Bacteriophages: A Possible Solution to Combat Enteropathogenic Escherichia Coli (EPEC) Infections in Neonatal goats

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Abstract

Due to the awareness and benefits of goat rearing in developing economies, goats' significance is increasing. Unfortunately, these ruminants are threatened via multiple bacterial pathogens such as enteropathogenic *Escherichia coli* (EPEC) and its pathotypes. In goat kids and lambs, EPEC causes gastrointestinal disease leading to substantial economic losses for farmers and may also pose a threat to public health via the spread of zoonotic diseases. Management of infection is primarily based on antibiotics, but the need for new therapeutic measures as an alternative to antibiotics is becoming vital because of the advent of antimicrobial resistance (AMR). The current study focuses on the prevalence of enteric diseases, their identification through various molecular techniques viz., SYBR green real-time PCR, conventional PCR based on bfpA gene, PCR based on uspA gene and isolation, purification of naturally occurring phages against three EPEC multi-drug resistant strains isolated from the neonatal goat. Bacteriophages are novel components that can be used to tackle bacterial infections and AMR, where host immune responses and antimicrobial agents become incompetent. It was observed that a PCR based approach is more effective and rapid as compared to phenotypic tests. It was also established that the isolated bacteriophages exhibited potent antibacterial efficacy in-vitro. Hence, bacteriophages, being a natural therapeutic biological agent docking bacterial host, may be explored as a potential alternative to antibiotics in managing public, livestock and environmental health in this troubling situation of AMR.

Introduction

Escherichia coli are known to be a natural commensal of warm-blooded animal gut flora. A new and more virulent generation of the MDR/XDR/PDR bacteria strain has been developed due to the indiscriminate use of antibacterial agents (Magiorakos et al. 2012). The acquisition of plasmid-borne resistant genes and unregulated antibiotic application has contributed to the emergence of resistance among the *Escherichia coli* strains. As a highly varied and adaptable pathogen, this peculiar feature of *Escherichia coli* has resulted and validated from its property of gene gain and loss (Croxen et al. 2013). The pathogenicity of *Escherichia coli* depends on its virulence factors, invasins, heat-labile and heat-stable enterotoxins, verotoxins and adhesin or colonisation factors (Relman and Falkow 2015). It is pathogenically classified into, diarrhoeagenic *Escherichia coli* and uropathogenic *Escherichia coli*. Diarrhoeagenic *Escherichia coli* is further subdivided into Enterotoxigenic *Escherichia coli* (ETEC), Enteropathogenic *Escherichia coli* (EPEC), Enteroinvasive *Escherichia coli* (EIEC), Enteroaggregative *Escherichia coli* (EAggEC) and Enterohemorrhagic *Escherichia coli* (EHEC), which causes diarrhoea and other associated illnesses (Garrine et al. 2020). *Escherichia coli* are present in both humans and livestock, where ruminants (sheep and goats) have been identified as a substantial pool and human infection source (Rahman et al. 2020). Since these ruminants not only harbour pathogenic strains, they can also cause asymptomatic infection in animals that can easily pass through the food web and contribute to human clinical diseases (zoonotic diseases). Serotypes like O91, O157 and O146 of EHEC isolated from sheep (Urdhal et al. 2003) and O157 isolated from goats is affiliated with human infections (Pritchard et al. 2000). Pathogenic variants of *Escherichia coli* affect humans and animals, causing diseases worldwide (Croxen et al. 2013), where EPEC is primarily responsible for fatal diarrhoea in developing countries (Bugarel et al. 2011). Cattles are the natural reservoir of O157 *Escherichia coli* containing verotoxin (CDC, 2019), and the ingestion of unpasteurised milk presents a high risk of O157: H7 *E. coli* infection (Disassa et al. 2017). Ruminant animals are conceptualised to pose as primary reservoirs of shiga toxin (Stx) producing *Escherichia coli* (STEC) where STEC infection resulted in 1 million diseases and 100 fatalities in 2010, as reported by the World Health Organization (Havelaar et al. 2015; Kim et al. 2020). Recent outbreak was reported from the United States where 25 people got infected due to STEC

O157:H7 resulting in 1 death (CDC, 2018) linked to leafy greens. A STEC O103:H25 outbreak in Norway due to fermented sausage consumption containing sheep has been reported to be related to the 2011 outbreak in Germany due to O104:H4 (Trine et al. 2012).

India ranks second in goat population with nearly 148.9 million approximately (NDDB 2019). In India, goats are considered poor people's bank or insurance scheme, distributed between landless and poor farmers, but the rapid surge in the animal farming sector has led to increased commercial goat farming (Kumar et al. 2010). Goat rearing in India has enormous demand as they are the main meat (chevon) producing animals. Goat farming is gaining traction in the commercial and domestic sectors over the last few years due to its popularity. However, as goat meat and milk are generally recognised worldwide, constraints on the growth of goat enterprises have also been observed due to its association with bacterial (Miller and Lu 2019) and parasitic infections (Ayaz et al. 2018) that are critical for causing human infections (Monteiro et al. 2018) and may pose a threat to public health by spreading zoonotic diseases. A retrospective study on goat from 1988 to 2012 showed that 43.67% of deaths were due to enteritis, followed by gastrointestinal parasitism and gastric diseases (Pawaiya et al. 2017). Medical science is currently entirely dependent on antibiotics to treat infectious diseases and strives to keep the human race healthy and protect animals.

Increase in drug-resistant bacteria has contributed to significant morbidity and mortality due to the inappropriate use of antibiotics (WHO 2020). Such conditions have contributed to the use of bacteriophages. The utilisation of broad-spectrum drugs affects the gut microbial community and its composition (Langdon et al. 2016). Structural modifications contribute to changes in the expression of genes, the activity of the protein, and the gut's overall metabolism, the results of which may or may not be temporary (Hasan and Yang 2019). Bacteria whose genes are altered by broad-spectrum antibiotics may serve as a gene pool for such resistant bacteria, contributing to the spread of antibiotic resistance (Peterson and Kaur 2018). This has prompted researchers to rethink a decades-old phage therapy approach as a viable new therapeutic alternative against bacterial infections. A randomised, double-blind, placebo-controlled clinical trial was performed where bacteriophage cocktails were administered intravesically to treat urinary tract infections (Leitner et al. 2020). Titze et al. (2020) conducted a study where antimicrobial activity was exhibited by phage mixture against *Staphylococcus aureus* from bovine mastitis. Since bacteriophages are highly selective, they are ineffective against the host non-pathogenic flora (Principi et al. 2019). Phages may be one of those multi-strategic instruments that could modulate microbial diversity and assist us in combating multi-drug resistant bacterial strains as we are about to reach the post-antibiotic period, where we are lagging in the fight against many diseases due to our same old classical approach (Aslam et al. 2018). Here in this article, we report the isolation of highly pathogenic strains of *Escherichia coli*, characterised by molecular methods, vis-à-vis their AMR characteristics that could pose a potential risk. Hence, suitable coliphages and their bactericidal activity on indicator organisms were screened to develop suitable phage therapeutic candidates.

Materials And Methods

Sample collection and isolation of *E. coli* strains from goat kids

A study was conducted in hebdomadic goat kids that showed acute diarrhoea with pasty greenish faecal matter soiling the perianal region. Faecal swabs were collected from 32 goat kids born between September-November (kidding season) from various unorganised herds in an organised goat farm. For bacteriological culture, swabs

were washed with 1.0ml of sterile PBS and vortexed before inoculation to culture media. They were cultured in MacConkey's agar (MCA) and Eosin Methylene blue agar (EMB) and incubated at 37°C. The colonies obtained of *Escherichia coli* were gram stained, and specific biochemical tests like Indole test and Triple sugar iron agar tests were conducted. The lactose fermenting colonies of MCA were selectively streaked on Congo-Red dye agar and incubated at 37°C for 72 hours, which resulted in the development of colonies with brick red colour.

Molecular characterisation of *E. coli* by PCR:

DNA extraction was performed using the Nucleopore® DNA Kit (Genetix) implementing the manufacturer's protocol from pure sub-cultured MCA colonies. The DNA is then used to detect EPEC and shiga-toxin-producing (Stx) producing *E. coli* (STEC) based on the conventional PCR (cPCR) detection using *bfpA* and *stx1* gene, respectively. The cPCR primers for *bfpA* were same as the SYBR green real-time primers designed in-house in the laboratory, while the *stx1* gene was amplified using *stx1F*: 5'CACAATCAGGCGTCGCCAGCGCACTTGCT3' and *stx1R*: 5'TGTTGCAGGGATCAGTCGTACGGGGATGC3' (Talukdar et al. 2013). The identification of *E. coli* molecularly was made by PCR amplification of the universal stress protein A (*uspA*) gene utilising species-specific primers (F-5'-CCGATACGCTGCCAATCAGT-3' & R-5'-ACGCAGACCGTAGGCCAGAT-3') (Fig: 1). The annealing temperature was kept at 55 °C for 1 min. The amplified *stx1* gene was sequenced by Sanger's dideoxy method using the BigDye terminator kit where the same sample was subjected to DNA isolation for the screening of EPEC by using *bfpA* SYBR green real-time PCR. The EPEC colonies with brick red colour in Congo-Red dye agar isolated were also used for molecular screening by *bfpA* SYBR green real-time PCR. Sequence identity plot was done to compare the nucleotide composition and point mutations in the coding region of the STx1 gene of the above-isolated strain.

Molecular screening of EPEC using *bfpA* gene-based SYBR-green real-time PCR

Bundle forming pilin protein A gene primers: Size of product 158bp

bfpA F: 5'-ATGGTGCTTGCGCTTGCTGC-3',

bfpA R: 5'-AATCCACTATAACTGGTCTGC-3'

Antibiotic susceptibility testing

EPEC was identified based on the modified Kirby Bauer Disk diffusion (Bauer et al. 1996) method, as per the CLSI (Clinical and laboratory standard Institute) guidelines. Antibiotic susceptibility was put up using antibiotic discs of Himedia- Mumbai, India. Set of drugs include Amikacin (30µg), Amoxy-Clavulanic acid (20/10 µg), Ampicillin (10µg), Ceftriaxone (30µg), Ceftazidime (30µg), Ceftazidime-Clavulanic Acid (30/10µg), Ciprofloxacin (5 µg), Co-trimoxazole (1.25/23.75 µg), Cefotaxime (30µg), Gentamicin (10 µg), Norfloxacin (10 µg), Aztreonam (30 µg), Cefepime (30µg), Meropenem (10 µg), Piperacillin-tazobactam (100/10 µg), Nitrofurantoin (300 µg), Colistin (10

µg) were tested for the three of the isolated EPEC strains (1873, 1845, B677). In-vitro presence of Extended-Spectrum Beta-Lactamase (ESBL) was also confirmed according to CLSI guidelines. Phenotypic evidence of ESBL development is confirmed when a difference of ≥ 5 mm is observed between the zone diameters of either cephalosporin (ceftazidime) disc and their respective cephalosporin/clavulanate (ceftazidime-clavulanic acid) disc (Wayne, 2011).

Sample collection and isolation of bacteriophages:

For the isolation of bacteriophages, water samples were collected from different Ghats (Assi Ghat, Tulsi Ghat and Manikarnika Ghat) of river Ganges from Varanasi in a sterile plastic container. A sample from the water specimen was treated with 1% chloroform (v/v) for 10 minutes with continuous vortexing/inversion for bacteriophage isolation. It was later centrifuged for 10 minutes at 10778xg. The supernatant, collected was flooded on the 90mm Mueller-Hinton Agar (MHA) lawn culture (4h old EPEC cultured in log phase). It was incubated overnight at 37°C. After 24h of incubation, the lawn culture is washed with 4 ml TMG (Tris HCL, Magnesium Sulphate, Gelatin pH 7.4) buffer and treated with 1% chloroform vortexing/inverting for 10 minutes. Soon after the chloroform treatment, it is centrifuged for 10 minutes at 10778xg. The supernatant is transferred carefully to another properly autoclaved 1.5 ml centrifuge tube without disturbing the sedimented lysed bacteria. The whole process of centrifugation repeated four times. Again a 4hr old lawn culture of the bacterial host (EPEC) was prepared. As mentioned earlier, the supernatant collected by the procedure was dropped on the plate and was incubated for 24h at 37°C. After incubating for 18-24hr, the surface with clear plaques was swabbed with TMG buffer by properly autoclaved swab buds and collected in 1.5 ml centrifuge or eppendorf tubes further processed. The same centrifugation process is repeated, as mentioned above, to pellet the bacterial and cell debris at 10778xg for 10 minutes. The purified supernatant was collected and was preserved at 4° C for further use.

Isolation of different bacteriophage strains from cocktails:

Cocktail isolated for all the three strains 1873, 1845, B677 is a mixture of different bacteriophages separated into different strains by soft agar overlay method (Kropinski et al. 2009). The soft agar is kept at a molten state at a temperature of not more than 40-43°C in the water bath. Bacterial and phage suspension is added in it which is further poured on the MHA plates and incubated for 18-24h at 37° C. After incubation, different morphological plaques of different sizes are observed which were later cut out and put into Luria-Bertani (LB) broth and was incubated overnight at 37° C. A total of 5 different plaques were cut out, and after incubation, they were treated with 1% chloroform (v/v) for 10 minutes and were centrifuged at 10778xg for 10 minutes. The supernatant obtained was transferred to another 1.5 ml centrifuge tube, and the process of centrifugation is repeated three more times.

Isolation of bacteriophage DNA:

Isolated phage lysate (10^{10} PFU/ml) was transferred in an eppendorf, and DNase (10mg/ml) was added and incubated for 30min at 37°C for degradation of bacterial DNA. After incubation, SDS (Sodium Dodecyl Sulfate) and proteinase K were introduced and was further incubated at 37°C for 1hr. Later, an equal volume of PCI (25:24:1) (Phenol/Chloroform/Isoamyl alcohol) was added and was centrifuged at 10778xg for 10 minutes. The

aqueous phase was collected, and then an equal volume of CI (24:1) (Chloroform/Isoamyl alcohol) was added and centrifuged at 10778xg for 10 minutes. RNase (10mg/ml) was added to the aqueous phase and was incubated for 30 minutes at 37°C. An equal volume of Isopropanol alcohol was added after incubation, and the solution was kept at room temperature. The suspension was centrifuged, and the pellet was washed with 70% ethanol and then again centrifuged at 10778xg for 10 minutes. Pellet was dried at 37°C for 1-2h, and was dissolved in TE (Tris-Cl-EDTA) and was stored at -20°C. The concentration and quality of isolated DNA were measured using a NanoDrop Bioanalyzer spectrophotometer (Thermo Scientific) at 260nm.

Characterisation of bacteriophages

Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) is a technique that is a quick, robust, precise and highly cost-effective fingerprinting method (Ranjbar et al. 2017). ERIC-PCR was applied against the harvested bacteriophage DNA for molecular genotyping where its PCR products were run on 1.2% gel electrophoresis for analysis. For DNA amplification, ERIC-PCR forward and reverse primers were used 5'-ATG TAA GCT CCT GGG GAT TCA C-3' (F) and 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (R) (Versalovic et al. 1991). The process was performed in the total volume of 25µl, including 5µl of template DNA. A total of 25µl was put up for PCR where 5 µl of the master mix constitutes, 1.6µl/pm of forward and reverse ERIC primer. Finally, the thermocycler (BioRad) was programmed with the annealing temperature at 41°C (Versalovic et al. 1994; Szczuka et al. 2004). The resultant product is then run on 1.2% gel electrophoresis for 45 minutes, loaded by EtBr (Ethidium Bromide), with a 100bp of a ladder (DNA marker) as a standard measuring means and the resultant bands were observed in UV light and photographed via gel doc CMOS camera for image capturing.

Results

Molecular confirmation and characterisation of *E. coli* isolates:

Around 28 kids (87%) showed the presence of EPEC by *bfpA* gene-based SYBR green real-time PCR (Figure: 2), *bfpA* gene-based conventional PCR (Figure: 3) and *uspA* gene conventional PCR (Figure: 4). Only one isolate of *E. coli* was *stx1* positive by conventional PCR (Figure: 5). The CIRG STEC strain showed 99% homologous with other reference strains. Phylogenetic analysis was conducted using the Minimum Evolution (ME) method (Figure: 6) of MEGA6 software (Tamura et al. 2013). It computed the optimal tree with the branch length sum = 0.05187866. The percentage of clustered replicate trees and the related taxa are subject to the bootstrap test (500 replicates). Evolutionary distances have been evaluated using the Maximum Composite Likelihood method, and the number of base substitutions per site is in units. The ME tree was scanned at a search level of 1 utilising a Close-Neighbour-Interchange (CNI) algorithm. To construct the initial tree, the Neighbour-joining algorithm was used. The evaluation included 13 sequences of nucleotides. The positions included in Codon were 1st+2nd+3rd+Noncoding. It excluded all positions containing gaps and missing data. In the final dataset, there were 520 positions overall.

Based on phylogenetic analysis, the STEC CIRG1:2017 strain was present in one of the two major branches with close association with STEC strains including ONT: H34, 4756/98, O6E01767 reference strains belonging to various subclasses. The strain 4756/98 was present in the same clade where STEC/CIRG: 2017 was placed. However, the DEC 10J strain was present in the other branch and SWUN 4124 and FD930 strains.

A sequence identity plot constructed using BioEdit V. 7.2.5 (Hall 1999) of various *E. coli* mapped for nucleotide variations is presented in Figure: 7. The most common point mutations observed in some of the strains, including CIRG1: 2017 Stx1, are G→A at the 1511 nucleotide position compared to *E. coli* strain' N1508 Stx1.'

Antibiotic susceptibility testing:

The isolated strains were multi-drug resistant (MDR) but non-ESBL producing strains of Enteropathogenic *Escherichia coli*, where all three (1873, 1845 & B677) were resistant to Amoxy-Clavulanic acid, Norfloxacin and Cefepime. Bacterial strain 1873 and 1845 were resistant to Meropenem, and Nitrofurantoin whereas bacterial strain B677 is sensitive to both; 1873 and B677 are resistant to Ampicillin whereas 1845 is Sensitive to same; 1845 and B677 both are resistant to Gentamicin whereas 1873 is sensitive.

Table 1
Antibiotic Susceptibility pattern of EPEC strains isolated from goat stool

S.No.	Antibiotics	1873	1845	B677
1.	Amikacin	S	S	S
2.	Amoxy-Clavulanic acid	R	R	R
3.	Ampicillin	R	S	R
4.	Ceftriaxone	R	S	S
5.	Ceftazidime	R	S	S
6.	Ciprofloxacin	R	R	S
7.	Co-trimoxazole	S	S	S
8.	Cefotaxime	S	R	S
9.	Gentamicin	S	R	R
10.	Norfloxacin	R	R	R
11.	Aztreonam	S	S	S
12.	Cefepime	R	R	R
13.	Meropenem	R	R	S
14.	Piperacillin-tazobactam	R	S	S
15.	Nitrofurantoin	R	R	S
16.	Colistin	S	S	S

Isolation of bacteriophages and its strains:

After dropping supernatant of water specimen on the 3hr old lawn culture of EPEC strain, ambiguous drops are visible after 24hr of incubation which become further comprehensible where complete clearing of the plate is seen (Figure: 8) after 120hr of incubation. Distinct morphologically plaque of different sizes was observed after implementing the overlay method (Kropinski et al. 2009) (Figure: 9).

Molecular characterisation of phages:

Five distinct patterns of bands were obtained via ERIC PCR amplification of phage DNA against EPEC isolates. For $\Phi 1$ 970bp, 560bp, 500bp, 460bp, 410bp, 290bp and 200bp bands; $\Phi 2$ 640bp, 450bp and 180bp bands; $\Phi 3$ 910bp, 640bp, 450bp, 420bp, 280bp and 180bp bands; $\Phi 4$ > 1kb, 920bp, 760bp, 720bp, 450bp, 340bp, 210bp and 180bp bands and $\Phi 5$ > 1kb, 1000bp, 720bp, 480bp, 450bp, 300bp, 320bp and 210bp bands were obtained, shown in the figure: 10. It can be inferred from different types of banding pattern obtained by the molecular characterisation that all the five phages are genomically distinct, i.e. diverse from each other.

Discussion And Conclusion

The prevalence of high morbidity and mortality among neonatal goats in the developing nations is associated with Enteropathogenic *Escherichia coli* infections ((Singh et al. 2018), which leads to severe, acute diarrhoea coupled with severe dehydration, extreme acid-base and electrolyte imbalance, and mild diarrhoea mortality without systemic disease, often in less than 12 hours (Gruenberg 2014). The present study explores the prevalence of enteric diseases in neonatal goats and their detection via different molecular techniques. Neonatal enteritis is caused by various etiological agents, with the incidence of mixed infections complementing each other and amplifying the condition due to immunity imbalance (Singh et al. 2018). EPEC is a bacterium with many serotypes of which only some are pathogenic, causing diarrhoea and septicaemia, which results in the death of goat kids if left untreated. EPEC isolate detection serves as an indicator for the presence of virulent *E. coli* in the herd, detected via *bfpA* gene-based SYBR green real-time PCR. In general the PCR- based detection methods for diarrheagenic *Escherichia coli* strains of veterinary importance are more adequate, sensitive and rapid in comparison to the classical phenotypic testing methods. The SYBR Green-based detection assays are widely alleged among copious chemistries available for real-time PCR assays (Tajadini et al. 2014). In general, compared to the conventional diagnostic methods, including isolation and culture techniques, molecular tests are highly sensitive, rapid, and less tedious (Franco-Duarte et al. 2019). Early identification, diagnosis, and development of a vaccine for EPEC infections, will not only protect neonatal goats from these infections but would also reduce the economic loss burden of the farmers.

Various forms of antibiotics are now indiscriminately used worldwide in the veterinary industry to encourage livestock growth and care (Sachi et al. 2019). Globally, $63,151 \pm 1,560$ tonnes of antibiotics are used in livestock every year, which are expected to rise by 2030 (Tiseo et al. 2020). Antibiotics are used in animal husbandry for therapeutic and prophylactic purposes, the global consumption of which is double in animals relative to humans (Kummerer 2008; Aarestrup 2012). Many studies have shown that significant portions of antibiotics are released into the environment without modification, i.e. with possible antimicrobial activity (Kummerer 2008). The requirement for animal protein (milk and meat) is increasing worldwide, facilitating the expansion of industrial farming and, with the injudicious use of antibiotics for treatment purposes, contributes to antimicrobial

resistance (AMR) (Sachi et al. 2019; Tiseo et al. 2020). One of the most common priority areas recognised by national and international agencies is AMR which is proliferating as a silent pandemic (Sharma et al. 2018). Hence, a renewed focus on research investments is needed to identify alternative, secure, cost-effective and creative solutions in parallel with discovering new antibiotics. One such biological entity that can be employed against antibiotic-resistant bacteria is bacteriophages (Sausseureau et al. 2014). Bacteriophages can infect and kill prokaryotes without any adverse effect on eukaryotes and are highly specific (Principi et al. 2019). Hence, in this study, we isolated phages against multi-drug resistant EPEC strains whose in-vitro analysis showed complete clearance of the bacterial lawn in 120h. Also, if they are employed for *in-vivo* treatment in goats, we isolated 5 different strains of phages that can be made into a cocktail and administered orally to the goats in which drugs are ineffective. Future research can be recommended based on the finding that phages can be used *in-vivo* and options for concentrations and different administration routes.

E. coli strains that are highly virulent and carry antimicrobial resistance are like 'double whammy' that need an alternate strategy. Hence, in the current study, we did thorough characterisation of the isolates targeting pathogenic genes and screened more isolates for enteropathogenicity by *bfpA* gene-based SYBR green real-time PCR. One isolate which carried both enteropathogenic (*bfpA*) as well as enterotoxigenic (*stx1*) was used as a model isolate (CIRG1:2017) for assessing the potential of isolated coliphages. Further, the same isolate was sequenced for *stx1* gene and found the G→A mutation and the phylogenetic tree showing taxonomically close to German origin strain *E. coli* strain 4756/98 (Zhang et al. 2002). In this scenario, it is further required to screen more isolates that carry the virulence factors without compromising AMR characteristics detection. A more detailed and extensive study would help devise a strategy in deciphering the AMR and virulence genes transmission dynamics in *E. coli* or other vital pathogens in the future. Nevertheless, the judicious use of antibiotics coupled with venturing into alternate therapeutics could help combat the menace of AMR strains that are fuelled with virulence factors putting the livestock production at risk and aggravating the zoonotic flow.

Declarations

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Conflict of Interest

None to declare

Availability of Data material

Not applicable

Code availability

Not applicable

Authors contribution

Neelam Jain (NJ), Gopal Nath (GN), G. K. Aseri (GKA), K.Gururaj (KG), and Kanika Bhargava (KB) contributed to the conception and design of the complete study. NJ, KB and G. K. Aseri (GKA) conducted literature search. NJ, KB, GKA and KG drafted and edited the manuscript. NJ, GN, and KB performed the experimental work, statistical analysis, data interpretation of bacteriophage studies and AST on EPEC. K.Gururaj (KG), designed and coordinated the EPEC research, AK Mishra (AKM) and Ashok Kumar (AK) planned the study on EPEC and aided in data interpretation. KG and RVS Pawaiya (RVSP) did the data analysis on EPEC. All the authors have reviewed and approved the manuscript.

Ethics approval

"There is no ethics involved in this current study, since no live animal experimentation has been conducted"

Consent to participate

Not applicable

Consent to publish

Not applicable

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Figures

Figure 1

Cycling conditions for the bfpA gene SYBR-green real-time PCR

Figure 2

bfpA gene SYBR green real-time PCR for EPEC screening: A: Cycle quantification plot (Log scale) for bfpA gene amplification (Purple- positive control, red- unknown), B: melt peak for the specific amplified product of 158bp

Fig. 2: PCR Amplification of bfpA Gene of EPEC
 Lane 1: DNA Ladder
 Lane 2 & 3: Negative Controls
 Lane 4: Amplified Product

Figure 3

bfpA gene conventional PCR for EPEC. Wells, 1: 100bp DNA ladder, 2: No template control, 3: Negative control, 3: 158bp bfpA gene amplicon

Figure 4

uspA gene PCR for E. coli. Wells, M: 100bp DNA ladder, 1, 3-7: 884bp uspA gene amplicon, 2: No template control

Figure 5

Gel picture showing the *stx1* gene amplification of the CIRG1: 2017 Shiga toxin-producing *Escherichia coli* (STEC) isolate (Lane 3) against no-template control (Lane 2) and 100bp DNA ladder (Lane 1)

Figure 6

Phylogenetic analysis of Stx1 gene of STEC strain CIRG1: 2017 with other standard reference cultures. The evolutionary history of the stx1 gene using the Minimum Evolution method

Figure 7

Illustration showing sequence identity plot for *stx1* gene of various STEC reference strains with the CIRG1:2017 strain. The dotted line represents homology with the first sequence. The nucleotides against the dotted line represent mutations in the sequence.

Figure 8

In Vitro Assessment of Antibacterial activity of phages against isolated EPEC strains

Figure 9

Overlay technique: Different types and sizes of plaques against EPEC Strain

Figure 10

Representative image of ERIC-PCR of bacteriophage DNA against EPEC isolates

Supplementary Files

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