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Isolation and Primary Characterization of Bacteriophages

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ABSTRACT:

Medical science has begun to acknowledge the emergency of drug resistant bacteria a fact made more amimous when one realizes that no fundamentally new antibiotic has been discovered for more than 30 years. Are we losing the battle? The emergence of pathogenic bacteria resistant to most, if not all, currently available antimicrobial agents has become a serious and critical problem in modern medicine, particularly in poultry[13]. Improvements in biosecurity on poultry farms are likely to be very expensive and difficult to maintain, so there is a need to find an acceptable, cost effective solution of preventing infection of poultry. The use of host specific bacteriophages has been promoted as an important and cost effective adaptable approach to control zoonotic bacteria. 32 fecal samples of poultry were collected from fourteen different poultry farms from different districts of state Gujarat, India. The samples were tested for the presence of phages of *E.coil* and *S. typhimurium* and *S. typhi* by double agar layer plaque method. Out of 32 samples, 5 samples showed the presence of *E. coli* phages and 4 samples showed the presence of *S. typhimurium* phage and 1 sample showed the presence of *S. typhi*. Ec-2, ADB-1, Ec-5 and ARJ-2 isolates of phage were found host specific while SM-3 and SM-5 were found polyvalent bacteriophage, SM-3 were having the capacity to infect E. coli, S. typhimurium and S. enteritidis. SM-5 infected on S. typhimurium and S. enteritidis. Antibiogram of natural host were performing by using 27 different types of antibiotics by paper disc method. The isolated host bacteria show high level of resistance against Lincomycin , Ampicillin , Ceftazidime , Cephadroxil , Bacitracin , Cephaloridine antibiotics and highly sensitive against Ofloxacin , Amoxycillin , Sparfloxacin , Lomefloxacin and Tetracycline.

1. Introduction

Bacteriophages are viruses that infect bacterial host and are estimated to be the most numerous biological entities in the biosphere. Phages exist wherever bacteria occur, and share a common ecology with their respective bacterial hosts[10]. They are the most abundant living entities on Earth - the estimates range from 10^{30} to 10^{32} in total - and play key roles in regulating the microbial balance in every ecosystem where this has been explored[13]. Since their independent discovery by Frederick Twort and Felix d'Herelle in 1915 and 1917, respectively, bacteriophages have been studied in numerous laboratories worldwide, and they have been used in a variety of practical applications.

Poultry meat is one of the most popular foods Worldwide. This paper concentrates on the safety aspects of poultry production and its products. Poultry and poultry meat are often found contaminated with potentially pathogenic. Microorganisms such as Salmonella, Campylobacter, *Staphylococcus aurous*, *Escherichia coli*, Listeria, *Yersinia enterocolitica*, Aeromonas and *Clostridium perfringens* have the potential to be important pathogens in poultry products[5].

Acute enteric infections caused by salmonellas remain a major public health burden worldwide. It has been estimated that approximately 13 million cases of salmonellosis occur worldwide annually, of which about 70 per cent reports come from China, India and Pakistan [3]. Typhoid fever, caused by Salmonella enteric serotype Typhi (*S. Typhi*) is a major public health problem, particularly in developing countries. Poultry, particularly chickens, are known to be the main reservoir for this zoonotic pathogen. Although some progress has been made in reducing Salmonella colonization of broiler chickens by using biosecurity and antimicrobials, it still remains a considerable problem. The use of host-specific bacteriophages as a biocontrol is one possible intervention by which Salmonella colonization could be reduced[15, 22]. Poultry is known to be the largest single reservoir of Salmonella. *S. gallinarum*, the causative agent of fowl typhoid, is the most prevalent host-adapted Salmonella strain in India[11]. *S. Typhimurium* and *S. Enteritidis* are not only involved in severe outbreaks of avian salmonellosis and economic losses to the poultry industry[11], these serovars also pose a definite zoonotic hazard as poultry are known to be the major transmitters of non host-adapted salmonellosis in humans[17]. Improvements in biosecurity on poultry farms are likely to be very expensive and difficult to maintain [7], so there is a need to find an acceptable, cost effective way of preventing infection of poultry with Salmonella[2]. Virulent phages cause bacterial host cell lysis and not only function to control bacterial populations, but can be used as indicators of bacterial (fecal) contamination [15, 22] and as tools for identifying (typing) specific bacterial strains [4, 14,20, 25]. Bacteriophages are natural predators of bacteria and are ubiquitous in the environment [19]. The use of host-specific bacteriophages has been promoted as a cost-effective and adaptable approach to control zoonotic bacteria [21].

During the last 20 to 30 years a great number of changes have taken place in processing mechanization and automation. From the point of view of hygiene, these changes have led to better microbiological quality and increased shelf-life of products. Attempts to decontaminate poultry have concentrated in three main aspects: 1). chemical methods (lactic acid, hydro-gen peroxide, trisodium phosphate), 2). Physical methods (-ionizing radiation, non-destructive heat treatments), 3). Novel methods (Use of natural predators, biopeptides and new preserving techno-logies).

2. Materials And Methods

2.1 CILLECTION OF SAMPLES AND ISOLATION OF PHAGES

2.1.1 Host Bacterial cultures

Salmonella strains and *Escherichia Coli* strain used as phage hosts were obtained from the Microbial Type Culture Collection and Gene Bank (Table. 1). Freeze-dried primary cultures were reconstituted in Luria-Bertani (LB) broth (Bacto Tryptone, 10 g/liter; Bacto Yeast extract, 5 g/liter; NaCl, 5g/liter) overnight at 37^0C .The strains are maintained in 1 to 2 ml LB broth with 20% glycerol at -70^0 C. Secondary culture were prepared from thawed primary culture, grown in LB medium overnight 37^0C, and inoculate on slants of Nutrient agar medium. The nutrient agar slants cultures were grown overnight at 37^0C, then sealed and stored in the dark at 5^0C. Subcultures were produced weekly, or biweekly as needed, from stored nutrient agar slant cultures inoculated to LB broth, grown overnight at 37^0C, and streaked on nutrient agar. Nutrient agar plates were checked for purity and uniformity and working cultures prepared by transfer of single isolated colonies to LB broth.

Table: 1 List of Bacterial strain collected from MTCC.

Serial No.	Host Name	MTCC No.
1.	*Salmonella Typhimurium*	98
2.	*Salmonella Enteritidis*	3219
3.	*Salmonella Typhi*	733
4.	*E.Coli*	1610
5.	*E.Coli*	739
6.	*E.Coli*	476
7.	*Enterobacter aerogens*	2822
8.	*Protease vulgeris*	742
9.	*Psudomonas aerugenosa*	741
10.	*Salmonella Paratyphi A*	735
11.	*Shigella flexneri*	1457
12.	*S.aerus*	96

2.1.2 Collection of Poultry Excreta

Samples of poultry excreta were collected from 9 different poultry farms in different district of Gujarat state in India. 32 samples were collected from various Poultry farm in sterile sample collector (Himedia) in December 2008, February 2009, May 2009 and June 2009. Samples of about 30-50 gram were collected in sterile Himedia sample collector. Samples were collected in various forms such as freshly laid fecal sample, dry fecal sample and semi solid fecal samples. All samples were held on ice in a cooler, returned to the laboratory, and held at 5°C for processing within a few hours of collection. Samples were collected with help of veterinary doctor and curator of poultry farm.

2.1.3 Isolation of Bacteriophage from samples

A suspension of fecal contents (1:9, w/v) was prepared in SM buffer (50mM Tris-HCl [pH 7.5], 0.1 M NaCl, 8 mM $MgSO_4$.$7H_2O$, and 0.01% gelatin) and incubated at 4^0C for 24 hrs with gentle agitation to allow phages to elute into the buffer. This suspension was then subjected to centrifugation at 13,000 × g for 10 min to remove bulk debris. The supernatant was then filtered through a 0.22 µm-pore –size membrane filter to remove any remaining bacterial cells. This filtered supernatant was added to equal quantity of double strength T-broth (Peptone,10g/liter; Meat extract, 3g/liter; NaCl, 5g/liter; Glucose, 1g/liter; $CaCl_2$, 0.2g/liter; $MgSO_4$, 0.5g/liter; pH 7.4(with HCl or NaOH). Inoculate 1ml of overnight grown culture of

host. Incubate at 37^0C for 24 hrs with shaking. Resultant growth culture were treated with chloroform, Chloroform is often used to break open infected cells to release phage encoded in it. Then centrifuge at 13,000 × g for 5 min, collect the supernatant and passed through 0.22-µm filter, this filtrate used in Double Agar Layer (DAL) plaque assay against the host bacterium.

2.1.4 Phage Lysate Production

Selected bacteriophages were increased by inoculation of log-phase broth cultures of the respective host strain and production of bacteriophage lysates in sufficient volume for additional characterization and analysis. Lysates were clarified by centrifugation (10 000 x g for 15 min at 5°C), decanted, and filtered through 0.45-mm cellulose acetate filters into sterile glass vials, treated with chloroform (to 2.5%), and stored at 5°C.

2.1.5 Double Agar Layer (DAL) plaque methods

Day-1: Enrichment of phage lysate

Phage lysate was added to the equal quantity of T-broth, inoculate 1 ml of overnight grown culture of *S. typhimurium* as a bacterial host and incubate at 37^0C for 24 hrs with shaking.

Day-2: Collection and utilization of enriched phage lysate

Add 1-to-2 ml of chloroform to culture flask with the help sterile pipette. Then this culture was centrifuge at 10,000 × g for 15 min, collect the supernatant and filter through 0.45 µm membrane filter. Centrifugation and filtration was done to remove the bacterial cell debris. This filtrate was used to prepare serial dilution of phage lysate with sterile saline dilution tube, prepare dilutions from 10^{-1}-to-10^{-8}. Transfer 0.5 ml of young culture of *S. Typhimurium* and 0.5 ml of each dilution to 10 ml of melted top agar tubes. Test suspensions were mixed thoroughly by vortexing and dispensed uniformly over the surface of 20 ml of hard nutrient agar (base agar) in 96-mm-diameter plates. Repeat the procedure for each dilution. Label the plates with dilution factor. Melted agar overlays were allowed to harden at room temperature then plates were inverted and incubated overnight at 37°C.

Day-3: Counting of plaque

Plaque (pfu) were counted or individually subcultured as appropriate to the enumeration or isolation protocol.

2.2 Enrichment Protocol

Fecal samples were reconstitute in SM buffer and treated with chloroform and stored at 4°C overnight to allow larger suspended sediments to settle out. These crudely clarified samples were then passed through 0.22-mm syringe filters. Several filter changes were needed to complete the filtration of each 10- to 15-mL of sample. Lytic phages were selectively enriched by mixing filtered effluent with double strength trypticase soy broth inoculated with an actively growing culture of host. (*E coli* MTCC 739, *S.typhimurium* MTCC 98 & *S.typhi* MTCC733). After overnight incubation at 35°C, chloroform was added and samples were stored at 5°C. Enriched samples were tested by double agar layer (DAL) plaque assay against individual Respective host. For isolation of phages, enriched samples were diluted in sterile saline in 10-fold series and used in DAL plaque tests at appropriate dilutions to yield separated individual plaques suitable for single-plaque transfer.

2.3 Antibiogram of Host bacteria

2.3.1 Isolation of *E. coli* and *S.typhi* as a Natural host of phages

Fecal samples were collected from different district of Gujarat state. Cloacal swabs were used during the collection of sample from diarrheic poultry from various organized, unorganized and government poultry farms in Gujarat. Samples were collected aseptically in sterile test tubes and immediately brought to the laboratory for processing. Kauffmann tetrathionate broth (TTB) containing brilliant green in the final concentration of 1:1, 00,000 was used as a selective enrichment broth for primary isolation of Salmonella. Brilliant green agar (BGA) was used as selective medium for primary isolation and MacConkey's lactose agar (MLA) was used for purification of suspected colonies. Characterization and preliminary identification of suspected Salmonella cultures were made on the basis of morphology, cultural characteristics, and biochemical reactions. Agglutinability of the suspected Salmonella culture with Salmonella polyvalent 'O' sera (SPAN Diagnostic Ltd, INDIA) was tested using slide agglutination test [22] Eosine Mythylene Blue and MacConkey's agar were used for the preliminary identification of suspected *E-coli* isolates. Further identification was carried out on the basis of morphology, cultural characteristics and biochemical reaction.

2.3.2 Determination of antibiogram of isolated natural host bacteria

Antibiotic sensitivity test: In vitro susceptibility of the organisms to various antimicrobial agents was determined by the disc diffusion technique[22]. The antimicrobial agents and their concentration (concentration in µg) used were shown in table no- 3. Antibiogram were performing by using 16 different types of antibiotics by paper disc method (Hi-Media Laboratories Ltd., single discs). Nutrient agar (N-Agar) is used as an antibiotic assay medium. 0.1 ml overnight grown culture of isolated bacteria mixed with 15 ml melted N-Agar at 45-50°C and poured on base agar containing petriplate and allow it to solidify. Than antibiotic discs were applied with sterile forceps to ensure even contact with the medium. All plates were incubated at 37° C for 24 hrs. After incubation the diameter of zone of inhibition, indicating a susceptibility of host for the respective antibiotic.

2.4 Host range Test

Phage isolates (E coli phage, S.typhimurium phage and S.typhi phage) recovered by the enrichment protocol were tested against 13 different strains of taxonomically related bacteria. The strains used in Host range testing were obtained from the Microbial Type Culture Collection and Gene Bank (Table. 1). Phage lysates were allowed to enrich into T-broth inoculated with respective bacterial test hosts. Collect the filtrate and DAL plaque assay method was performed for each test hosts and incubated overnight at 37°C. Plaque formation, indicating a susceptible host for the respective phage, was assessed after 24 hrs. Polyvalence of isolated Bacteriophage were also tested by Spot assay method.

3. Result and Discussion

There are more than 2500 Salmonella serovars distributed throughout the world; some of these viz. *S.typhi, S. gallinarum, S. typhimurium* and *S. choleraesuis* are host specific, the majority are non adapted and can cause infection in man and animals alike [9] . Non-typhoidal salmonellosis is an important enteric infection in humans and poultry [11]. It has been estimated that approximately 13 million cases of salmonellosis occur worldwide annually, of which about 70 per cent reports come from China, India and Pakistan [3]. Poultry is known to be the largest single reservoir of Salmonella[12]. In the present investigation, 32 samples were collected from various poultry farm of Gujarat. S. Typhimurium and S. Enteritidis are not only involved in severe outbreaks of avian salmonellosis and economic losses to the poultry industry [24, 16]. These serovars also pose a definite zoonotic hazard as poultry are known to be the major transmitters of non host-adapted salmonellosis in humans [17]. The samples were tested for the

presence of phages of *E.coil* and *S.typhimurium* and *S.typhi* by double agar layer plaque method. Out of 32 samples, 5 samples showed the presence of *E-coli* phages named as EC-2, EC-4, EC-5 , MHEC – 2 and MHEC-5 and 4 samples showed the presence of *S.typhimurium* phage named as SM-3, SM-5, LHSM- 4 and LHSM – 5 and out of 14 samples 1 sample showed the presence of *S.typhi named as ARJ-2 as show in Table no -2*.

Table – 2: Isolation of Coliphage from poultry fecal samples.

Name of Place / Poultry farm	**Sample name**	**E coli phage Isolation**	**S.typhimurium phage isolation**	**S.typhi phage isolation**
Dhebakuva – 1	DK-1	-	-	ND
	DK-2	-	-	ND
	Dk-3	-	+	ND
Dhebakuva –2	DK-4	-	-	ND
	Dk-5	-	+	ND
Dharmaj	DJ-1	-	-	ND
	Dj-2	-	-	ND
Ankalav	Ak-1	-	-	ND
	AK-2	-	-	ND
	AK-3	-	-	ND
Anand Agriculture University	LH-1	-	-	-
	LH-4	-	+	-
	LH-5	-	+	-
	CH-2	-	-	-
Rajkot	RJ-1	-	-	ND
	RJ-2	-	-	ND
	RJ-3	-	-	ND
	RJ-4	-	-	ND
Mustafa Poultry farm AMRELI	AM-1	-	-	-
	AM-2	+	-	-
	AM-3	-	-	-
	AM-4	+	-	+
	AM-5	+	-	+
	AM-6	-	-	-
Mahuva Poultry farm – 1	MH-1	-	-	-
	MH-2	+	-	-
	MH-3	-	-	-
	MH-4	+	-	-
Mahuva Poultry farm – 2	MH-5	-	-	ND
	MH-6	-	-	ND
	MH-7	-	-	ND
	MH-8	-	-	ND

The sharing of phage types among various species indicated the interspecies transmission of organism[18, 23] and re-emphasized the need to control salmonellosis at every step. In recent years, antibiotic resistance in Salmonella has assumed alarming proportions worldwide[1, 8]. Monitoring drug resistance pattern among the isolates not only gives vital clues to the clinician regarding therapeutic regime to be adopted against individual cases, but is also an important

tool to devise a comprehensive chemo prophylactic and chemotherapeutic drug schedule on herd basis within a geographical area.

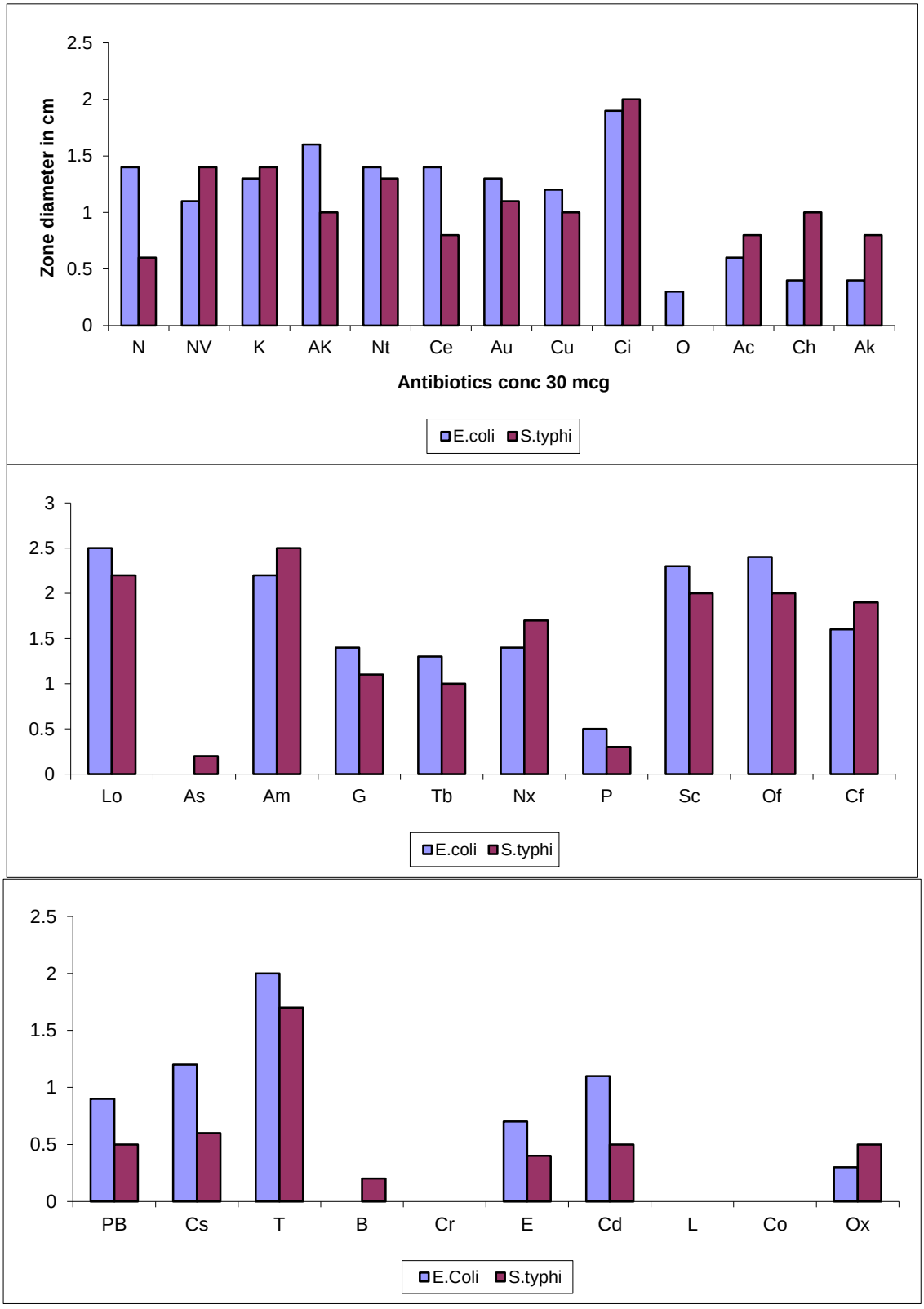

Figure 1 :Antibiogram of Isolates *E. coli ADB-2* and S.typhi ARJ-*2*

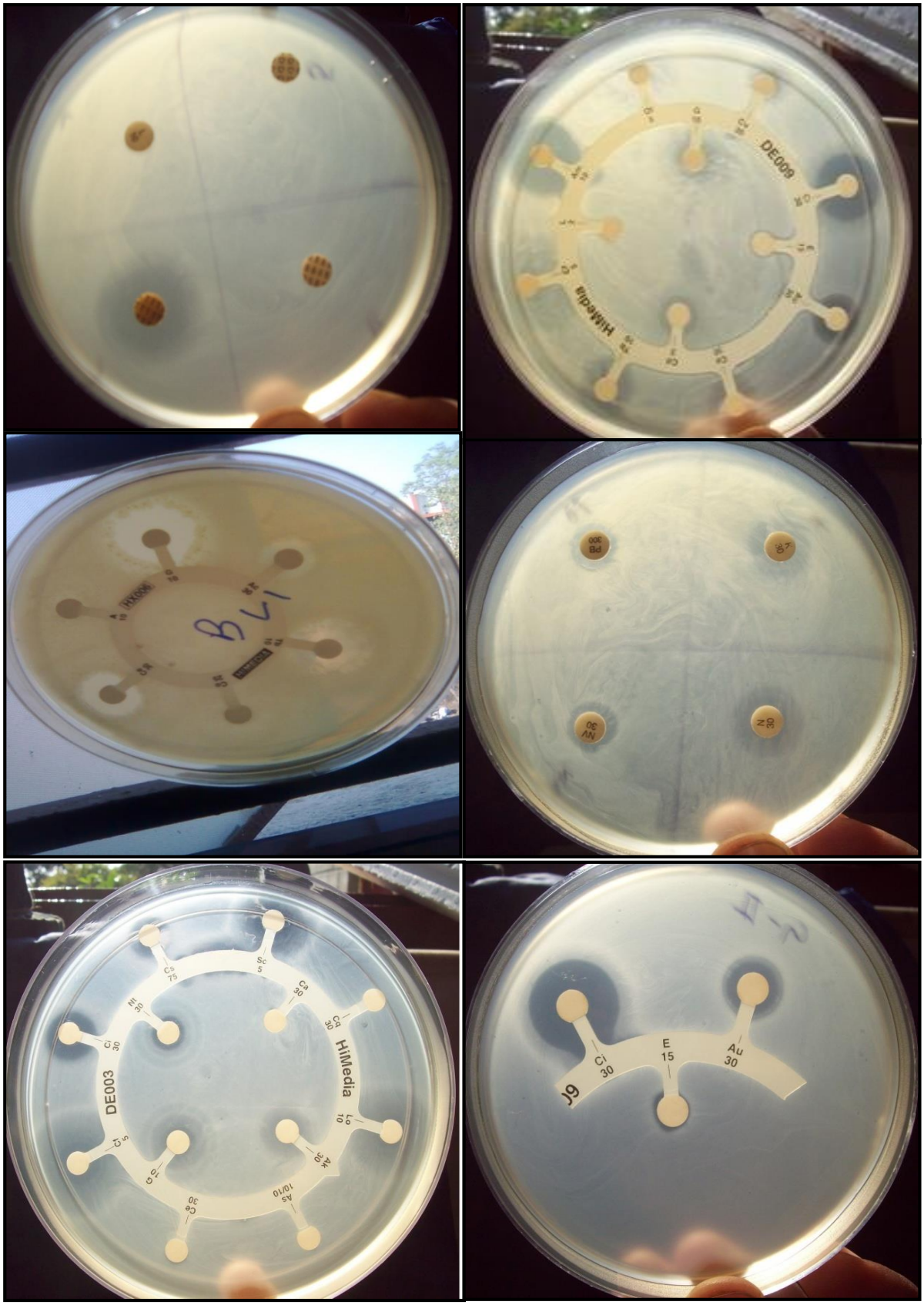

Figure 2 : Antibiotic sensitivity test of Isolates *E. coli ADB-2* and *S. typhi-2*

On the basis of Antibiogram data , E.coli ADB-2 was observed to be sensitive against Tetracyline, Lomefloxacine, Sparfloxacine, Amoxyciline, Ofloxacine (The zone of dimeter is

≥ 2.0), it showed intermediate resistance against Oxytetracyline, Penicillin, Oxacillin, Cephalothin, Amkalin (The zone of dimeter is ≤ 0.5 c.m.) and finally E. coli ADB-2 was observed higher resistance against Bacitracin, Cephaloridine, Cephadroxil, Ceftazidime, Ampicilline, Lincomycin, Co-trimoxazole. S. typhi ARJ-2 was observed to be sensitive against Lomefloxacine, Sparfloxacine, Amoxyciline, Ofloxacine, Ceftriaxone (The zone of dimeter is ≥ 2.0) it showed intermediate resistance against Bacitracin, Polymyxin, Ampicillin , Erythtromycin, Clindamycin, Penicillin, Oxacillin, (The zone of dimeter is ≤ 0.5 c.m.) and showed higher resistance against Cephaloridine, Cephadroxil, Ceftazidime, Lincomycin, Co-trimoxazole, Oxytetracylin.It has been extensively studied that the major cause of antibiotic resistance is the poultry feed as supplements and uncontrolled antibiotic usage. Determination of antibiogram and drug resistance pattern of the isolates gives the idea to the clinician regarding therapeutic schedule in an individual cases. The data of antibiogram also useful tool to devise a comprehensive chemo prophylactic and chemotherapeutic drug schedule and doses of antibiotic within a geographical area.

Thirteen bacterial strains, listed in Table 4, were tested for sensitivity against the isolated phages. The Spot Test [6] and DAL method were used to determine the host range of the phage. Lytic activity was examined following overnight incubation at 37°C and recorded on a scale as shown in table 4. To obtain an accurate estimate of relative phage lytic activity, all host range determinations were carried out simultaneously using a single high-titer stock of purified bacteriophage. Ec-2, Ec-4, Ec-5 and ARJ-2 isolates of phage were found host specific while SM-3 and SM-5 were found polyvalent bacteriophage, SM-3 were having the capacity to infect *E coli, S.typhimurium* and *S.enteritidis*. SM-5 infected on *S.typhimurium* and *S.enteritidis*. Due to the ability to infect various species of bacteria, it has been concluded that the isolated bacteriophages SM-3 & SM-5 can become an effective agent in the effort to reduce the incidence of salmonellosis in poultry. The study of SM-3 & SM-5 also provided the basis for future experiment of bacteriophage for indicator and biocontrol agent in poultry.

Table – 4: Host range of isolated bacteriophages

Name of Host	**E. coli phage**			**S. typhimurium phage**		**S. typhi phage**
	EC-2	**ADB-2**	**EC-5**	**SM-3**	**SM-5**	**ARJ-2**
E-Coli 1610	+/-c	-/+t	+/-c	-/+t	-	ND
E-Coli 739	+tc	+c	+tc	ND	ND	-
E-Coli 476	+c	+c	+tc	-	-	ND
*E-Coli **	-	+tc	ND	ND	ND	ND
En.aerogens	-	-	-	-	-	-
S.typhimurium	-	-	-	+/-c	+/-c	-
S.enteritidis	-	-	-	+/-t	+/-t	ND
S.typhi	-	-	-	-	-	+tc
S.peratyphi A	-	-	-	-	-	ND
Shigella flexneri	-	-	-	-	-	ND
P.vulgaris	-	-	-	-	-	ND
P.aeruginosa	-	-	-	ND	ND	ND
S.areus	-	-	-	-	-	ND

+ Sign indicates plaque formation; +/- indicates small or diffuse plaque; -/+ indicates a very small plaque; - indicates no plaque formation; c indicates a clear plaque; t indicates a turbid plaque; tc indicates a turbid plaque with a clear center; ND indicates ‘‘no description’’ due to apparent loss of infectivity in storage following isolation.

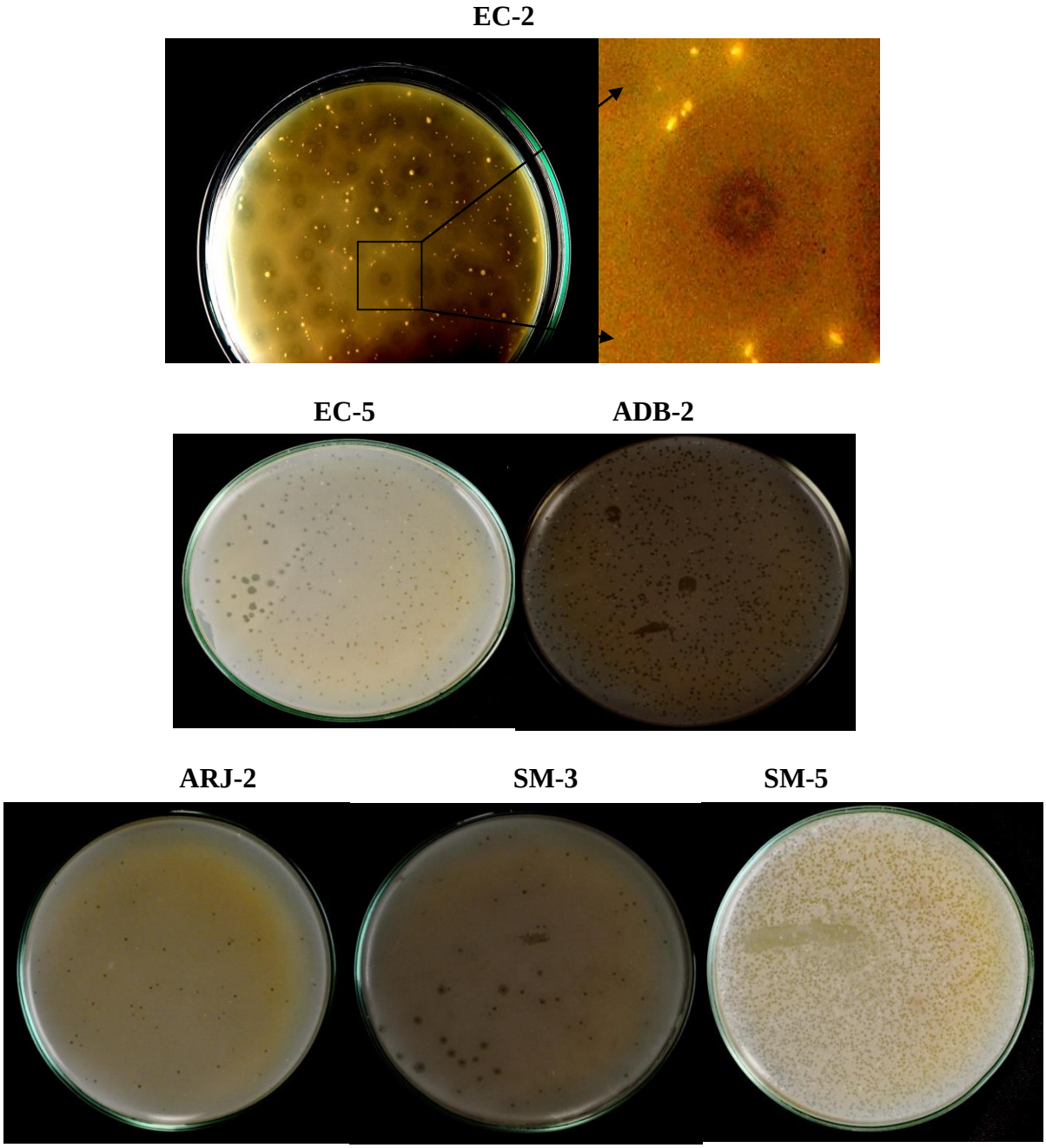

Figure 3 :Isolation of bacteriophage

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