RESULTS
All animals in this study were healthy throughout the trial and showed no ill effects of carrying either cholera disease or phage. All animals were culture-negative for *Vibrio cholerae* prior to experimental challenge with bacteria.

**Clinical and histological findings in RITARD model**

Rabbits infected with *Vibrio cholerae* O1 strain MAK 757 produced diarrhoea. Grades II–IV type diarrhoea was observed in the control rabbit. However, in the experimental rabbit treated with phage, only Grade I and subsequently Grade II was observed. *Vibrio cholerae* O1 was recovered by direct plating on TCBS agar from the rectal swabs of control and experimental rabbits. Visual examination at 24 h after challenge revealed a lower amount of haemorrhagic fluid accumulation and reduced peristaltic movement in the phage-treated rabbit compared with the control rabbit, which had considerably more fluid accumulation as well as massive peristaltic movement at the site of inoculation (Fig. 5).

Histological results showed that in the control rabbit (MAK 757- treated), villi lost their normal shape and contour (Fig. 6). The villi were grossly shortened and widened with inflammatory cellular infiltration in the villous lamina propria. Blood vessels were congested and large numbers of neutrophils were present in the superficial lamina propria and in the lumen. Bacteria were present in large numbers on the surface and within the luminal epithelial cells, indicating acute diarrhoea. On the other hand, villi of the intestinal mucosa appeared almost normal in the experimental rabbit (phage-treated). Cellular infiltration was observed in the villous lamina propria but inflammatory cells were minimal. From these observations, it appeared that the phage-treated rabbit was significantly protected compared with the control rabbit.

**Analysis of Vibrio cholerae MAK 757 and Vibrio cholerae O1 phages in RITARD model**

Both in vitro and in vivo experiments were conducted to investigate the therapeutic use of phage cocktail for controlling *Vibrio cholerae* infection. After 24 h of infection, the untreated rabbit was visibly ill, lethargic and scruffy. However, the experimental rabbit was found to be healthy (absence of Grades III and IV diarrhoea). Thereafter, colonies and plaques were counted following serial dilution of the
intestinal homogenate from both the rabbits (Table 1). The control rabbit had a mean of $1.5 \times 10^{11}$ CFU/mL of MAK 757, which was 100 times greater than the administered dose. The phage-treated rabbit at 24 h post-infection produced 100-fold less infectious cells ($1.3 \times 10^9$ CFU/mL) than the untreated rabbit but also showed the presence of 100-fold more phages ($0.9 \times 10^{10}$ PFU/mL) than the administered dose. The mean and S.D. of bacterial counts were calculated for both the administered dose (mean $7.86 \pm 0.47$ CFU/mL) and intestinal contents (mean $5.78 \pm 0.35$ CFU/mL). The difference in bacterial counts in both rabbits was statistically significant as determined by Student’s t test ($P = 0.01$), Wilcoxon–Mann–Whitney rank test ($P = 0.01$) and Kolmogorov–Smirnov test ($P = 0.003$). In the i in vitro model, similar conditions and parameters were used as in the in vivo model. The control set-up, which received $1 \times 10^9$ CFU/mL bacterial cells, showed $0.5 \times 10^{11}$ CFU/mL at 24 h post-infection. Simultaneously, the experimental set-up (phage treated) infected with $1 \times 10^8$ PFU/mL cocktail phages (Vibrio cholerae O1 phages), showed 100 times less bacterial cells than the control set-up and 100 times more phages ($0.75 \times 10^{10}$ PFU/mL) than the infected one.

**Lytic activity of individual and phage cocktail against Vibrio cholerae**

The lytic activity of vibriophages (ATCC B1, B2, B3, B4, and B5) and the cocktail were evaluated in in vitro. Vibrio cholerae O1 MAK 757 was mixed with the phages or the phage cocktail separately at an MOI of 0.1 and incubated at 37°C with shaking. Results showed that the phage cocktail was more effective as a lytic agent than the individual phages (Fig. 7).

**Faecal shedding of phage cocktail in the absence of host in rabbit**

Survival and stability of the “phage cocktail” was determined in faeces of uninfected animals after oral inoculation by gastric tube with $1 \times 10^8$ PFU. Faecal samples from each of the animals were collected at 1, 3, 6, 12, 24, 36 h and phage count was enumerated. It was seen that in the faecal samples phage count decreased steadily from the 1st ($6 \times 10^6$ PFU/gm of faeces) to 12th ($6.7 \times 10^1$ PFU/gm of faeces) hour after which the presence of phage could not be detected (Fig. 8).
Clinical findings for oral treatment in rabbit

After the oral administration of $1\times10^9$ CFU *Vibrio cholerae* MAK 757 grade II - IV type diarrhoea was observed in two control rabbits (one in each set of experiment). In two rabbits (one in each set of experiment) treated with phage cocktail 6 and 12 h after the bacterial inoculation, only grade I diarrhoea was seen. Two rabbits (one in each set of experiment) which were administered with phage cocktail 6 and 12 h prior to *Vibrio cholerae* inoculation, developed grade II to III diarrhoea. *Vibrio cholerae* O1 was recovered from the rectal swab of control and phage treated rabbits by direct plating on TCBS agar (Eiken Chemicals, Tokyo, Japan). Two control rabbits (one in each set of experiment) administered only with the phage cocktail was critically observed for any abnormal temperature change, behavioural change or any other symptoms of adverse effects. No abnormal temperature change, behavioural change or any other symptoms of adverse effects was seen.

Shedding of *Vibrio cholerae* and phages in rabbit

Animals which were administered the phage cocktail 6 h after the bacterial challenge shed 100 fold lesser number ($1.2\times10^3$ CFU/gm of faeces) of *Vibrio cholerae* at the 60th hour compared to 12th hour ($2.8\times10^5$ CFU/gm of faeces) whereas rabbits administered with phage cocktail 12 h after the bacterial challenge shed 10 fold lesser number ($3.8\times10^5$ CFU/gm of faeces) of *Vibrio cholerae* at 60th hour compared to 12th hour ($6.4\times10^6$ CFU/gm of faeces) (Fig. 9a and b). A 6 h post-treatment had been found to be effective in reducing the number of *Vibrio cholerae* by 100 times (p < 0.01) but the 12 h post treatment is not effective statistically in treating the infection (p > 0.05).

No reduction in bacterial shedding by the rabbits that were administered phage cocktail 6 and 12 h prior to the bacterial challenge was seen, compared to the control (Fig. 9a and 9b). Unlike untreated controls (one rabbit in each set) treated rabbits did not show grade IV diarrhoea. Result showed that the phage count declined rapidly in both the cases. In case of 6 and 12 h post treatment, phages could be detected upto 36th hour. The counts were 138 and $1.1\times10^3$ PFU/gm of faeces respectively. In case of the 6 and 12 h pre-treatment, phages were detectable only upto the 24th hour. The
counts were 424 PFU/gm of faeces for 6 h pre-treatment and 56 PFU/gm of faeces for 12 h pre-treatment (Fig. 9c).

**Phage genome analysis**

To evaluate the phage genome size, phages DNA were separated by PFGE. It was found that vibriophage B1, B2, B3, B4 has a genome size ~ 40 kb. The genome of vibriophages B5 was seen to be of ~ 100 kb (Fig. 10).

**Influence of temperature on vibriophages**

Vibriophages (ATCC B4 –B5) were found to be relatively heat stable as over a period of 1 h, between a temperature range of 25-60°C, not a significant loss in phage activity was observed. As they were detected viable at a titre of $1.96 \times 10^6$ PFU/ml and $1.6 \times 10^6$ PFU/ml respectively. Only 2 log$_{10}$ reduction or 25 % decline in viability was found compare to the control ($1.4 \times 10^8$ and $1.25 \times 10^8$ PFU/ml) respectively at 4°C (Fig. 11a). In case of vibriophages B1, B2, B3 the viable counts at 60°C were $9.6 \times 10^4$ PFU/ml, $4\times 10^4$PFU/ml and $9.3 \times 10^5$ PFU/ml respectively which showed almost 50% reduction in their viability compare to the control ($1.66 \times 10^8$ PFU/ml, $1.6 \times 10^8$PFU/ml, $1.2 \times 10^8$PFU/ml at 4°C.

**Influence of organic solvent on vibriophages:**

Almost no effect on phage activity was seen after 1 h of incubation with chloroform but diethyl ether causes reduction in phage counts upto 1 log$_{10}$ PFU/ml. However, after ethanol treatment complete loss of phage activity was seen (Fig. 11b).

**Influence of pH on vibriophages:**

Vibriophages were found to be resistant to low pH as they can retain their activity at low pH 2. Vibriophage B1 and B2 ($5.1 \times 10^3$, $5.5 \times 10^3$ PFU/ml) showed more sensitivity to low pH compare to the vibriophage B3, B4, B5 ($2.8 \times 10^4$, $3.3 \times 10^4$ and $5 \times 10^4$ PFU/ml). In case of basic pH vibriophage B1 and B2 ($7.5 \times 10^2$, $8.3 \times 10^2$ PFU/ml) showed high sensitivity at pH 12 than phage B3, B4, B5 ($4.6 \times 10^3$, $6.2 \times 10^3$, $3.2 \times 10^3$ PFU/ml). In this experiment vibriophages never lost its infectivity completely (Fig.
11c). Whereas, within a pH range of 6–8 no difference in the phage titers with respect to control was observed. Optimum stability of vibriophages was found at pH 7.0.

**Survival and stability of phage cocktail in adult mice in the absence of host**

Within first hour of oral administration of phage cocktail into mice a titer of $5.65 \times 10^7$ PFU/ml in GI tract increased to a titer of $1.6 \times 10^8$ PFU/ml within next 6 h was observed. However, by 12th hour of administration, titer had fallen to $2.3 \times 10^5$ PFU/ml and afterwards no plaques were found. Likewise, phage cocktail titer in liver and spleen was also estimated. Titers in liver at 1, 3, and 6 h after phage inoculation were $2.86 \times 10^4$ PFU/ml, $1.6 \times 10^6$ PFU/ml, $1.1 \times 10^8$ PFU/ml respectively. However, phage titers in spleen at respective time periods were $6.3 \times 10^3$ PFU/ml, $1.53 \times 10^5$ PFU/ml and $4.3 \times 10^7$ PFU/ml (Fig. 12). There was a gradual fall in titer thereafter and ultimately, after 24 h of administration, phage became undetectable. Even though there was a difference in the phage titers of GI tract, liver, and spleen but the difference was not statistically significant ($P > 0.05$). All mice, during the experiment were healthy and active with normal body temperature.

**Efficacy of phage cocktail in controlling Vibrio cholerae infection in mice**

In the animal model used for the present study, *Vibrio cholerae* is believed to reside mainly in the gastrointestinal tract. We therefore evaluated the effect of phage cocktail (ATCC B1-B5) (MOI- 0.1) on bacterial (MAK 757) colonization of the intestine. Each mouse was inoculated with $1 \times 10^9$ CFU/ml of *Vibrio cholerae* and GI specimens were obtained from day 1 day 4 after the phage cocktail treatment. In mice (phage treatment group) given phage cocktail (once daily) from day 1 – day 3 after the administration of *Vibrio cholerae*, the number of viable bacteria in tissue homogenate of treated from day 1($7.1 \times 10^6$ CFU/gm) to day 4 ($9.1 \times 10^3$ CFU/gm) was significantly($P<0.05$) less than that in the tissue samples of the control group i.e. day 1($3.4 \times 10^7$ CFU/gm) to day 4 ($9.8 \times 10^9$ CFU/gm) respectively(Fig. 13).
Efficacy of Ciprofloxacin as an antibacterial agent in mice

To measure the therapeutic activity of ciprofloxacin in vivo small intestine of adult mice was taken as the experimental organ. The MIC for ciprofloxacin was estimated as 0.1 µg/ml (Table 2). The fluoroquinolone drug ciprofloxacin was charged against Vibrio cholerae MAK 757 strain, at a dose of 40 mg/kg once a day, resulted in a decline of about 5 log10 CFU/gm of tissue from the control levels (Fig. 13). The reduction from day 1(4.5×10^6 CFU/gm) to day 4 (3.7×10^1 CFU/gm) was statistically significant (p<0.05) than the untreated controls which was 3.4×10^7 CFU/gm at day 1 and 9.8×10^9 CFU/gm at day 4 respectively.

Efficacy of Oral Rehydration Solution against Vibrio cholerae infection in mice

Oral rehydration solution (ORS) was given as a daily basis for 3 days to ORS treated group after infection. ORS treatment was unable to reduce the viable bacterial count in tissue homogenate from day 1 (1.9×10^7 CFU/gm) to day 4 (7.1×10^8 CFU/gm) compare to the infected control; day 1 (3.4×10^7 CFU/gm) and day 4 (9.8×10^9 CFU/gm) respectively (Fig 13). However, ORS treatment limited the rate bacterial multiplication as evident from the result. The ORS treatment was found to be statistically insignificant (p>0.05).

Cytokine levels in serum samples

The levels of the pro-inflammatory cytokines (IL-6 and TNF-α) in serum samples of untreated Vibrio cholerae infected (via oral route), phage cocktail treated (via oral route), antibiotic treated (via oral route) and ORS treated (via oral route) mice were determined at day 1, day 2, day 3, and day 4. The results presented in fig.14 (IL-6) and fig.15 (TNF- α) indicates a gradual increase in the level of cytokines in the sera of untreated infected mice (p<0.05). A significant reduction in the levels of IL-6 and TNF-α in sera was detected (p<0.05) in phage-treated and antibiotic treated mice. But in case of ORS treated mice a rise in the cytokine levels were detected which is not significant statistically (p<0.05).
Histological finding

Histologically, the villus height and crypt depth were determined for 6-10 villus crypt units/mouse with a calibrated eye piece graticule and the villus: crypt ratio were calculated. At least 3:1 villus to crypt ratio was considered as normal. Complete villus crypt units were defined on the basis of a uniform intact epithelial lining and a rounded villus tip [McKay et al., 1990]. The mean value of the measured parameters for each animal were evaluated and entered as raw data (data not shown). No significant variation was observed except some alteration in villous architecture in control (Vibrio cholerae, MAK757) mice. Dilated villus lamina propria in few of the intestinal villi was seen in control group, but congested crypts with cellular infiltration in villus lamina propria and submucosa with mild degree neutrophilic response < 10/hpf (high power field) was observed in all the sections (Fig.16A).

Villous contour was within normal limit with mild neutrophilic response in villous lamina propria in phage cocktail treated mice after bacterial infection (Fig.16B). Antibiotic treated mice after bacterial infection showed almost normal villus configuration but increase cellularity with moderate neutrophilic response (10%-25% neutrophils/hpf) in villus lamina propria and submucosa (Fig.16C). Intestinal mucosa was normal with mild cellular response in ORS treated mice after bacterial infection (Fig.16D).
Fig 5: Ventral views of gross pathological features of rabbits in the removable intestinal tie–adult rabbit diarrhoea (RITARD) model. (A) *Vibrio cholerae* MAK 757-infected rabbit, showing much more haemorrhagic fluid accumulation. (B) Phage-treated rabbit with lesser degree of haemorrhagic fluid accumulation.

Fig 6: Histopathological changes to rabbit intestine at 24 h post-challenge. (A) In the control rabbit, villi lost their normal shape and contour, with inflammatory cellular infiltration in the villous lamina propria. (B) In the experimental rabbit, villi of the intestinal mucosae are almost normal in shape, with minimal inflammatory cells.
Table 1: Intestinal *Vibrio cholerae* colony counts and plaque counts in the removable intestinal tie–adult rabbit diarrhoea (RITARD) model without and with phage treatment.

<table>
<thead>
<tr>
<th>In vivo experiment</th>
<th>Parameters used</th>
<th>In vitro experiment</th>
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<tr>
<td>Control</td>
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</tr>
<tr>
<td>12</td>
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Fig 7: In vitro lytic activity of the phage cocktail and individual phages against *Vibrio cholerae* MAK 757.
Fig 8: Survival and stability of phage cocktail in faeces after administration at a dose of $1 \times 10^8$ PFU/ml.
Fig 9: Faecal shedding of *Vibrio cholerae* in rabbits treated with a phage cocktail: (a) Bacterial counts 6 h before and 6 h after inoculation; (b) 12 h before and 12 h after inoculation; (c) Phage counts in faeces at various time periods before and after inoculation with $1 \times 10^9$ CFU *Vibrio cholerae* O1 MAK 757.
**TABLE 2: Ciprofloxacin MIC for selected strain *Vibrio cholerae* MAK 757.**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>MIC of Ciprofloxacin (µg/ml)</th>
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<td><em>Vibrio cholerae</em> MAK 757</td>
<td>0.1</td>
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Fig 10: Pulse Field Gel Electrophoresis of whole genome of bacteriophages of *Vibrio cholerae*: lane 1 lambda low range PFG marker; lane 2 lambda HindIII digest; lane 3 100 bp ladder; lane 4 B1; lane 5 B2; lane 6 B3; Lane 7 B5, Lane 8. B4.
Fig 11a: Vibriophages (ATCC- B1, B2, B3, B4 and B5) sensitivity to various temperatures. The phage cocktail preparation held at 4°C in sterile PBS was taken as control for evaluating the phage cocktail efficacy to various temperatures phage.
Fig 11b: Vibriophages (ATCC- B1, B2, B3, B4 and B5) sensitivity to various organic solvents. The control for organic solvents, phage preparation was held at 4°C in sterile PBS.
Fig 11c: Vibriophages (ATCC- B1, B2, B3, B4 and B5) sensitivity to various pH. The control for sensitivity to various pH phage preparation held at pH 7.0 acted as control.
Fig 12: Stability of vibriophages in GI tract, liver and spleen homogenates of mice at varying time periods after phage administration (1× 10^8 PFU/ml).
Fig 13: Comparison of the ability of active phage cocktail, antibiotic and ORS treatments in reducing the viable bacterial count in mice intestine.
Fig 1: Cytokine (IL-6) levels in sera of *Vibrio cholerae* infected, bacteriophage treated, antibiotic treated, and ORS treated adult mice.
Fig 15: Cytokine (TNF-α) levels in sera of *Vibrio cholerae* infected, bacteriophage treated, antibiotic treated, and ORS treated adult mice.
Fig 16: Histopathological changes to mice intestine at 24 h post-challenge. Magnifications 40X. (A) In the control group of mice, dilated villus lamina propria of the intestinal villi was seen. (B) In the phage cocktail treated mice, villous contour was within normal limit with mild neutrophilic response. (C) Antibiotic treated mice after bacterial infection showed almost normal villus configuration but increase cellularity with moderate neutrophilic response. (D) Intestinal mucosa was normal with mild cellular response.