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PREFACE

The International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) is an autonomous, international, philanthropic and non-profit centre for research, education and training as well as clinical service. The Centre is derived from the Cholera Research Laboratory (CRL). The activities of the institution are to undertake and promote study, research and dissemination of knowledge in diarrhoeal diseases and directly related subjects of nutrition and fertility with a view to develop improved methods of health care and for the prevention and control of diarrhoeal diseases and improvement of public health programmes with special relevance to developing countries. ICDDR,B issues two types of papers: scientific reports and working papers which demonstrate the type of research activity currently in progress at ICDDR,B. The views expressed in these papers are those of authors and do not necessarily represent views of International Centre for Diarrhoeal Disease Research, Bangladesh. They should not be quoted without the permission of the authors.

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ABSTRACT

Of the typing methods of bacteria used for the epidemiological investigation of the disease, bacteriophage typing appears to be the most sensitive. Many authors have published papers on the use of cholera phage in the epidemiological studies of cholera and differentiation of biotypes of V.cholerae. Studies on shigella phages are limited to S. flexneri and S. sonnei phages. During the routine research for bacteriophage from stool of the patients or bacterial cultures from them we isolated a phage which produce round creanated pluck, has a burst time of about 18-22 minutes, is neutralised by its homologous antiphage serum and lyses all the S.dysenteriae type I and all the S. flexneri type 2, part of type 3 and 4 and none of type 1, 5 and 6. It does not attack any of the other Shigella sp. Salmonella, E.coli, Paracolon, Proteus and V.cholerae or other Vibrios and Aeromonas sp.

INTRODUCTION

Bacteriophages are a group of bacteria specific viruses which have the ability to bring about lysis of growing bacterial cultures. Since 1917 when d' Herelle published his independent discovery of bacteriophage the interest on the phages grew more. d' Herelle showed that when the filtrate of the stool from patient is added to a growing shiga bacillus the growing culture was killed and there is an increase in the titer of the lytic principle in the broth. This behaviour suggested by him as an ultra virus, pathogenic for bacteria, destroying its host cells as it multiplies. Because of the susceptibility of pathogenic bacteria to phages, and because of the wide distribution of phages in the nature, d' Herelle suspected that they played a role in resistance to and recovery from the disease. The role of bacteriophage in the characterisation of bacteria and its genetic behaviour have been studied by many authors. Anderson et al (1956) worked on the bacteria phage typing of enteric pathogens and showed clearly that the typing pattern is of use in the epidemiological investigation of the disease.

Most of the published works in South Asia has been on cholera phage and this has been studied with greatest care on many epidemic strains. The number of publications on phages

acting on shigella are very limited at this time. During the late 1960's a few studies were done on the use of bacteriophage along with colicine for the typing of shigella, specially *S. flexneri*. Stefan Slopek and his associates in Wrockland, Poland (2) divided 767 strains of *Shigella flexneri* into 69 types using 12 dysentery bacteriophages. Vera Lazlo et al (3) in Budapest worked on the phage typing of *Shigella flexneri* and got 90 phage types for 4606 *S. flexneri* strains. In their opinion phage typing proved to be suitable in epidemiological practice for subdividing the serotypes, for tracing the infection routes and for distinguishing between cases from different foci (4). Pruneda and Farmer from CDC, (5) while trying to establish a standardized schemes for differentiation shigella strains, typed 265 strains of *Shigella sonnei* into 87 different lysis pattern.

The works reported so far in different literature were limited to typing a single serotypes of shigella by some cluster of phages. In CRL we isolate every year large number of shigella from dysentery patients reporting to our hospital. In order to find out a good classifying set of phage for all or a few sero-types of shigella, we started looking for new isolates of phage from stool of patients reporting to our hospital. All these new isolates of phages are being tested against a set of shigella organisms to observe the lytic pattern. In the course of the search for new phage we isolated from a culture plate of

patient no. OPD-153950 a phage which caused lysis to the pure growth of *S.flexneri* on the plate. The phage plaque were picked up, purified and found to attack a range of host bacterial species (shigella) but do not act upon any other bacteria.

MATERIALS AND METHODS

Media used: For all the studies on the phage we initially started using two different broth and plating media. When Trypticase Soy Broth (TSB) BBL was used as enriching media the plating media were Trypticase Soy Agar (TSA) (BBL) and when T_1N_1 (Trypticase 1% sodium chloride 1%, pH 7.2) broth used as enriching media the plating media was T_1N_1 Agar. It has been found on comparison that T_1N_1 media which is used in our laboratory for Cholera phage work gives comparable result to Trypticase Soy medium. Hence all the works on this phage was carried out in T_1N_1 medium.

Isolation of phage preparation

The host culture no. P-18167 were inoculated into 5 ml. T_1N_1 broth and incubated for 4-6 hours to get a visible turbidity. One defined isolated plaque from the isolation plate were inoculated into the growing culture in the tube and further incubated for 6-8 hours at 37°C and kept overnight at room temperature. The residual bacteria in the phage enriched broth were removed by filtering the broth through 0.45 M millipore

filter. The titre of the enriched preparation of phage was enumerated by a) Miles and Misra method: Making serial 10 fold dilution of the phage broth and dropping them onto a lawn made of 6 hours grown culture of the host strain and incubating over-night at 37°C. The lysis in the area of the drop were recorded as Confluent lysis, (CL), Semiconfluent lysis (SCL), 3+, 2+, 1+. The count of phage plaque on the last countable dilution may be taken to quantitate the phage titre in the preparation.

b) Agar overlay method: 0.1 ml of the respective 10 fold dilution of the phage preparation were mixed with 0.5 ml of the 6 hour grown culture and the mixture is put in a small tube containing 3.5 ml. of the ½ strength T_1N_1 Agar (T_1N_1 broth containing 0.75% of Agar kept at 45°C). The whole mixture is mixed well and poured onto well dried T_1N_1 Agar plate. After the media is set the plates were incubated for 24 hours and read. The total number of plaques in the highest dilution is taken to quantitate the phage tire in the preparation. (Adams, 1959)(6). From the plates the size and shape of the plaques were recorded.

PURIFICATION OF PHAGE PREPARATION

Method (b) described above is used to purify the phage preparation. A single plaque of the type seen on the plate is picked up and enriched again as per above method. The bacterial culture picked along with the plaque acts as the enrichment

culture. The method may be repeated to get a homogeneous plaque type in the phage preparation.

Determination of Burst Time:

Overnight grown culture of p-18167 were inoculated into 5 ml. T_1N_1 broth and incubated at 35°C in a well controlled water bath. Bacterial count was made at 0 hrs. from the inoculated tube and then at minutes 10, 15, 20, 25 and so on at 5 minutes interval up to 75 minutes. Serial tenfold dilution of the broth culture was made and appropriate dilutions were plated onto T_1N_1 Agar plate to get countable colony. The total number of colonies recorded and burst time of the bacteria calculated.

Preparation of Antiphage Serum:

Antiphage serum was prepared by immunising rabbits weighing 1-2 kg. Four rabbits were chosen and kept aside in 4 cages for 1 week before starting the injection. Three injections I.V. of 0.3 ml. 0.4 ml. and 0.6 ml. of pure phage preparation of titre 109 pfu/ml. was given on alternate days. After one week test blood drawn. As the titre was not very high 3 more injections of 0.5 ml., 0.7 ml. and 1.0 ml of the above phage were given every alternate day and test bleeding done after 1 week's rest. The antiphage titre of serum were found sufficiently high and all the rabbits were bled to death using a septic precautions and after separation the serum was kept at 4°C in refrigeration without any preservative.

The ability of the serum to neutralize the phage preparation was measured using serum diluted to 1/10, 1/100 and 1/1000 and known amount of phage by using methods used by (Islam, 1967)(7). The titration of phage from phage serum mixture in each tube was made at 5, 15, 30, 45, 60, 75 and 90 minutes by using agar over-lay method.

The lytic pattern of this phage against other members of Enterobacteriacae, V.cholerae, pseudonas and Aeromonas was observed by dropping the dilutions of the phage on the lawn of respective cultures on T_1N_1 agar plate. Clear visible lysis of the bacteria by the phage preparation forming distinct plaques with diluted phage recorded.

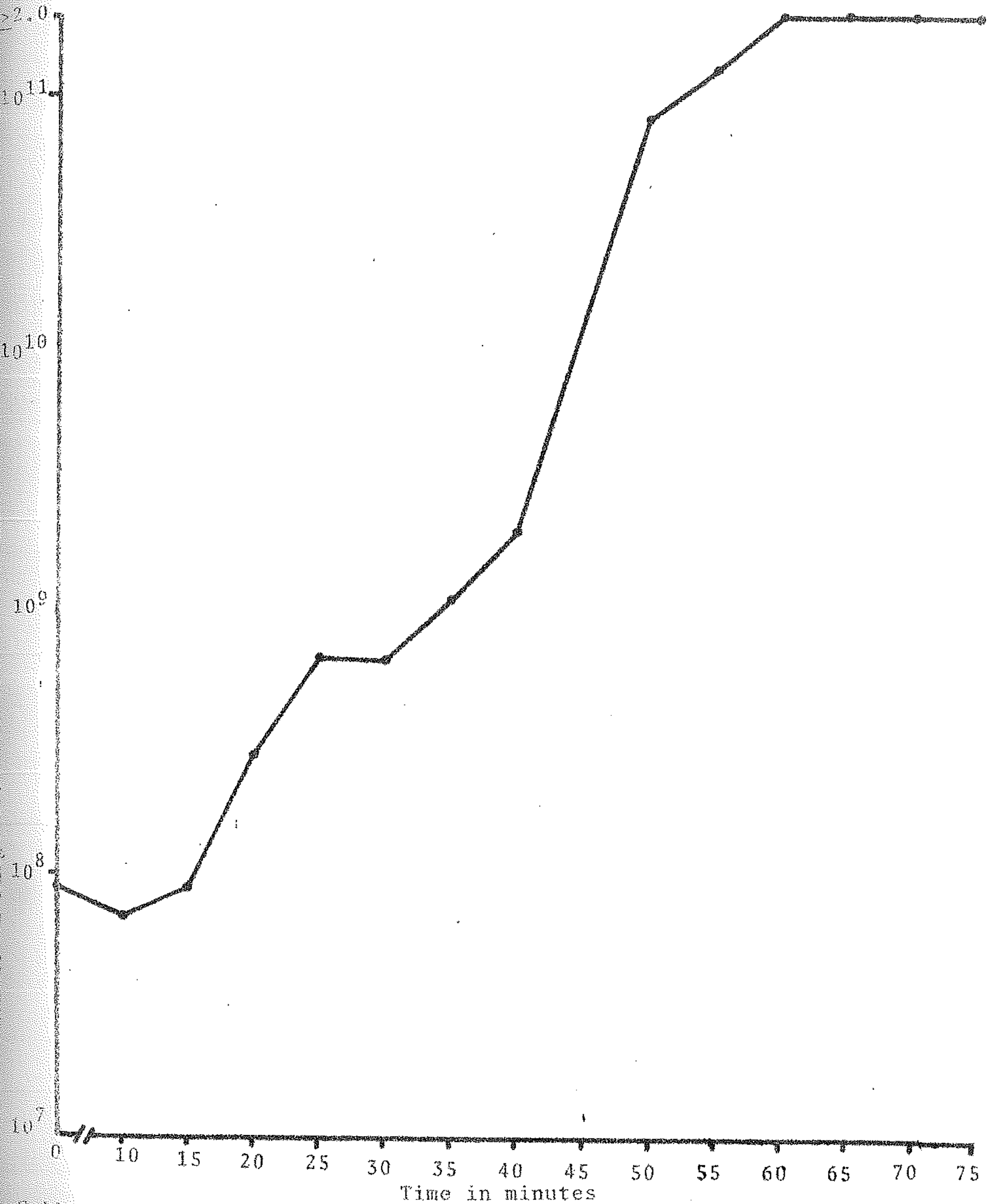
RESULTS

Graph I shows the multiplication pattern of the phage preparation when added in the broth in presence of the homologous bacteria. It can be seen that first bursting properly took place within 18-22 minutes; the second within 35-40 minutes and third within 54-58 minutes.

Table 1 shows the results of neutralization test done with the antiphage serum and the different dilution of phage at different intervals.

Figure I

Multiplication Pattern of the Phage Preparation



Subheading - include methods of experiment

TABLE 1

NEUTRALISATION OF PHAGE BY ANTIPHAGE SERUM

Serum dilution	1/10		1/100		1/1000	
Expected No. of Phage in the mixture	160	1600	160	1600	160	1600
Duration of incubation	Observed		p.f.u. per plate			
5 mins	18	270	74	SCL	SCL	CL
15 "	2	72	21	SCL	CL	CL
30 "	0	11	9	>SCL	SCL	CL
45 "	0	0	0	SCL	SCL	CL
60 "	0	0	0	>1000	SCL	CL
75 "	0	0	0	560	SCL	CL
90 "	0	0	0	410	CL	CL

p.f.u. = Plaque forming unit

SCL = Semi confluent lysis

CL = Confluent lysis

Table 2 shows the lytic pattern of the phage when tested against various strains belonging to Enterobacteriacae, V. cholerae and Aeromonas sp. It can be seen that the phage attacked all the S.dysenteriae type 1, all the S.flexneri type 2 and a part of type 3 and 4 but not type 1, 5 and 6. None of the other shigella serotypes, salmonella, E.coli, Para colon, proteus, V.cholerae and Aeromonas sp. was attacked.

DISCUSSION

The use of phage in typing the bacterial strain in looking the epidemiological patterns of the disease has been documented by various authors in the past. Recent work by Slopek et al has indicated that bacteriophage typing could become the method of choice for studying outbreaks from S.sonnei. Most of the later work has been done on cholera phages which has been characterised and patterns well established (8). The work on shigella phage are mainly on the S.flexneri phages (2-4) and S.sonnei (5, 9). In order to look for an universal phage which may attack almost all the important prevalent shigella species we carried out a routine search for phages which lyse all shigella sp. The phage reported in this paper has been found to have wide ranging lytic activity. With the usual enrichment procedure, using plain broth, we could enrich the phage to 2.1×10^{10} pfu/ml. which is different from other shigella phages.

TABLE 2

RESULTS OF LYTIC PATTERN OF SHIGELLA PHAGE AGAINST VARIOUS TYPES AND ENTEROBACTERIACAE V.CHOLERAE, AND OTHER GRAM NEGATIVE BACTERIA

Name of the bacteria	No. tested	Lytic Pattern
Shigella dys. type 1 (Shigella shiga)	171	All attacked
Shigella dys. type 2	22	None attacked
Shigella dys. type (3-10)	11	None attacked
Shigella flexneri type 1	24	None attacked
type 2	31	All attacked
type 3	23	Attacked
	15	Not attacked
type 4	16 17	Not attacked
type 5	12	Not attacked
type 6	8	Not attacked
Shigella boydii	20	Not attacked
Shigella sonnei		
Phage 1	16	Not attacked
Phage 2	18	Not attacked
Salmonella	49	Not attacked
E.coli	52	Not attacked
El Tor Vibrio	73	Not attacked
NAG vibrio	39	Not attacked
Paracolon	24	Not attacked
Aeromonas	8	Not attacked
Proteus	14	Not attacked

This phage preparation does not lose its titre appreciably when kept at 4°C for about 2 months. They form distinct round crenated pluck with a slightly clearer centre. As can be seen in Table 2 the phage attacks all the strains of S.dysenteriae type 1 tested in the series. The lytic pattern is variable with S.flexneri where all of type 2 and part of type 3 and 4 are attacked. No satisfactory explanation could be given for this selective action on the Shiga bacillus and partial action on S. flexneri. However none of the other shigella sp. or E.coli Salmonella, para colon and proteus sp. were attacked. The V. cholerae and NAG vibrios were also not attacked.

As S.shiga and S.flexneri type 2, 4 and 6 constitutes almost 75-80 percent of the shigella isolates in our laboratory we feel it strongly that biochemically confirmed shigella sp. may be further confirmed by testing them with this phage preparation thus avoiding in most cases the use of very costly antisera which are to be imported, from outside countries.

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