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LEADING ARTICLE

USE OF BACTERIOPHAGE AS A MARKER FOR IDENTIFICATION OF FRESHLY-ISOLATED INDIVIDUAL ESCHERICHIA COLI STRAINS

K.A. MONSUR, A. LATIF MIAH, M.I. HUQ, FARIDA HUQ[1], G. PODDAR, M.A. SALEK AND YASMIN ARA BEGUM

International Centre for Diarrhoeal Disease Research, Bangladesh, GPO Box 128, Dhaka-2, Bangladesh;
[1]Head, Microbiology Branch, Institute of Public Health, Dhaka, Bangladesh

Abstract

One or more of 24 selected bacteriophages at 100 and 1000 times routine test dilution (RTD), spotted on lawns of individual *Escherichia coli* strains were found to attack over 90% of 297 *E. coli* strains tested. It was found that in most cases, it may be possible to use the phage susceptibility pattern of an individual *E. coli* strain as an identifying marker for that strain. In four cases, *E. coli* strains with the same phage pattern, isolated from different individuals, were found to belong to the same serogroup. Similarly, of 16 enterotoxigenic *Escherichia coli* (ETEC) isolates from 75 contacts of cases with watery diarrhoea, 7 were found to have the same phage pattern as ETECs from their respective index case. The toxigenic character of each pair was also found to be identical. The practicality of using phage patterns for studying the dynamics of *E. coli* population was demonstrated by its ability to detect changes in the *E. coli* population in a single patient.

Key Words: Bacteriophages; *Escherichia coli*; Enterotoxins; Diagnosis, Laboratory; Serotyping; Diarrhoea.

Introduction

The *Escherichia coli* population in the gut of man and animals is a changing mixture of many different strains (3, 15, 16, 18). Epidemiological studies of *E. coli* infection must take into account this turnover. This has become all the more important because of the growing understanding of the importance of *E. coli* as a diarrhoeal pathogen all over the world.

Although extensive studies have been carried out on the academic and genetic aspects of *E. coli* phages, a systematic study on the use of phage for clinical and epidemiological investigations of *E. coli* appears to have been neglected. A number of publications indicate that such studies may also have useful applications. Thus a bacteriophage Ω 8 which is specific for *E. coli* 08 has been isolated from sewage (10, 19). Gross, Cheasty and Rowe isolated a bacteriophage specific for the K1 polysaccharide antigen of *E. coli* (7). More recently Gupta *et al.* (9) have described a bacteriophage specific for K5 antigen. This phage may be of help in the diagnosis of this serologically elusive antigen, which is among the most frequent K-antigen in strains from extra-intestinal diseases. It is, therefore, not unlikely that through a more intensive study it may be possible to isolate more and more phages which can identify other serogroups or may correlate with other characteristics of *E. coli* such as toxigenicity, enteropathogenicity, invasiveness.

In order to follow *E. coli* strains associated with infection, a tool is needed which is simple, easy to use, and is at least capable of differentiating individual *E. coli* strains from the different ones present in the feces. Although serotyping is a well-established method for identifying individual *E. coli* strains, only a few laboratories worldwide are capable of performing complete serotyping for *E. coli*. Commercially prepared antisera are available only for a limited number of antigens and are, therefore, not able to serotype all conceivable *E. coli* strains. Some investigators (2, 13, 17) felt that testing the bacteriophage susceptibility pat-

Correspondence and reprint requests should be addressed to: Dr. K.A. Monsur.

tern of individual *E. coli* strains against a suitable collection of phages might offer a potential alternative tool. However, little work appears to have been done to explore this possibility.

During an earlier study the authors observed that phages which attacked a number of *E. coli* strains were also found to attack some *Shigella* strains and vice versa. This study was undertaken to evaluate the effectiveness of using a battery of 24 bacteriophages, selected either from *E. coli* or *Shigella*, to differentiate between *E. coli* strains. We attempted to determine if *E. coli* isolated from different individuals, but having the same phage pattern, would also belong to the same serogroup (where this could be identified) and also to find out if the phage pattern associated with an enterotoxigenic *E. coli* (ETEC) strain from an index case could be used as a marker for detecting the ETEC strain from subsequent contacts.

Materials and methods

1. *Isolation and selection of phages*

Sewage water or fecal suspensions were sterilized with chloroform, centrifuged, and 1 ml of the supernatant, after evaporation of the chloroform, was added to 5.0 ml of an early log phase growth of randomly-selected *E. coli* or *Shigella* strains in heart infusion broth. After incubation at 37°C for 6 h the culture was sterilized with chloroform. The following day the chloroform was evaporated from 1 ml of this culture and 10^{-1} and 10^{-2} dilutions were spotted on well-dried lawns of a 2-h growth of bacterial cultures selected for phage enrichment and incubated overnight at 37°C. Phage plaques were observed on many of the inoculated spots, not only on the homologous strain but sometimes also on heterologous strains. This phage detection process was repeated many times and from more than 2000 possible plaques, 371 were arbitrarily selected for further study. Each of these selected phages was purified by 3 successive picks of single plaque from the propagating strain by the agar layer method (1).

The further selection of the phages was carried out in stages by testing each of the 371 phages against a battery of 100 or more *E. coli* strains (from a variety of sources and times in Bangladesh). Phages selected for further studies were those which demonstrated a unique host range pattern against the *E. coli* strains used. In general, the following criteria were used as the basis of phage selection :

(a) Only those phages which attacked nearly 10% or more of a battery of *E. coli* strains were accepted, using the dilution which produced confluent lysis against the propagating strain, termed as the routine test dilution (RTD).
(b) Selected phages had the widest host range, showing the least degree of overlapping reactions and attacking strains different from the others which were previously selected.
(c) *E. coli* strains which were not attacked by any of the previous phages, were tested repeatedly against fresh sewage samples in an effort to isolate phages specific for them, so as to broaden the overall host range of the phage bank to be used for the test.
(d) The phages had to be stable, and produce a titer of at least 10^9 plaque forming units (pfu)/ml.
(e) Preference was given for those phages which produced clear and distinct plaques. On the basis of these criteria, a selection of 24 phages was made.

2. *Preparation and preservation of phages*

Appropriate dilutions of the purified phages were mixed with the propagating bacteria and plated by the agar layer method to give a nearly-confluent lysis after overnight incubation. The plates were then frozen overnight at −40°C. Upon thawing the next day, the frozen agar contracted, expressing about 4 ml of clear fluid per plate. This fluid usually contained about 10^{11} pfu/ml of phage. The authors had found this technique useful when they needed small quantities of high titer phage.

Fluid from 2 plates were pooled, and centrifuged at 5000g for 20 minutes. The supernatant was collected. To increase stability, $CaCl_2$ and $MgCl_2$ was added to give a final concentration of 1 mM each. The fluid was then passed through a 0.22μ membrane filter. Chloroform (0.05ml) was added to each 2ml aliquot which was then stored at 4°C until used. The stocks were tested periodically for loss of titer. Most were found to be stable for about a year.

3. *Performance of the test*

It was observed that the host range of each phage increased if multiples of the RTD were used. It was, therefore, decided to use the phage in concentrations of 100 and 1000 times the RTD.

0.1ml of an overnight broth culture of a single colony was inoculated into 3ml of heart infusion broth and incubated for 1 hour. This young culture was then used to flood a well-dried heart infusion agar plate containing 20 ml agar per 9.0cm petri-dish. The excess fluid was removed by suction and the plate was then dried at 37°C for 1 h. Appropriate dilutions of the 24 phages were then spotted on this plate by using a multiple inoculator (Craft Machine Inc. 1-95 & Concord Road, Chester, Pennsylvania). A broth negative control was always included on each plate. Phage lysis was usually visible within 4 h but for standardization the plate was read after overnight incubation.

The presence or absence of phage lysis was recorded for each phage against each *E. coli* strain tested.

Testing for Toxins :

Tests for heat-labile toxin (LT) and heat-stable toxin (ST) were carried out by the use of CHO cells (8) and the infant mouse assay (4) respectively. Two pure isolates and a pool of 5 colonies were examined from each *E. coli* plate for the detection of toxigenic strains (12).

Serological identification :

E. coli colonies were grown overnight at 37°C on sheep blood agar plates. Growth was removed and suspended in saline, boiled for 1 h, centrifuged at 3000g for 15 minutes and the sediment resuspended in saline. Serotyping was done using standard techniques (5) with commercially-prepared *E. coli* "O" antisera (Difco Laboratories, Detroit, Michigan, U.S.A.).

Maintenance of stock cultures :

Stock cultures were maintained both in stab and agar slants made with blood agar base medium (Difco Laboratories, Detroit, Michigan, U.S.A.)

Results

Evaluation of the set of 24 phages for epidemiological investigation

The 24 selected phages were tested against 297 *E. coli* strains from different sources (all from Dhaka, Bangladesh). 280 (94.3%) were attacked by one or more of the phages while 17 (5.7%) were attacked by none. Of these, 280 susceptible strains, each of 244 gave a unique phage pattern of its own. In the remaining 36 strains there were 14 different pairs, each pair with its own phage pattern, and 2 different groups, each group having 4 strains with similar pattern.

Phage evaluation with enteropathogenic E. coli (EPEC)

The initial specimens for this study were collected from an ongoing case-control study carried out at the International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B) on patients with watery diarrhoea and their contacts. In all, 41 samples were examined, 27 from patients and 14 from contacts of 6 patients. Between 10-15 colonies were tested from each subject. Colonies showing identical or nearly-identical phage patterns were tested serologically in an attempt to determine if a relationship existed between the phage pattern and serogroup.

Sixteen different strains of EPECs belonging to 6 different 'O' serogroups were isolated. Some of the serogroups showed more than one phage pattern. Four pairs of *E. coli* strains could be isolated, each pair with its own phage pattern, different from those of other three. Two of these pairs could not be serotyped. The members of each of the other two pairs of *E. coli* were from different individuals but both strains of a pair belonged to the same serogroup, viz, 0111ab, 0111ac (Table, Example 4) and 0125ab, 0125ac. On two occasions, EPECs were isolated from patients along with *V. cholerae* 01. In one of them the EPEC strain with a unique phage pattern persisted along with *V. cholerae* 01.

Enterotoxigenic E. coli (ETEC)

A subsequent study was carried out to trace ETEC strains from the index case to their contacts using the phage pattern as a marker. Specimens for this study were obtained from a case-control study at ICDDR,B which was designed to determine if ETECs from an index case could be isolated from their contacts by testing for toxigenic *E. coli*. Isolated *E. coli* colonies from the stools of 20 cases and 75 of their contacts were examined for phage patterns and toxigenicity. Between 10-15 colonies from each index case and 5-10 colonies from each contact were examined. Simultaneously the colonies from the cases and contacts were tested for detection of toxigenic

OBSERVATIONS ON THE RELATIONSHIP BETWEEN PHAGE PATTERN, SEROGROUP AND TOXIGENICITY OF *E. COLI* STRAINS

Examples	Source of specimens	Colonies giving the pattern/ colonies tested	Phage Nos. against which the strain is susceptible																							Toxins		Serogroup	Remarks about phage pattern
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	ST	LT		
1.	Index case	15/16		C	C			C	C	C		C	C								C	2						+	+	untypeable	
	i) Contact	6/6		S	S			C	C	C		C	C								S	1						+	+	untypeable	Identical
	ii) Contact	1/6		C	C			C	C	C		C	C								S	2						+	+	untypeable	
2.	i) Index case	10/10	4	C			2	1	C	C			C		C	C	S	C		C		2			C	C		+	−	untypeable	Not identical
	Contact	2/5				S*			C	S			C													C		−	+	untypeable	
	ii) Index case	10/10				4				4		1	C		S	S		S		S			C					+	−	untypeable	Not identical
	Contact	4/5						1					S						C							S		−	+	018ab,018ac	
3.	Index A	10/10			C	1			S	C		C	C							1	C	2						+	−	untypeable	
	Index B	15/15			C				S	C		2	S							1	C	2						+	+	untypeable	Identical
	Contact B	1/10			C				S	C		2	C							1	C	1						+	+	untypeable	
4.	Unrelated–1	10/10				S			2													S			2	C				0111ab,0111ac	Identical
	Unrelated–2	1/15				S			2													S			2	C				0111ab,0111ac	
5.	1st Specimen	8/10		S	S	1			S	S		S	S								S	2						+	−	untypeable	Sudden change
	2nd Specimen	8/10	1	3	S	1			S	S		S	2								S	1						+	−	untypeable	in the 3rd
	3rd Specimen	10/10								S									C	S		1						−	−	020a,020b	specimen

The Nos. 1 to 4 indicates 1= +(⩽10), 2 = + + (11-25), 3 = + + +(26-50) and 4 = + + + +(>50) plaques.
C = confluent lysis.
S = semiconfluent lysis.
* = control.

strains as described earlier. ETECs from contacts having a phage pattern similar to that of index case were considered to be the same strain. Strains with similar patterns were further tested together as a group against the same phage dilutions. This was done to minimize errors due to experimental variations.

Using the conventional tests (4, 8), ETECs were detected in 16 of the 20 cases of diarrhoea. ETECs were also found in 16 of 75 contacts, all asymptomatic. In 7 of these contacts the phage pattern of the ETECs and also their toxigenic characteristics were similar to those of the index cases (Table, Example 1). The phage pattern of the remaining 9 contacts differed from those of their respective index cases. These strains also differed in their toxigenic and, on one occasion, serologic characteristics [Table, Examples 2(i) and 2(ii)]. Three individuals were found to have ETEC belonging to the same phage pattern though an LT component was missing in one of them (Table, Example 3). In one case the predominant *E. coli* population in the first and second specimens from one patient had the same phage pattern and both were ST-positive. However, there was a sudden unexplained change in the 3rd specimen from this patient, in whom all the 10 colonies examined belonged to an entirely different phage pattern and serogroup (Table, Example 5).

Repeat examinations of samples from index cases showed that the predominant initial strain persisted for 3 or 4 days and occasionally for 1 or 2 weeks.

The phage susceptibility pattern of the strains were found to remain stable in repeated tests within a period of 3 months. All the ETEC isolates were susceptible to at least three or more phages, which enabled the strain to be identified with a reasonable degree of accuracy.

In 7 of the 16 contacts in whom ETECs could be identified, the phage pattern and toxigenic characteristics of the strains matched with those of their respective index cases. Where the phage pattern differed, the toxigenic characteristics were also found to be different from that of the index case. It was found that the toxigenic strains of the index case could be followed by identifying their phage pattern. This indicates that the phage pattern is able to trace an individual *E. coli* strain.

Discussion

Our findings support the evidence that *E. coli* have numerous different phage patterns. Consequently, complete phage typing of all *E. coli* may not be possible or practical. However, it seems possible to use the phage pattern of a strain as a marker for identifying that particular strain from amongst many others. Thus :

i) Though the phage pattern and serogroup may be independent of each other, our assumption that the phage pattern can identify a particular strain is supported by the observation that frequently strains belonging to the same phage pattern also belonged to the same serogroup.
ii) In 7 out of the 16 ETECs which were isolated from the contacts of 20 index cases with ETEC diarrhoea, the ETEC had the same phage pattern and toxigenic characteristics as that of the respective index case. In the remaining 9 instances, although an ETEC was found in the contact, it had different toxigenic characteristics and also a different phage pattern, confirming that phage had been able to differentiate between ETEC strains.
iii) In spite of the continuously changing flora of the *E. coli* population in the gut, the toxigenic strain in the index case could be followed by identifying their phage pattern.

Merson *et al.* (11) isolated ETEC from 7% and Gorbach *et al.* (6) from 15% of healthy persons. In our study ETEC could be isolated from about 20% of contacts, all asymptomatic. The mere isolation of ETEC from the index and contact cases may, therefore, be incidental and does not establish the identity of the strains. This study suggests that the phage pattern may identify the strain more reliably. The fact that one of the 3 ETECs with the same phage pattern was ST + LT- while the other two were ST + LT + (Table, Example 3) is probably due to the loss of plasmid coding for LT and not related to phage characteristics.

Many potential uses of phage patterns as an identifying marker can be conceived. It may be possible to use it for studying the dynamics of the *E. coli* population in the gut, a subject about which there is very limited information. In this study, following a strain by observing its phage pattern made it possible to demonstrate that in one patient an initial ST + ETEC present in the stool in predominant numbers for two consecutive days, was suddenly replaced by another strain with a different phage pattern, toxigenic characteristics and serogroup (Table, Example 5)

This change would normally have been missed unless attention was drawn by the sudden change in the phage pattern. Again, as frequently happens, if, from the phage pattern, a strain of *E. coli* is found to predominate in the stool, and it is neither an EPEC nor ETEC yet is suspected as being a pathogen, it may be worthwhile to test the strain for its enteroinvasive and enterohemorrhagic capabilities (14, 20) or other virulence characteristics. Rather than a blind search, the phage pattern, in such a case may prove a more successful way of identifying these elusive strains. In our studies, on one occasion, in a mixed infection of *V. cholerae* 01 and EPEC, the phage pattern of the EPEC made it possible to trace this strain, along with *V. cholerae* 01, all throughout the course of illness.

In many laboratories the use of a pool of 5 or 10 colonies of *E. coli* from the primary plate is a standard practice for detecting the presence of ETECs (12). Accordingly, we are in the process of evaluation of this technique in our laboratory. We feel the identification of the phage pattern of the different strains used in the original pool examined against the patterns which may be subsequently isolated from the pool to be of significance. Finally the phage pattern may prove helpful in developing collections of different *E. coli* strains having specific characteristics such as ETEC, EPEC or EIEC.

In view of the innumerable different strains of *E. coli* and because of the very limited host range of the individual phages which have so far been isolated, a complete phage typing of all the *E. coli* strains appears virtually unmanageable and beyond the capabilities of any individual worker. Perhaps, because of these reasons, except for sporadic attempts (2, 13, 17) no serious efforts have so far been made to follow up this approach as a tool for epidemiological investigation of *E. coli* infection. This study indicates that even by using a small number of phages, it may be possible to develop a simple, workable tool which would, in most cases, enable one to establish the identity of two or more *E. coli* strains, from amongst a group of many others having different patterns. This promising technique is currently in its preliminary stage of development. Further studies are needed for better standardization of the test and to validate the results with other investigations.

Though the phage sensitivity patterns of strains have been found to remain stable over a short term period, to what extent this may change over time remains to be studied. Qualitative and quantitative changes in some of the surface components, possible over a period, may change the sensitivity pattern of the strains against some of the phages to which these surface components may serve as receptors. Phages which attach themselves to stable receptors, always exposed, may not show such variation, and with time, it may be possible to pick up more and more of these phages.

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