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Isolation and therapeutic efficacy of enteropathogenic *E. coli* phage cocktail against colibacillosis in neonatal goats

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Phage based therapeutics have shown promising results against the infections caused by the drug resistant bacteria. To combat the problem of antibiotic resistance posed by diarrhoeagenic *E. coli*, here, we identified and characterized 38 *E. coli* phages which were isolated from 70 solid sources (goat-faeces and soil). The *in vitro* lytic range of phage isolates (n=38) against 439 isolates of *E. coli* was found between 16 and 53%. Three phage isolates with highest host range showed lytic efficacy against 53, 48 and 46% of *E. coli* isolates, respectively. A preparation with above three phages was developed, and the phages of the preparation were found stable at wide range of temperature, pH and chloroform treatment. Endotoxin content of the preparation was found below the threshold level and it also passed safety and sterility tests. a total of 40 diarrheic goat kids were administered orally with the therapeutic phage preparation for two days twice daily. Total 21 diarrheic goat-kids were successfully treated using the therapeutic phage preparation, whereas 19 kids could not be treated (success rate: 52.5%; 21/40). The results of the current study provide insight for using lytic bacteriophages for therapeutic interventions against drug resistant *E. coli* responsible for colibacillosis in neonatal goat kids.

Keywords: Depression score, Diarrhoea, Faecal Score, Goad kids, Host Range

Plants respond to diverse environmental conditions without changing the DNA content by epigenetic Bacteriophages are possibly the oldest (~3 billion years old) and the most ubiquitous (~10^{30} – 10^{32}) known biological entities on the Earth[1] which and play a crucial part in maintaining microbial balance in nature[2]. They can be found in sufficient numbers in any place where their hosts are present[3]. Obligate lytic bacteriophages are used in the treatment of bacterial infections, and as biocontrol agents in the food industry, because they result in the lysis of bacterial cells[4]. In the last 20-30 years, many bacterial strains have shown resistance to antibiotics and the infections caused by these strains are very difficult to treat[5,6]. The problem of the antibiotic resistance is increasing day by day and has taken pandemic form worldwide[7]. In the past few years, the emergence of multi-drug-resistant (MDR), extreme-drug-resistant (XDR), and pan drug-resistant (PDR) bacterial strains has increased the problem immensely[8]. Hence, there is an urgent need to tackle the problem of antibiotic resistance. In this context, bacteriophage could be an alternative to antibiotics in treating the infections caused by the resistant bacterial strains.

Phage therapy is gaining popularity among researchers all over world[9]. Phage therapy has several advantages over antibiotics, such as (i) fast and promising antibacterial activity; (ii) no/little resistance, toxic effects and bacterial dysbiosis; and (iii) effective against L-forms of bacteria as well as MDR, XDR and PDR bacterial pathogens[4,10]. Phage therapy has been found useful against several bacterial infections in human and veterinary medicine[11]. Phage cocktails have shown promising *in vitro* activity against the bacteria[12]. Korf *et al.*[13] reported that the phage cocktail showed the lytic activity against extended-spectrum beta-lactamases producing *E. coli* isolates. Sanchez *et al.*[14] developed the phage cocktail with broad host range lytic activity against *E. coli* strains. Thus, in the current study, we planned to isolate and characterize the phages virulent to enteropathogenic *E. coli*, and to develop and assess the therapeutic potential of the phage cocktail having lytic enteropathogenic *E. coli* phages against colibacillosis in neonatal goat kids.

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Materials and Methods

Isolation of the host bacterium

Enteropathogenic *E. coli* (EPEC) isolated from neonatal goat kid affected with colibacillosis was selected as indicator (host) bacterium for isolation, purification, propagation, and characterization of the *E. coli* phages. Isolation and identification of the indicator bacterium was carried out as per standard cultural, morphological and biochemical protocols[15]. Confirmatory identification was done by conventional polymerase chain reaction (PCR) using specific primers; 5´-ATGGTGCTTGCGCTTGCTGC-3´ (forward primer)[16] and 5´-AATCCACTATAACT GGTCTGC-3´ (reverse primer)[16] targeting the bundle forming pilus-A (*bfp*-A) gene. The PCR mixture consisting of 50 ng template DNA, 10 pmol of each primer and 12.5 µL of 2 × PCR master mix (Takara, India) was diluted up to a 25 µL volume with nuclease-free water. PCR was performed with an initial denaturation at 95°C for 2 min followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. To detect gene size, the PCR product was analyzed using 1% TAE buffered agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, USA) along with 100 bp marker (MBI Fermentas, USA). Thereafter, the bacterium was sent to National Centre for Veterinary Type Cultures (NCVTC), Hisar, Haryana (India) for obtaining an accession number.

Bacteriophage isolation

For isolation of the *E. coli* phages, a total of 70 solid sources (goat-feces and soil) of the phages were collected from different sheds of the Institute (ICAR-Central Institute for Research on Goats) and various places of Mathura, Uttar Pradesh (India). The phage isolation was done as described by Mishra *et al.*[17]. About 10 g of the sample was homogenized in 100 mL of an SM buffer [0.1 M NaCl, 10.0 mM MgSO4. 7 H2O, 0.05 M Tris HCl (pH = 7.4), 1% Gelatin (w/v)] and thereafter the suspension was centrifuged (10000 rpm for 10 min) to remove the debris. The supernatant was then filtered through 0.22 µ syringe filter (Millipore, USA) and the filtrate was added to an equal amount of double strength BHI broth supplemented with 0.1% MgSO4.7 H_2O (HiMedia, India) and inoculated with the mid log phase host culture (EPEC). After incubation at 37°C for 16-18 h, the medium was centrifuged at 10000 rpm for 10 min. The supernatant obtained could be called bacteria free filtrate (BFF), was filtered through 0.22 µ syringe filter and tested for the presence of the phages. Four tubes each containing 5 mL of soft nutrient agar (0.8% agar) were kept at 50°C after melting to prevent the agar from solidifying. The broth culture of the host bacterium was incubated overnight and then, 50 µL of the overnight culture was poured in to a sterile tube for preparing the control plate. Then after vortexing, the molten soft agar was spread evenly on the surface of the hardened bottom agar and the plate was allowed to stand undisturbed for 15 min. Similarly, to the second, third and fourth tube; 200, 500 and 1000 µL of BFF was added to each tube, 50 µL of a pure overnight culture of the host bacterium was also added to each tube and plates were allowed to stand undisturbed for 15 min. After that, all plates including the control ones were incubated at 37°C for 18-24 h. Following the incubation, the plates were inspected for the presence of plaques.

Bacteriophage purification and propagation

Purification of the phages was done as per the standard plaque purification method[18]. An individual plaque was picked with the help of a sterile straight loop by just touching the plaque, and was streaked on the newly prepared control plate in form of the parallel lines. To harvest the phages, 2 mL of SM diluent was poured on to the plate and it was kept at 4°C for 4 h and thereafter the phages were harvested with help of the clean glass beads. Gross agar shreds were removed by the slow speed centrifugation and the supernatant (phage suspension) was filtered through 0.22 micron filter. Multiplicity of infection (MOI) was determined as per the method described by Mishra *et al.*[17]. Propagation of the phages was done by conventional liquid culture method and agar wash method[19]. The serial tenfold dilutions of the phage suspension in SM buffer were prepared, thereafter the agar overlay assay was used to quantify the phages. The numbers of visible plaques were counted between 30 and 300 which were expressed as plaque forming units per mL (pfu/mL). For long term storage of the phages, the phage suspensions in SM buffer were kept at 4°C, and were also stored as 50% (v/v) glycerol solution at –70°C. Alternatively, the phage suspensions can also be stored over chloroform (5% v/v) at 4°C.

Host range determination and selection of phages for therapeutic trial

In vitro lytic activities of the phages against *E. coli* isolates were determined by the spot inoculation

method of Park *et al*.[20]. Briefly, each culture was inoculated into sterilized Brain Heart Infusion broth (HiMedia, India) and incubated at 37°C. A 16-h old pure broth culture of each *E. coli* isolate was spread plated onto the nutrient agar plate and 3 to 5 µL of the phage suspension was aseptically placed on the dried surface of the agar. After 16 h of incubation at 37°C, the sensitivity of the isolates to the phages was observed by formation of clear circular zone. Top three phage isolates showing the highest lytic range against the *E. coli* isolates were selected for the preparation of the phage cocktail intended for the therapeutic trial. The equal proportion of the phage isolates were taken to make the preparation.

Morphological and molecular characterization of phages

Morphology of the phages was determined using Transmission Electron Microscopy (TEM) at the Plant Pathology Division, Indian Agricultural Research Institute, New Delhi (India). About 10 µL of the phage suspension was put on the grid and left for 30 s. The suspension was negatively stained using 2% (w/v) uranyl acetate on carbon-coated grids. Thereafter, the grid was observed for the morphological characterization of the phages.

For molecular identification of the three isolated *E. coli* phages, the phage glycoprotein-23 (*gp*-23) gene, which encodes for major capsid protein of the phages, was targeted in the PCR using the specific primers: 5´-TGTTATIGGTATGGTICGICGTGCT AT-3´ (forward primer) and 5´-TGAAGTT ACCTTCACCACGACCGG-3´ (reverse primer)[21]. The PCR mixture consisting of 50 ng template DNA, 10 pmol of each primer and 17 µL PCR master-mix (Takara, India) was diluted up to a 25 µL volume with nuclease-free water. PCR was performed with an initial denaturation at 98°C for 2 min followed by 30 cycles of denaturation at 98°C for 30 s, annealing at 52°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 5 min. The PCR product was analyzed using 1% TAE buffered agarose gel with 100 bp marker (MBI Fermentas, USA). The PCR amplified products were purified by gel elution, and sequenced using BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems Thermoscientific, USA) on both the strands using the PCR primers. The chromatogram data was proofread for errors and the forward and reverse strands were aligned using the ClustalW algorithm of BioEdit Version 7.0 tool[22]. A sequence identity plot was also drawn to highlight the point mutations in the open reading frame (ORF) of the coding region of *gp*-23 gene of *E. coli* phage isolate obtained from the current study using the aforesaid tool. The obtained DNA sequences of the above gene were submitted to NCBI-GenBank for the accession numbers. Phylogenetic analyses were computed using the Minimum Evolution method and the evolutionary distances were analyzed using he Maximum Composite Likelihood method. The Neighbor-joining algorithm was used to generate phylogenetic tree using MEGA 7.0[23]. Further, phylogenetic analysis was conducted for the sequenced gp-23 gene of current bacteriophage isolates and compared for evolutionary data with other standard bacteriophage strains from NCBI-GenBank.

Stability of phages

Resistance expressed by the *E. coli* phages to physical and chemical agents was determined using the method of Chow and Rouf *et al*.[24]. An amount of 0.1 mL of the phage suspension with titer of approximately 1×10^3 pfu/mL was mixed with 4.9 mL of BHI broth. This 5 mL was redistributed as 1 mL into 4 separate sterile test tubes. The test tubes were kept in water bath at various temperatures ranging between 30 and 60ºC at 10ºC intervals for 30 min. After 30 min, 0.1 mL of a tube was mixed with 50 µL of 14-16 h old broth culture of indicator organism in 5 mL of soft agar. The same procedure was performed with rest three tubes. The plaque count (pfu) was done following standard agar overlay method.

Likewise, phage stability was also assayed at pHs ranging from 5.5 to 8.5 and at chloroform treatment. For assessing the long-term stability of the phages at 4 and 37°C, 5 mL of each phage-preparation was kept at the mentioned temperatures and the lytic activity of each preparation was assessed by the spot inoculation method of Park *et al*.[20] at a weekly interval and the results were expressed as the phages' survivability percentage.

Pyrogenicity, sterility and safety testing of therapeutic phage preparation

The estimation of the endotoxin content was done to assess the pyrogenic potential of preparation using ToxinSensorTM Chromogenic LAL Endotoxin Assay Kit (GeScript/Genxbio, Canada). Sterility of the therapeutic phage preparation was done on blood agar. In sterility testing, 100 µL of the preparation was spread over the blood agar and then kept at 37°C for 4 days aerobically and anaerobically. To assess the safety of the preparation, it was given orally to 4

healthy neonatal goat kids and thereafter the kids were monitored for 2 days for any adverse reactions such as fever, depression, hypersensitivity reactions, etc.

Assessment of *in vivo* efficacy of therapeutic phage preparation

To assess therapeutic efficacy of the phage preparation against colibacillosis in neonatal goat-kids, the fecal score[25] (Table 1) and depression score[26] (Table 2) were taken into consideration. Three groups of neonatal goat kids affected with white scours were designated as control, treatment group and positive treatment control, respectively. The selected diarrhoeic kids were having the fecal score of either '3' or '4', and depression score of either '1' or '2'. On the phage treatment, when the kids showed fecal score of '1' and depression score of '0', and maintained them for at least 7 days, the treatment was considered successful. For the control study, a total of 8 diarrheic neonatal goat-kids were orally fed with the preparation without phages twice in a day for two days. In the treatment group, a total of 40 diarrhoeic goat kids were orally administered with the therapeutic phage preparation twice per day for two days. For the positive treatment control, 8 diarrheic kids were given gentamicin at dose rate of 4 mg/kg twice per day for 3 days. Sodium bicarbonate solution (7.5%) was orally fed to the kids of the control and treatment groups prior to administration of the respective preparations. The kids of all groups were observed for a week. The diarrhoeic kids were kept in the separate pens under the intensive rearing system as per standard practices. The kids were allowed to suckle the mother for 15 min twice per day.

Table 1 — Correlation of faecal score with diarrhoea

Fecal score	Consistency of fecal matter	Severity of diarrhoea
1	Solid/Normal (N)	No Diarrhoea (Healthy Condition)
2	Soft/Pasty (S)	Mild Form of Diarrhea
3	Runny (R)	Moderate Form of Diarrhea
4	Watery (W)	Severe Form of Diarrhea

Table 2 — Correlation of depression score with diarrhea

Score	Attitude (Demeanor)	Suckling Behaviour
0	Normal-alert; ambulatory-runs away	Present-vigorous
1	Sternal recumbency but stands when approached and walks away; does not run	Present–seeks nipple, but not actively
2	Lateral recumbency–turns to sternal recumbency when coaxed but does not stand	Present–very weak, holds nipple with difficulty
3	Lateral recumbency-comatose	Absent

Results

Isolation of host bacterium

The organism (host bacterium) was identified as Gram-negative short rods and showed catalase positive, oxidase negative and IMViC reactions as +ve, +ve, –ve and –ve. The organisms showed lactose fermentation on MacConkey agar and typical green metallic sheen on Eosine Methylene Blue agar. PCR Amplification of the *bfp*-A gene resulted in a single amplicon of 150 bp (Fig. 1) which tentatively confirmed that it was EPEC. The bacterium was confirmed as EPEC by getting accession as VTCCBAA1160 by NCVTC, Hisar, Haryana (India).

Isolation, purification, and propagation of *E. coli* bacteriophages

Detection of the phages was determined by the presence of plaques (Fig. 2). Purification was done by plaque purification method in which a single plaque was streaked on the soft agar in form of parallel lines. After overnight incubation at 37°C, the clear

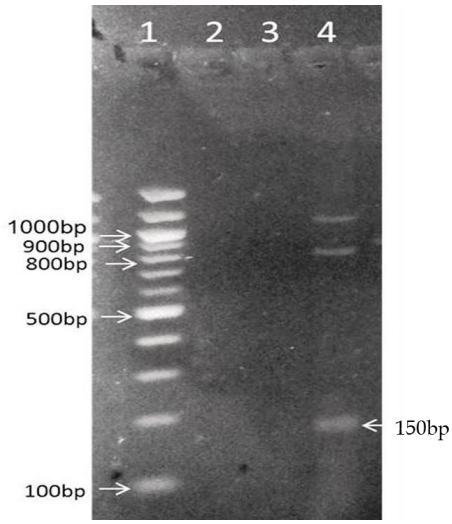

Fig. 1 — Amplification of *bfp*-A gene of EPEC. [Lane 1: Molecular weight marker, Lane 2-3: Negative controls, and Lane 4: Amplified gene product]

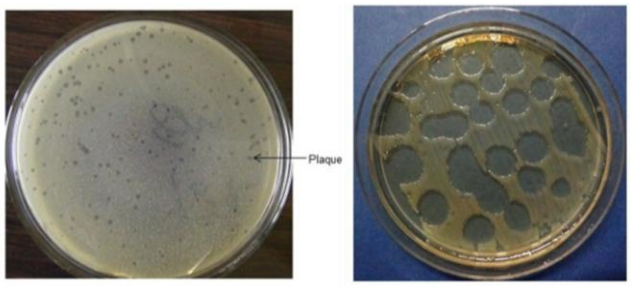

Fig. 2 — Presence of phages in the form of plaques and clear circular areas in lawn cultures of *E. coli*.

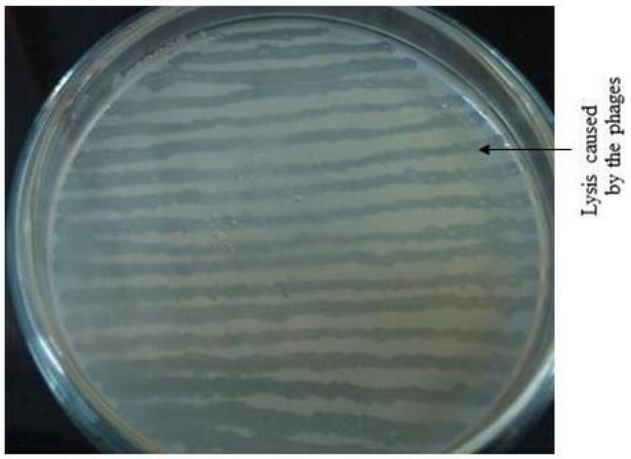

Fig. 3 — Purification of phages by streak plate method.

areas around the parallel lines were observed (Fig. 3). The optimal MOI was found as 0.01. Out of 70 samples, 38 isolates of the *E. coli* phages were isolated, purified and propagated. The concentration of the phages in the harvest of agar wash method was found higher (10^{10} to 10^{12} pfu/mL) than the conventional liquid culture method in which it was found in a range of 10^7 to 10^8 pfu/mL. Each purified phage suspension (n=38) was kept at 4°C, over chloroform (5% v/v) at 4°C and into 50% (v/v) glycerol solution for further use.

Host range determination and selection of phages for therapeutic trial

The sensitivity of the target organism (*E. coli*) against the test phage was observed by the formation of a clear circular zone in the spot test (Fig. 2). The *in vitro* lytic ranges of the phages are given in Table 3. The *in vitro* lytic activity of the phage isolates (n=38) against 439 isolates of *E. coli* ranged from 16 to 53%. Top three phages with the highest lytic range showed lytic activity against 53, 48 and 46% of *E. coli* isolates, respectively. Hence, these three phages (in form of phage cocktail) in concentration of 10^{11} pfu/mL were selected for therapeutic trial against colibacillosis in neonatal goat-kids. The three selected phages were taken in the equal proportion to make the final cocktail.

Morphological and Molecular characterization of phages

Dimensions and shape of the *E. coli* phages seen under TEM are shown in Fig. 4. The electron microscopy showed that the phages had an icosahedral head and a contractile tail. The tail length was about 148.24 nm whereas diameter of the head was about 74.37 nm. On the basis of the

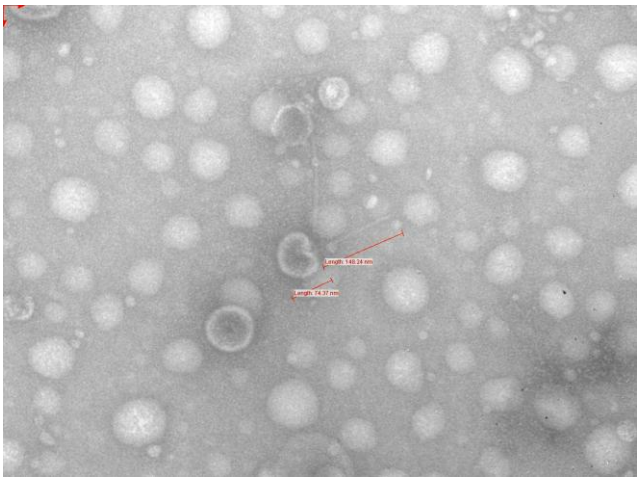

Fig. 4 — *E. coli* phage under transmission electron microscope

Table 3 — Lytic potential of *E. coli* bacteriophages

Serial no. of phage isolate	Total no. of *E. coli* isolates tested	No. of *E. coli* isolates lysed	Lytic efficacy (%)
1	439	134	30
2	439	145	33
3	439	212	48
4	439	123	28
5	439	192	43
6	439	132	30
7	439	110	25
8	439	102	23
9	439	112	25
10	439	140	31
11	439	233	53
12	439	202	46
13	439	90	20
14	439	130	29
15	439	110	25
16	439	149	33
17	439	146	33
18	439	107	24
19	439	115	26
20	439	144	32
21	439	135	30
22	439	137	31
23	439	82	18
24	439	155	35
25	439	125	28
26	439	112	25
27	439	199	45
28	439	189	43
29	439	144	32
30	439	139	31
31	439	135	30
32	439	91	20
33	439	74	16
34	439	123	28
35	439	80	18
36	439	118	26
37	439	124	28
38	439	143	32

morphological characteristics, the *E. coli* phages were classified as members of *Myoviridae* in the order *Caudovirales*.

PCR amplification of the *gp*-23 gene of the selected three phages resulted in to an amplicon of 850 bp size (Fig. 5) and the accessions received from NCBI GenBank of the submitted *gp*-23 gene sequences were MK358142, MK836292 and MK836293, respectively. The sequence analysis indicated point mutations of A→T at 35th nucleotide position for Phage-30 (Phage No. 3), and A→C at 108th nucleotide position for Phage-59 (Phage Number 12) in the *gp*-23 gene among the selected phage isolates (n=3) (Fig. 6). We compared our sequences with 10 sequences of other phages of *Myoviridae* family. The sequence identity plot portrayed multiple point mutations in the open reading frame of the gp-23 gene of the current phage isolates (Fig. 7). In phylogenetic analysis, there were two major branches, with the first branch having two sub-clades and the second one having a single clade with two taxa (Fig. 8). The first branch has three CIRG-phage isolates, whose taxa are closer to *Shigella* and *Yersinia*-phages, while the other subclade contained *Salmonella*, *Citrobacter*, *Staphylococcus*, and *Enterobacter* phages. The reference coliphage is located in the distant taxon when compared to CIRG *E. coli* phage isolates.

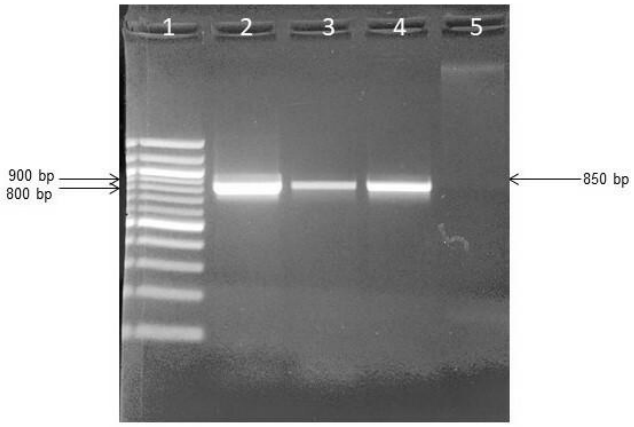

Fig. 5 — Amplification of *gp*-23 gene of *E. coli* phage. [Lane 1: Molecular weight marker, Lane 2-4: Amplified gene products, and Lane 5: Negative control]

Stability of the phages

The thermal tolerance study revealed that the phages of the cocktail showed 100% survivability at 30 and 40°C and it declined sharply at 60°C (Table 4). Likewise, at pH 6.5 and 7.5, the survivability was found 100% (Table 4). On the chloroform treatment, 5% reduction was observed in the survivability of the phages of the cocktail. The phages of the cocktail showed 90% survivability when kept at 37°C for one month. When kept at 4°C, the phages were found stable for a very long time (>2 years).

Pyrogenicity, sterility and safety testing of therapeutic phage preparation

The endotoxin content of the preparation was found as 1.04 EU/mL. In sterility testing, no bacterial growth on blood agar was observed after 4 days of incubation. Hence, the preparation was found sterile. Under safety testing, no visible adverse effects were noticed after administration of the preparation during the observation period (2 days). Thus, the therapeutic preparation was found safe for use against colibacillosis in the diarrheic neonatal goat kids.

Therapeutic efficacy of phage preparation

No therapeutic efficacy of the preparation (without phages) was observed in the kids of the control group and all the kids showed no signs/symptoms of improvement after receiving the preparation twice in a day for 2 days. In the treatment group, a total of 21 diarrheic goat kids were successfully treated using the therapeutic phage preparation whereas 19 kids could not be treated (success rate: 52.5%; 21/40). The treated kids did not show diarrhea during the observation period, i.e., 7 days. All kids of the positive treatment control were treated successfully within two days.

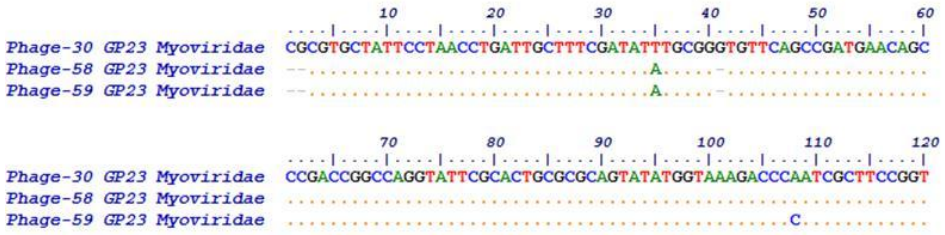

Fig. 6 — Point mutations in glycoprotein-23 gene among the selected phage isolates

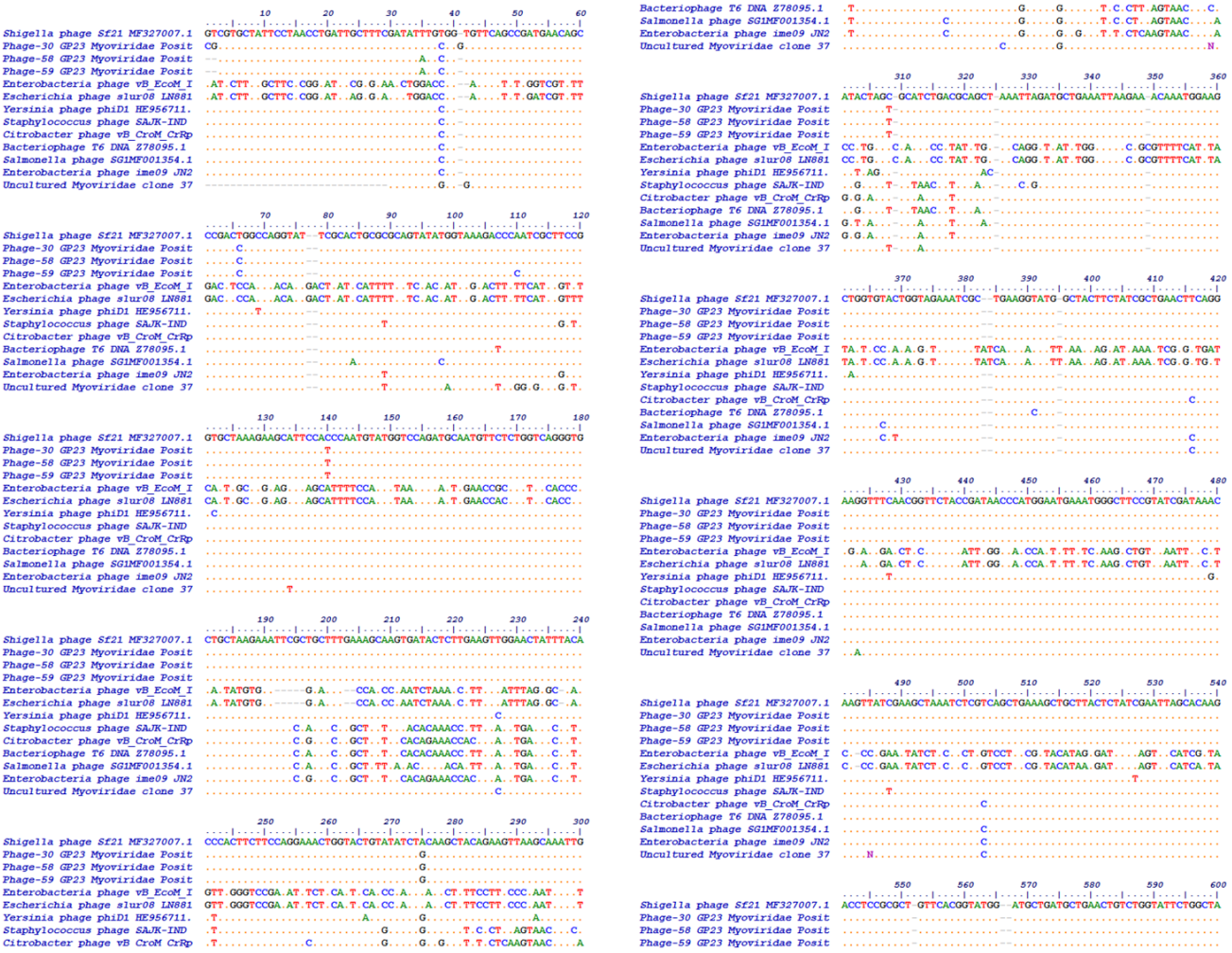

Fig 7 — Multiple point mutations in open reading frame of various *gp*-23 sequences

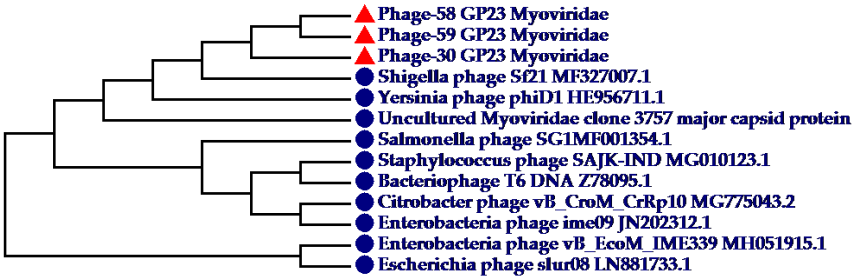

Fig. 8 — Phylogenetic tree showing genetic relationship of selected *E. coli* phages with other phages

Table 4 — Stability of *E. coli* phages at various temperatures and pH

Temperature (°C)	% Survivability of phages	pH	% Survivability of the phages
30	100	5.5	85
40	100	6.5	100
50	65	7.5	100
60	0	8.5	90

Discussion

Diarrhea is a predominant cause of mortality in neonates of most animal species including goats[27] and the most common one among the diarrheal diseases is colibacillosis (white scours)[28]. In the current study, the kids having white scours caused by EPEC were selected for the phage therapeutic trial. The fecal and depression scores were used to assess therapeutic

efficacy of the phage preparation, because they were directly related to severity of diarrhoea[25,26]. EPEC was selected as an indicator (host) bacterium for the isolation of *E. coli* phages, because it was found the most frequent cause of colibacillosis in neonatal goat-kids in the earlier study conducted at the Institute (ICAR-CIRG, Makhdoom)[29]. In feces and soil, the host bacterium is present in the abundant quantity, hence goat-feces along with soil was selected as source for the isolation of *E. coli* phages. A cocktail having three phage isolates with the highest lytic ranges was selected for the therapeutic use against colibacillosis in neonatal goat kids as the phage cocktails are preferred over single phage for therapeutic use against variety of bacterial infections[30,31].

Electron microscopy is done to assess morphological characteristics of bacteriophages and based on the morphological characteristics; classification of the *E. coli* phages could be done[19]. The tailed phages are classified under order *Caudovirales* and the phages with long contractile tails are subclassified in the family *Myoviridae*[32]. In the current study, the phages were having icosahedral head with long contractile tail. That is why, we considered that the phages could belong to the family *Myoviridae* in the order *Caudovirales*. Further, phage genome sequencing is required to ensure phage classification[33].

For molecular identification of the *E. coli* phages by PCR, the *gp*-23 gene was targeted which encodes for major capsid protein of the phages[21]. A high throughput sequencing from water samples of the lake Baikal revealed high genetic diversity in the *gp-23* gene of the T4-like bacteriophages whose indicator hosts were members of the *Enterobacteriaceae* family[34]. The current study found some interesting features of taxonomical closeness between CIRG *E. coli* phages, *Shigella* phage, non-*Enterobacteriaceae* member and *Yersinia* phage compared with the reference *E. coli* phage '*Slur08*' which appeared as a distant taxon. This may be an indication of the shared niches of these phages and the presence of various host bacterium leading to an emergence of diverse strains of phages hosting them. Another study[35] conducted on *E. coli* Myophages ST32 isolated from lakes in China revealed a double-stranded DNA genome of ~53 Kilobases and when compared with *Enterobacter* phage, only 47 of the 79 ORFs shared >90% identity. This can be the reason why the reference *E. coli* bacteriophage appeared as a distant taxon to CIRG *E. coli* phage isolates. The sequencing analysis could relate to various associations including antimicrobial resistance in indicator host as reported earlier[33] that carbapenem-resistant *Klebsiella pneumoniae* phage viz., vB_Kpn_F48 could be a promising candidate for use in phage therapy applications[36]. A similar approach in the current study revealed that the CIRG *E. coli* phages when characterized by gene sequences and other properties (lytic efficiency, physicochemical properties, etc.) showed a promising candidate for the phage therapeutic preparation which was subsequently prepared and tested in goat kids. On the contrary, the study[37] also targeted the protein sequence similarity of *Myoviridae* members and their subfamilies including *Peduovirinae*, *Teequatrovirinae* and *Spounavirinae*, and found that 4 groups belonging to the 'T4-like viruses' shared >70% proteins based on the CoreExtractor distance measure.

Characterization of the phages intended for the therapeutic trials is necessary, because the therapeutic use of the uncharacterized phages mostly result in to the failure[38]. Therefore, in our study, the stability of the phages intended for therapeutic use was assessed at various temperatures, pH and chloroform treatment. The phages of the preparation showed tolerance to wide range of temperature, pH and chloroform treatment. Due to stability to a wide temperature and pH range, the phages of the preparation would capable to withstand hot climatic conditions of India and the pH of the various sites inside the body[39]. To eliminate any possibility of transfer of toxic as well as resistance genes present in the phages intended for therapeutic use, the chloroform treatment is done as it removes the bacterial cells infected with lysogenic phages[40]. Assessment of the long-term stability is required for any phage preparation to be used therapeutically[39]. The stability of the phages of the cocktail was found quite satisfactory at 4 and 37°C. Estimation of endotoxin content in the therapeutic phage preparation is mandatory as it is produced due to lysis of host bacteria cells by the phages which may cause endotoxic shock. The endotoxin content of the phage preparation was found below the recommended level[41]. Hence, the preparation was found safe.

The preparation was found sterile in the sterility test, that is, viable cells of EPEC were not present in

the preparation. Hence, there was no possibility of occurrence of diarrhea induced by *E. coli* already present in the preparation. In the safety test, the preparation was found safe for therapeutic use hence, it could be used in the therapeutic trials without any detrimental effects on the health of the kids. Sodium bicarbonate solution (7.5%) was fed to the kids prior to the administration of the phage cocktail, because it is a biologically safe alkali and provides protection to the phages against acidic environment of the stomach[42]. Out of 40 kids, 21 kids were successfully treated using the phage preparation showing 52.5% success rate. The untreated kids (n=19) might have been infected simultaneously with other infectious agents. Hence, the kids did not respond to the phage treatment. The findings of the current study are supported by many other workers[31,43], who also demonstrated *in vivo* therapeutic efficacy of the phages against *E. coli* infections including colibacillosis. Hence, the developed therapeutic phage preparation could be used against neonatal colibacillosis in goat kids.

Conclusion

Out of 38 identified *E. coli* phage isolates, three isolates having the highest host ranges were selected for the therapeutic treatment against colibacillosis in neonatal goat kids. A phage cocktail was prepared using the above three phages and it passed stability, sterility, pyrogenicity and safety tests. The prepared phage cocktail was found effective in the treatment of neonatal colibacillosis in goats with success rate of 52.5%.

Ethical approval

The permission for the animal-experimentations was obtained from the Institutional Animal Ethics Committee (IAEC) and the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Department of Animal Husbandry and Dairying, Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

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Conflict of Interest

Author declares no competing interests.

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