Isolation of MDR Pathogens From Sewage and Birds Droppings and Their Possible Treatment Using Lytic Phages

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Abstract- *With the rising prevalence of multiple-antibiotic resistant-bacteria (MDRs) and the lack of development of new antibiotics by the pharmaceutical industries, there is an urgent need to develop novel approaches to combat MDRs, especially Pseudomonas aeruginosa, Escherichia coli and Staphylococcus aureus. Bacteriophage therapy has been applied for decades as a means of treating bacterial infections in some parts of the world and numerous encouraging results have been documented. In the present study waste water sample from hospital sewage treatment plant was used as sample. Selective media like were used for isolating pathogens such as Staphylococcus, Escherichia coli and Pseudomonas. Their identification was carried out using morphological and biochemical characteristics using Bergey's manual of determinative bacteriology, 9th edition. Their antibiotic resistant status checked using antibiotic multidisc. From isolates MDR bacteria characterized and used for further study.Enrichment of sewage sample followed by isolation of bacteriophages was carried using agar overlay method.These bacteriophages can be used as possible treatment for diseases caused by MDR bacteria. [6]Isolation of pathogens from hospital effluent was successful . Their characterization and MDR status determination was done. Isolation of lytic baceriophages which grow on these pathogenic bacteria was successfully carried out. Phage therapy can be used for both chronic and acute infections. The potential use of lytic phage on various infections is the future aspects of the presented research. Phage therapy to treat human MDRs is possible solution to the burning issue of MDR bacteria.*

Keywords- MDR bacteria; Phage therapy; hospital effluent; antibiotic sensitivity tes

I. INTRODUCTION

Antibiotic resistance is a worldwide problem. The emerging crisis of antibiotic resistance and the uncertain outlook for development in new antibiotics have dramatically altered landscape of MDRs, generating renewed interest in phages as a means of eradicating drug-resistant microorganisms.[1] In addition, phage therapy is the therapeutic use of lytic bacteriophages to treat pathogenic bacteria. In this study, Enrichment of sewage sample followed by isolation of bacteriophages against the MDR pathogens was carried using agar overlay method. Extensive study need to be done to determine if there is any effect of chronic and acute infections by phage therapy. The potential use of lytic phage on various infections is the future aspects of the presented research. Phage therapy to treat human MDRs is possible solution to the burning issue of MDR bacteria[2].

II. MATERIALS AND METHODOLOGY

The waste water sample was collected from drainage and broiler.This sample was checked for physico-chemical parameters like color, odor, temperature, pH.Loop full of it was directly streaked on sterile selective media plates such as MacConkey's agar, SS agar, Mannitol salt agar, Cetrimide agar to isolate pathogenic strains of *Escherichia, Salmonella, Staphylococcus, Pseudomonas* respectively.The colonies showing specific characters and which were more in number restreaked on to another plates of same media to obtained pure isolates.The biochemical tests of the obtained isolates were then carried out using various sugars and reagents.This was then followed by the antibiotic sensitivity testing .

Protocol for Antibiotic Sensitivity Test (AST)

- The discs for AST were selected according to CLSI guidelines. Selection of discs was based on the Gram nature of isolate. For Gram positive isolate discs used were HEXA G PLUS 1, for Gram negative isolates HEXA G MINUS 18
- 4-5 colonies of pure isolate were inoculated in respective sterile broth (Nutrient broth).
- It was allowed to incubate approximately for 3 to 4 hours at room temperature, until it attended an Optical Density (O.D) in-between 0.08 to 0.11 at 570 nm on colorimeter.
- After attending a specific O.D, using sterile buds broth inoculated isolates were spread on sterile Mueller-Hinton Agar.
- Spread plates were kept for drying for some time.
- Considering the Gram character of the isolate anti-biotic disc was placed with a flame sterilized forcep.

- The plates were incubated for 48 hours at room temperature (approximate 28°C).
- The zones were measured using HiAntibiotic ZoneScale™-C (PW297).
- The isolates were screened to identify the MDR strains.

Enrichment, isolation and detection of phages Enrichment [4]

To increase the number of phage virions in a collected sample, it is necessary to add 5 ml of double strength phage broth (DSPB), 5 ml specific media broth and 5 ml of respective isolates to 45 ml of raw sample.The specific broth medium such as Brilliant bile green broth for *Escherichia*, dettol broth for *Pseudomonas*, disinfectant test broth for *Staphylococcus* and salmonella broth for *Salmonella* was used to enrich the phages against specific host.This mixture is incubated at 37° C for 24 hours.

Centrifugation and filtration

Rapid filtration to separate the phage from host in the enrichment mixture requires adequate centrifugation first.To minimize filter clogging, a triple centrifugation at 2500 rpm for 10 min at 4°C was used. Supernatant obtained was then treated with chloroform and again centrifuged.To save time in the event that filter clogging does occur, an extra filter assembly and an adequate supply of sterile membrane filters should be available.These membrane filters have a millipore filter paper with size of 0.45 µm, which holds back all bacteria, allowing only the phage virions to pass through.

Agar overlay

Evidence of phage in the filtrate was checked by production of plaques by agar overlay method. The medium used was soft nutrient agar.The soft agar along with 0.1ml of organism and 0.1ml of phage filtrate was mixed well and poured over the top of prewarmed hard nutrient agar.Prewarmed plates result in a smoother top agar surface.The plates were then incubated at 37°C at 24 hrs.

III. OBSERVATIONS AND RESULTS

Table no.1 Physico-chemical analysis

The same sampling procedure was carried out for sewage as well as for chick droppings. Collected samples were immediately checked for pH, temperature, color and odor.

Sr No.	Name of sites	Temp	pH	Color	Odor
1	Hospital effluent	22°C	6.3	Blackish	Pungent
2	Prayeja drainage	22°C	5.6	Blackish	Pungent
3	Broiler droppings	38 °C	6.1	Brownish	Pungent

The isolates were characterized for colony characters and Bio-chemicals were performed according "Bergey's Manual of Determinative Bacteriology- 9th edition". Showing the following result in table no. 2 and 3.

Table no.2 Colony Characters of sewage isolates

Colony characters	Isolate code			
	ECp	**PAp**	**SAp**	**SALp**
Size	2 mm	1 mm	2 mm	1 mm
Shape	Circular	Circular	Circular	Circular
Color	Pink	yellowish	Yellowish	Black with peripheral yellow
Margin	Regular	Regular	Regular	Regular
Elevation	Convex	Umbonate	Convex	Convex
Opacity	Opaque	Opaque	Translucent	opaque
Consistency	Mucoid	Mucoid	Smooth	Mucoid
Gram character	Gram negative rods	Gram negative rods	Gram positive cocci	Gram negative rods
Motility	Motile	Motile	Non motile	Motile

EC p : *E. coli* (prayeja sample); PA p : *P. aeruginosa* (prayeja sample); SA p : *S. aureus* (prayeja sample); SAL p : *Salmonella* (prayeja sample)

Table no. 3 Colony characters of birds droppings isolates

Colony characters	Isolate code			
	ECc	**PAc**	**SAc**	**SALc**
Size	1 mm	2 mm	2 mm	2 mm
Shape	Circular	Circular	Circular	Circular
Color	Pink	Orange	Whitish	Yellowish
Margin	Regular	Regular	Entire	Regular
Elevation	Convex	Flat	Convex	Convex
Opacity	Translucen	Opaque	Translucen	Opaque

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Consistency	Mucoid	Mucoid	Mucoid	Mucoid
Gram character	Gram negative rods	Gram negative rods	Gram positive cocci	Gram negative rods
Motility	Motile	Motile	Non motile	Motile

EC c : *E. coli* (chick sample); PA c : *P. aeruginosa* (chick sample); SA c : *S. aureus* (chick sample); SAL c : *Salmonella* (chick sample) From the Biochemical test performed the identification of MDR organisms was done as per "Bergey's manual of Determinative Bacteriology 9th Edition", these isolates may belong to corresponding genus as mentioned in table No.4

Table no. 4

Sr.No	Isolates code	Genus (Most probable)
1.	EC p	*Escherichia*
2.	PA p	*Pseudomonas*
3.	SA p	*Staphylococcus*
4.	SAL p	*Salmonella*
5.	EC c	*Escherichia*
6.	PA c	*Pseudomonas*
7.	SA c	*Staphylococcus*
8.	SAL c	*Salmonella*

The disc was selected on the basis of two criteria;1. There should be no repetition of antibiotic in selected combination. 2. The antibiotics used should be of recent generations. If the isolates were found to be resistance to more than three antibiotics they were considered as Multi Drug Resistance (MDRs).

Table no. 5 Antibiotic Sensitivity Testing of gram positive isolates and screening of MDR

HEXA G PLUS 1		
Antibiotics	**Isolate code**	
	SA p	**SA c**
Penicillin (10 mcg)	R	R
Oxacillin (1 mcg)	R	R
Cephalothin (30 mcg)	30 mm	R
Clindamycin (2 mcg)	R	10 mm
Erthormycin (15 mcg)	R	R
Amoxyclav (30 mcg)	29 mm	R
	MDR	**MDR**

Table no. 6 Antibiotic Sensitivity Testing gram negative isolates and screening of MDR

HEXA G MINUS 18						
Antibiotics	**Isolate code**					
	ECp	PAp	SALp	ECc	PAc	SALc
Amikacin (30 mcg)	R	R	20 mm	R	R	23 mm
Cefaclor (30 mcg)	R	R	R	16 mm	R	19 mm
Ceftriaxone (30 mcg)	R	R	R	R	R	8 mm
Cefuroxime axetil (30 mcg)	R	22 mm	26 mm	R	R	10 mm
Cefoxitin (30 mcg)	R	R	24 mm	24 mm	R	10 mm
Tetracillin/clavunic acid (10/30mcg)	23 mm	R	R	R	R	R
	MDR	**MDR**	-	**MDR**	**MDR**	-

ISOLATION AND DETERMINATION OF PHAGES

Plaques were determined against the isolated MDR Pathogens by the agar overlay method.

Table no. 7 Plaque morphology and size are as follows

Phage isolate from sample	**Size of plaque (mm)**	**Plaque morphology**	**PFU/ml**
EC p	1 mm	Clear	40 ×10/ml
PA p	2 -3 mm	Clear	4 ×10/ml
SA p	1 mm	Clear	96 ×10/ml
EC c	2 mm	Clear	48×10/ml
PA c	1 mm	Clear	72 ×10/ml
SA c	1 mm-2 mm	Clear	112 ×10/ml

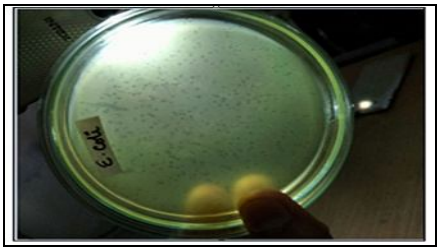

Figure no. 1 Plaques of EC c isolate

IV. DISCUSSION AND CONCLUSION

In this study, lytic phages against the pathogens were isolated from sewage and broiler waste sample. The phages were detected by using agar overlay method and specific host cells.[3] Plaque assay of filtered phage lysate on the lawn of MDR strains exhibited clear plaques suggesting a highly lytic activity of phages. The electron microscopic examination needs to be done to identify the phage morphology.The isolated phages against the MDR strain of *Pseudomonas* spp can be used in treating bacteremia and other infections caused by *Pseudomonas* spp.The possibility of bacterial resistance to phage may be an obstacle in the development of an effective phage therapy system.[4]Data from the literature helps to understand that even if the bacteria develop phage resistance new phage that may have lytic activity against that particular bacteria can be utilized.[5] Some of the reasons that phage therapy has not been globally recognized and applied may be due to three major concerns: 1) The rapid lyses of a large numbers of microbes, especially Gram negative that may release endotoxin (i.e. LPS). 2) The second important concern is where phages may destroy other non target microbes and disturb the normal flora. This is not real concern since phages are highly receptor-specific and no such data has been reported elsewhere. 3) Thirdly, there is a possibility that phage preparations may contain residual bacterial antigens or endotoxins. To address this, bacteriophage production for clinical trials have to follow specific Good Manufacturing Practice (GMP) guidelines with appropriate quality controls, and to meet specific standards for purity and sterility.[6] In summary, this study provides the clear evidence that Phage therapy can be used to treat human MDRs which is the possible solution to the burning issue of MDR bacteria.

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