maclowry (1974); Wilson and Miles (1975).

For the assessment of pathogenicity, two methods were employed i.e., appearance of growth on Congo red medium and ileal loop ligation method. The test loop

Table 2: Detail of recovered Coliphages with small plaque (SP) from different sewage sources

Sampling areas	No. of samples	+ve samples	Mean plaque (No./Plate)	Mean PFU/ml ±SD×10^7
Rajawala	10	4	70	7.0±9.70
Satyana Road	10	4	88	8.8±2.87
Firdous colony	10	4	63	6.3±5.7
UAF	10	3	86	8.6±11.50

Table 3: Detail of recovered Coliphages with large plaque (LP) from different sewage sources

Sampling areas	No. of samples	+ve samples	Mean plaque (No./Plate)	Mean PFU/ml ±SD×10^7
Rajawala	10	4	30	3.0±5.70
Satyana Road	10	5	38	3.8±17.05
Firdous colony	10	4	43	4.3±17.5
UAF	10	3	46	4.6±5.70

Table 4: An *in vitro* activity of Coliphages (SP) against pathogenic *E. coli* on tryptone agar plates

E. coli to phage ratio	Appearance of CFU/ml after adsorption time		
	5 minutes	10 minutes	15 minutes
1:1 (a)	7	2	0
1: ½ (b)	10	5	0
1: ¼ (c)	14	7	0

SP = Small plaques

Table 5: An *in vitro* activity of Coliphages (LP) against pathogenic *E. coli* on tryptone agar plates

E. coli to phage ratio	Appearance of CFU/ml after adsorption time		
	5 minutes	10 minutes	15 minutes
1:1 (a)	5	0	0
1: ½ (b)	9	2	0
1: ¼ ©	12	4	0

LP = Large plaques

showed two times increase in diameter when compared to the control loop and the magnitude of pathogenicity index was calculated as 0.5, which may indicate that the organism was moderately pathogenic. On Congo red medium red colonies indicated that it was pathogenic strain. These results were comparable with those described by Altwegg and Bockemubi (1998).

Isolation of coliphages: Two types of phages were recovered from sewage water of different areas of Faisalabad, Pakistan including Rajawala, Firdous Colony, Satyana Road and University of Agriculture, Faisalabad. Two types of plaques were observed on the basis of their size i.e. large plaque (LP) and small plaque (SP). Small plaques measured 1-1.5 mm in diameter while large plaques measured 3.4 mm in diameter. As for as their shape was concerned, both had round edges. The detail about small and large plaques with their mean plaque forming unit (PFU) contents are shown in Table 2 and Table 3, respectively. The difference in the number of plaques obtained from the sewage water of different sources may be due to the fact that the area yielding more PFU contents might be having bacterial host in greater number as compared to the others. The presence of host cells in greater number may be attributed to the presence of more organic matter in the sewage. These results are comparable with those observed by Dhillon *et al.* (1970) who investigated sewage from urban and rural areas. When the characteristics of isolated phages were compared with those described in the literature, it was found that small plaque forming phages belonged to class of T_4 phages while those forming large plaques belong to the class of T_1 phages. The phase lysates were separately preserved in broth with glycerol, at 4°C for one month and found active equally and there was no significant decrease in their efficiency of plating (EOP). This finding was substantiated by those of Clark (1962) and Keogh and Pathingill (1976), who reported that their viability persisted upto two years under such conditions.

***In vitro* and *In vivo* assessment of phage activity:** For *in vitro* test, same protocol was followed for both large and small plaques. Both types of phages were equally able to lyse bacteria *in vitro*. The concentration of phages and *E. colli* was chosen as 10^7 PFU and 10^7 CFU per ml, respectively. *E. coli* to phage ratio was used as 1:1, 1: ½ and 1: 1/4 , while time of adsorption was 5, 10 and 15 minutes. The results indicated that with an equal concentration of *E. coli* and phages, no colonies of bacteria appeared after 10 minutes, but when phage concentration was reduced, colonies continued to appear even after 10 minutes. It was noted that no bacteria was able to grow on the plates after 15 minutes at any dilution of phages used. The results are shown in Table 4 and Table 5.

Table 6: An *in vivo* Coliphage (T_1 and T_4) activity against pathogenic *E. coli* in broiler chicken

Group	No. of birds	*E. coli* 10^7/ml	Phages 10^7/ml	Morbidity	Mortality
A	25	D.W (3 days)	D.W (3 days)	3	0
B	25	1/P (1 day)	D.W. (3 days)	4	0
C	25	I/M (1 day)	I/M (1 day)	0	0
D-1	12	D.W (3 days)	N.T.	12	7
D-2	13	N.T.	D.W. (3 days)	0	0

I/M = Intramuscular, I/P = Intraperitoneal, D.W. = Drinking Water, N.T. = No Treatment

As one of the objective of this study was to investigate the strength of lytic action of bacteriophages *in vivo*. The emergence of multi drug resistant bacterial pathogens motivated to attempt the therapeutic efficacy of bacteriophages in birds. The therapeutic application of phages as antibacterial agents in previous decades was impeded by several factors such as the failure of phages to recognize a broad range of bacteria and presence of toxins in the crude lysates of phages. In the present study, the narrow host range problem was alleviated and dealt with by isolating single strain of bacteria and virulent phage specific for it. Purifying the phage lysate preparations diminished toxin levels.

The estimation of whether the phages being active *in vivo* or not, was made by administering the broiler birds of 14 days with coliphages through different routes including oral, intraperitoneal and intramuscular simultaneously with *E. coli* given through same route with same concentration i.e. 10^7/ml. The dose of phages and bacteria given orally was 1 ml, but when parenteral routes were used, amount was reduced to 0.1 ml. The cultures of *E. coli* and phages were prepared in nutrient broth and diluted in normal saline before use.

The control birds (D_1) receiving only *E. coli* showed the signs of illness after 18 hours. They became lethargic, standing with dropping wings and eventually collapsed. Out of 12 morbidity was seen in all but mortality occurred in 7 birds after 48 hours. The other 13 control birds (D_2) that were given only the phages, neither showed the signs of disease nor mortality. The detailed results are shown in Table 6.

The results of group A, B and C revealed that morbidity occurred when phages were given orally but no morbidity recorded after intramuscular inoculations. This indicated that gastric juices might have had some inhibitory action upon phage activity and no such retarding force encounters when they are injected. But protective capability of phages even after oral administration through feed has been documented by Barrow and Soothil (1997) and Park *et al.* (2000).

It is concluded that phages may be looked for as good alternatives of those antibiotics to which bacteria have become resistant. This first ever effort in the country would definitely be a landmark in the field of phage therapy. It may provide further impetus for a better future of phage remedy in our circumstances also, as being taken in the other countries of the World.

References

Altwegg, M. and J. Bockemubi, 1998. Topley and Wilson's Microbiology and microbial infections. Arnold, London, 2: 946-947.

Awan, J.A. and S.U. Rahman, 2002. Microbiology Manual. Unitech Communication, Faisalabad, Pakistan.

Baily, W.R. and E.G. Scott, 1966. Microbiological methods. Butterworth and Co., London.

Barrow, P.A. and J.S. Soothil, 1997. Bacteriphage therapy and prophylaxis: Rediscovery and renewed assessment of potential. Trends Microbiol., 5: 268-271.

Bell, R.G., 1979. The limitation of the ratio of faecal coliform to total coliphages as a water pollution index. Water Res., 10: 745-748.

Bergh, O. and K.Y. Borsheim, 1989. High abundance of viruses found in aquatic environments. Nature, 340: 467-468.

Bruynoge, R. and J. Maisin, 1921. Essais de therapeutique an moyen du bacteriophage du staphylocoque. C.R. Sci. Biol., 85: 1120-1121.

Buxton, A. and G. Frazer, 1977. Animal microbiology. Blackwell Scientific Publications, Oxford, 1: 354-355.

Caccupccino, J.G. and N. Sherman, 1999. Microbiology: A laboratory manual. 4th Ed. Addison Wasley Longman, Inc., Harlow. England, pp: 219-229.

Clark, W.A., 1962. Comparison of several methods for preserving bacteriophages. Appl. Microbiol., 10: 466-471.

Cowan, S.T., 1974. Bergy's Manual of determinative bacteriology. The William and Wilkins Company, Batimore.

Cruickshank, R.J., P. Duguid, B.P. Marmion and R.H.A. Swain, 1975. Medical Microbiology. Vol. II, London.

Davis, B.D., R. Dubleco, N.S. Gisberg and W.B. Wood, 1973. Microbiology. Harper and Row, Publishers, Maryland, New York.

Dhillon, T.S., Y.S. Chan, S.M. Sun and W.S. Chau, 1970. Distribution of coliphages in Hong Kong Sewage. Appl. Microbiol., 20: 187-191.

Duke, P.B. and J.D. Jarvis, 1972. The catalase test: A cautionary table. Med. Lab. Tech., 29: 203-204.

Ewert, D.I. and M.J.B. Oaynter, 1980. Enumeration of bacteriophage and host bacteria in sewage and activated sludge treatment processes. Appl. Environ. Microbiol., 39: 376-383.

Fimlt, G.W. and I.J.Y.C. Fimlt, 1975. A comparative study of API, encise and conventional method. Med. Tec., 5: 1-8.

Hanbin, E.H., 1896. L'action bactericide des Eaux de la Jumna et due Gange Sur le Vibrion due Cholera. Ann de Inst. Pasteur, 10: 511.

Harrigen, W.F. and M.E. McCance, 1976. Use of coliphages as indicators of water pollution. Can. J. Microbio., 19: 747-751.

Healle, F., 1917. Sur un microbe invisible antagonistic des beilles dyrenterique. C.R. Acad. Sci. Paris, 1965: 373-375.

Hilton, M.C. and G. Stotzky, 1973. Use of coliphages as indicator of water pollution. Can. J. Microbiol., 19L: 747-751.

Janin, P.R., 1975. Rapid methods and automation in microbiology. Analytical products. Inc., Plainview, New York, 1-10.

Keogh, B.P. and G. Pathingill, 1976. Long term storage of bacteriophages of lactic streptococci. Appl. Microbio., 14: 421-424.

Kolter, R., 1992. Life and death in stationary phase. ASM NEWS., 58: 75-79.

Kreuger, W.W., 1953. Principles of Microbiology. W.B. Saunder's Co., Philadephia.

Kutter, E., 1997. Phage therapy: Bacteriophage as antibiotic. Evergreen State College T_4 phage a elwha evergreen edu.

Lederberg, J., 1996. Smaller fleas and infinitum. Therapeutic Bacteriophage. PANS, 93: 3167-3168.

Levine, B. and J.J. Bull, 1996. Phage therapy revisited: the population and biology of a bacterial infection and its treatment with bacteriophage and antibiotics. Amer Naturalist, 147: 881-888.

Mckinny, R.E., 1962. Microbiology for sanitary engineers. Dept. of Civil Engineering, University of Kansas. McGraw Hill Book Co., Inc., New York.

Merckant, C.A. and I.A. Pecker, 1966. Veterinary Bacteriology and Virology. The Iowa State Uni. Press, Ames. Iowa.

Ogunseitan, O.A., G.S. Sayler and R.V. Miller, 1990. Dynamic interactions of *Pseudomonas aeruginosa* and bacteriophages in lake water. Microb. *E. coli*. 19: 171-185.

Park, C.I. Shimamura, M. Fukanaga, K. Mori and T. Noki, 2000. Isolation of bacteriophages specific to a fish pathogen *Pseudomonas plecoglossicida* as a candidate for disease control. Appl. Exiron. Microbiol., 66: 1416-1422.

Proctor, L.M. and J.A. Fuhrman, 1990. Viral mortality of marine bacteria and cyanobacteria. Nature, 343: 60-62.

Radetsky, P., 1996. Return of the good virus. Discover, 17: 50-58.

Robertson, E. and J.D. Maclowry, 1974. Mathematical analysis of API enteric 20 profile register using a computer diagnostic model. Appl. Microbiol., 28: 691-695.

Twort, F.W., 1915. An investigation on the nature of ultramicroscopic viruses. Lancent, 2: 1241-1243.

Willson, G.S. and A.A. Miles, 1975. In Topley and Wilson's principles of bacteriology, virology and immunology. Vol. 1, 2nd Ed. Ward Arnold, Ltd. London.