CHARACTERIZATION AND IN VITRO EFFICACY STUDIES OF WIDE HOST RANGE LYTIC BACTERIOPHAGE ΦDMEC-1 INFECTING ESCHERICHIA COLI ISOLATED FROM PYOGENIC SKIN INFECTIONS


*DBT - Research Laboratory, Department of Microbiology, Gulbarga University, Gulbarga-585 106, Karnataka, India
**Department of Biotechnology and Research Centre, Babuji Institute of Engineering and Technology, Davangere -577 004, Karnataka, India
***Department of Dermatology, S.S Institute of Medical Sciences and Research Centre, Davangere -577 004, Karnataka, India
****Department of Microbiology, Health Plus Food Stuff Private Limited, Mysore -570 008, India
*****Drug Discovery Research Lab, Skanda Life Sciences Private Limited, Bengaluru -560 091, India.
******(Corresponding Author: Manjunath N.S., E-mail: nsm.microb1973@gmail.com)

ABSTRACT

The main objectives of the present study was to characterize the bacteria *Escherichia coli* isolated from patients infected with pyogenic skin infections and bacteriophage isolated from the cocktail of sewage samples. The study was also focussed to evaluate the stability and in vitro efficacy of bacteriophage lytic activity against multidrug resistant *E. coli*. A total of 83 bacteria among 114 samples were isolated from the patients of pyogenic skin infections of three major hospitals of Davangere city, examined over a period of twelve months. *Escherichia coli* were found to be one among four major causative agents including *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Proteus mirabilis*. Four *E. coli* strains were isolated by following standard conventional microbiological methods and screened for multidrug resistance by disc diffusion method. The taxonomical identification by conventional microbiological studies of *E. coli* was confirmed by 16S rRNA gene sequencing and phylogenetic analysis. Among the tested *E. coli* isolates 3 were resistant to most of the antibiotics and one against all the antibiotics tested. The mixture of sewage originated from hospital, municipal and domestic waste was used as source for the isolation of potent bacteriophage ΦDMEC-1 having lytic activity against multidrug resistant *E. coli*. The phage was identified as belonging to the family *Siphoviridae* and characterized for host range and stability in terms of lytic after different time intervals of storage. The phage retained lytic activity against the host for 3 months period. The present study reveals that *E. coli* strains are one among the major bacterial causative agents of pyogenic skin infections which are multidrug resistant. Highly potent and stable Bacteriophage ΦDMEC-1 isolated from the sewage cocktail showing good lytic activity on multi drug resistant DMEC-1 and has a broad host range on *E. coli* strains isolated. The study shows the prevalence of multidrug resistant *E. coli* in pyogenic skin infections and further insists to study on other multidrug resistant bacteria. The study also has an impact on isolation of bacteriophages as an alternative to antibiotics, to fight on the threatening act of various multidrug resistant bacteria.

KEY WORDS: Antibiotics, bacteriophage, *Escherichia coli*, multidrug resistance, skin infection

INTRODUCTION

Skin and soft tissue infections (SSTI) are one of the most common infections in patients of all age groups. Infections mostly are self-limiting or can be treated with antibiotics (Jones et al., 2007). However, several reports associating the *Escherichia coli* with SSTI have been published: *E. coli* was found to be the causative agent of cellulitis localized to lower or upper limbs (Brzozowski and Ross, 1997; Corredoira et al., 1994; Yoon et al., 1998), surgical site infections (Tourmoussoglou et al., 2008), infections after burn injuries (Rodgers et al., 2000), and others. A study monitoring SSTIs during a 7-year period and encompassing three continents (Europe, Latin America, and North America) showed *E. coli* to be an important causative agent, since it was the third-most prevalent isolated species, preceded solely by *S. aureus* and *P. aeruginosa* (Petkovšek et al., 2009). Mounting concerns about drug-resistant pathogenic bacteria (Cohen, 1992) have rekindled interest in alternative treatments of bacterial infections. Prominent among these alternatives is phage therapy, the use of bacteriophages to kill or otherwise control the bacterial populations in infected hosts. Prior to the discovery and widespread use of antibiotics, it was suggested that bacterial infections could be prevented and/or treated by the administration of bacteriophages (Sulakvelidze et al., 2001). Undue scepticism and unfounded optimism are both misplaced as regards the rediscovery of phage therapy. The technology holds at least the prospect of practical solutions to some urgent public health problems, and not just those caused by *E. coli*. The development costs of phage therapy are much lower than for a new antibiotic. In view of the emergence of new infectious diseases at an unpredicted pace and the escape of well-known bacterial diseases from antibiotic control, we
Early studies suggested that phage therapy may be effective against a broad range of human infections caused by members of the genera *Staphylococcus* spp, *Salmonella* spp, *Klebsiella* spp, *Escherichia* spp, *Proteus* spp, and *Pseudomonas* spp. (Alisky et al., 1998, Barrow and Soothill, 1997). Phages may have immense potential for controlling *E. coli* infections in cattle and could be cured or prevented enteropathogenic *E. coli* (10⁶ CFU) diarrhoea in calves with strain-specific phages administered in a single oral dose (10⁷ PFU) or sprayed on the litter PFU (Smith et al., 1987). A cocktail, “Pyophage”, containing phages targeting *Staphylococcus* spp, *Streptococcus* spp, *Pseudomonas* spp, *Proteus* spp, and *E. coli* is routinely employed in the treatment of various purulent skin or wound infections. Infected skin ulcerations can be chronic and resistant to antibiotic treatment. Phages can be topically applied with impressive success and would appear to be quite promising (Abedon et al., 2011). In the present study, we have characterized multidrug resistant *E. coli* isolated from patients with pyogenic skin infections. Lytic phage ΦDMEC-1 against multi drug resistant *E.coli*, isolated from the domestic and hospital sewage was characterized for morphology, host range, and stability at various conditions. *In vitro* lytic activity of potent bacteriophage against multidrug resistant *E. coli* was also evaluated.

**MATERIALS AND METHODS**

**Microbiological media and Chemicals:**

Luria Bertani (LB) Agar (Hi-media, India), LB broth, LB soft agarose (0.7%), EMB Agar, Mac Conkey’s agar, EMB agar (Hi-media, India), SM buffer with gelatin (50mL/L of 1M Tris of pH 7.5, 5.8 g NaCl, 2g MgSO₄.7H₂O, 5mL/L of 2% gelatin in distilled water) were prepared and used (Sambrook et al., 1989). Agarose (Hi-media, India), Chloroform (Merck, India), Glycerol(Merck, India), Ethidium bromide (Hi-media, India).

**Clinical *E. coli* isolates from pyogenic skin infection patients:**

A total of 4 *E. coli* isolates from the patients of pyogenic skin infections of three major hospitals were used in the study and the data has been previously published (Manjunath N.S et al., 2013). Clinical Isolates were characterized for antibiotic susceptibility test by standard microbiological methods and drug resistant organisms were identified in accordance with CLSI guidelines (CLSI 2005).

**Characterization and antibiotic susceptibility of *E. coli***

**Antibiotic sensitivity test**

The clinical isolates were screened for antibiotic susceptibility by Kirby Bauer’s disc diffusion method (Bauer et al, 1966) using the antibiotic discs (Hi-media Ltd,India) on Mueller-Hinton agar, according to Clinical and Laboratory Standard Institute (CLSI) guidelines (CLSI, 2005) and as per the hospital antibiotic policy. Bacterial suspension of 0.5 Mc Farland standard was used for the test. Antibiotic discs Amikacin (30mcg), Amoxicillin (30mcg), Cefazidime (30mcg), Cephotaxime (30mcg), Ciprofloxacin (5mcg), Cefoparazone (75mcg), Levofloxacin (5mcg), Tobramycin (10mcg) were used for Gram negative bacteria including *E.coli*. Finally the results were recorded by measuring the diameter of zone of inhibition of each antibiotic disc and scored as sensitive or resistant. *E. coli* (MTCC 739) was used as a control strain.

**Extraction of DNA**

The genomic DNA was extracted by following the method of Daniel et al., (1994). A 0.25-ml aliquot of the organism suspended in nutrient broth was centrifuged at 12,000 x g for 5 min. The supernatant was discarded, and the pellet was re-suspended in 100 µl of cell lysis solution {250 U of lysozyme (Sigma Chemical Co., St. Louis, Mo.) per ml, 25 U of lysostaphin (Sigma) per ml, 10 mM EDTA, 10 mM Tris (pH 8) in deionized H₂O}. This suspension was incubated for 30 min at 37°C, placed in a 95°C heat block for 10 min, cooled to room temperature, and then diluted in 900 µl of sterile water.

**Polymerase chain Reaction:**

**16S rRNA gene amplification and phylogenetic analysis**

The identification of the test strains was carried out at molecular level employing the 16S rRNA gene amplification and sequencing. The 16S primers and conditions used in PCR amplification of 16S rRNA gene are as described previously (Sauer et al., 2005) with slight modifications. The primers used were as follows UNI IL forward primer (5’-
GTTGAGCATGTGTTA-3’), UNI_IR reverse primer (5’-CCATTGTACACGTGTGTTA-3’) (synthesized at Sigma Aldrich, Bangalore). The master mix of total volume of 25 μl was composed of the following: 1 μl of each primer (10 μM concentration), 0.2 mM dNTPs (Bangalore GeNei), 1 X PCR buffer (Sigma Aldrich, USA), 2.5 mM MgCl₂ (Bangalore GeNei), 4 μl containing 10 ng of genomic DNA as template and 1 U of Taq DNA polymerase (Sigma Aldrich, USA). PCR was performed with following temperature conditions: Initial denaturation at 94°C for 5 min followed by 35 cycles of 94°C for 45 s, the annealing temperature of 40°C for 30 s and synthesis at 72°C for 40 s. The final extension was carried out at 72°C for 5 min. The PCR amplifications were performed in a Thermocycler Gene Amp PCR system 9700 (Applied biosystems, USA) in 0.2 ml reaction tubes. Expected amplicon of 287 bp was visualized with Ethidium bromide after gel electrophoresis in 1% agarose gel.

Gene sequencing and phylogenetic tree construction

For determining the nucleotide sequences of 16S rDNA and antibiotic resistance determinants, the PCR amplified products were purified using PCR purification kit (Sigma Aldrich), DNA sequencing of the purified product was performed by dideoxy chain termination method using gene specific primers at the sequencing facility of Vimta Labs (Hyderabad, India). The gene sequences obtained were analyzed by using the BLAST search programme (Altschul et al., 1997). The sequence of the PCR product was compared with known 16S rRNA gene sequences in the Gene Bank by multiple sequence alignment using the CLUSTAL W Program (Thompson et al., 1994) and the phylogenetic tree was constructed using Mega 5 program (Tamura et al., 2011).

Isolation and characterization of Bacteriophage

Multidrug resistant *E. coli* strain (DMEC-1) isolated from the pyogenic skin infections as a host, the cocktail of sewage originated from hospital, municipal and domestic waste as a source were used for the isolation of the phage Φ DMEC-1. Initially, sewage sample was pre-treated with 200µl of chloroform per 25ml of sample for 15 minutes at room temperature (Sambrook et al., 1989). LB broth (2 ml of 10X concentrated, Hi-media), 2 ml of 16-18 hours grown host bacterial suspension containing 10⁵ cfu in LB broth and 18 ml of sewage were mixed in a 100ml capacity sterile cotton plugged conical flask. The flask was incubated overnight at 37°C for 15-16 hours. Subsequently, 200 µl of chloroform (Merck, India) was added and the flask was further incubated at 4°C for 1 h. The lysate was centrifuged in 1.5 ml eppendorf tubes at 4000 x g for 10 minutes and the supernatant was aspirated with a sterile 5 ml syringe and passed through a 0.22 μm membrane filter (Minisart, Sartorius, Germany). The filtrate stock was assessed for bacteriophage titre by double-agar layer overlay method (Adams., 1959) with modification (Merabishvili et al., 2009). Phage stock was serially diluted 10-folds (10⁻¹ to 10⁻⁵) using SM buffer with gelatin (50mL/L of 1M Tris pH 7.5, 5.8 g NaCl, 2g MgSO₄.7H₂O, 5mL/L of 2% gelatine in distilled water) (Jamaludeen et al., 2007) and from each 100µl of sample is aspirated, dispensed into sterile 15ml screw capped tubes and mixed with 100µl of host bacterial suspension of 0.5 Mac Farland standards. The content was mixed and incubated at 37°C for 20 minutes with gentle shaking. Luria bertani agarose (0.7%) of 3ml maintained at 47°C in water bath was dispensed into each 15ml tubes, mixed gently and poured on dried LB agar plates of 90mm as top layer to spread uniformly (Sambrook et al., 1989). A control tube containing host bacterial suspension and 4ml of top agarose without filtrate was cultured on LB agarose plates (Jamaludeen et al., 2007). Plates were allowed to set and incubated at 37°C for 15-16 hours. A sterile gel cutter with a rubber bulb was used to cut the gel along with the underlying agar. A few prominent and well isolated plaques were taken through sterile gel cutter with a rubber bulb was used to cut the gel along with the underlying agar. A few prominent and well isolated plaques were taken through

Preparation of Phage stock

A phage plate lysate stock was prepared by mixing 100μL of bacteriophage suspension with 100 μL of the host bacterium suspension of 0.5 Mac Farland standard in 15ml screw capped tube (Sambrook et al., 1989) and incubated for 20 min at 37°C. The top LB agarose (0.7%) of 3 mL maintained in molten (47°C) condition was added, mixed and poured onto LB agar plates, allowed to set and incubated for 6–8 h at 37°C. The phage was recovered by adding 3 mL of SM buffer to each plate and the plates were stored at 4-8°C for several hours with gentle shaking on rocking platform to allow bacteriophage to diffuse from the agarose. SM buffer containing phage particles was transferred to a polypropylene tube, 0.2 mL of chloroform was added and again 2ml of SM buffer was also added and drained to recover the maximum phage particles. The mixture was gently vortexed, then centrifuged at 4000 x g for 15 min at 4-8°C (REMI, CPR-24 Centrifuge, India). The supernatant was aspirated, a drop of chloroform was added, and the phage
A drop of purified and fixed phage lysate preparations were also incubated at pH 2, 4, 5, 6, 7, 8, 9 and 10 for 1 h and 4 hours at room temperature and 37°C and also in the presence of 20% (v/v) glycerol at -40°C and -20°C. Phage titres were determined after 3, 6, 9 and 12 months of storage by double agar layer technique (Adams., 1959).

Stability of the phage ΦDMEC-1 to pH, temperature and chloroform
A purified phage filtered through 0.22μm Minisart Sartorius filter of 10⁸ pfu/ml strength was incubated at room temperature and 60°C with shaking. The preparations were also incubated at pH 2, 4, 5, 6, 7, 8, 9 and 10 for 1 h and 4 hours at room temperature and 37°C with shaking. An equal volume of phage (10⁸ pfu/ml) was mixed with chloroform and incubated for 2 and 24 h at room temperature with shaking (Zuanna et al., 2011). After exposing the phage sample at various conditions, phage titre was assessed by double agar layer technique (Adams., 1959).

RESULTS
Characterization of multidrug Resistant E. coli
The antibiotic susceptibility data of E. coli isolates are presented in Table 1. Among the E. coli strains, 3 isolates were found to be resistant to most of the antibiotics and the isolate DMEC-1 was resistant to all the antibiotics tested.

Table 1 Antibiotic susceptibility of E. coli isolates

<table>
<thead>
<tr>
<th>Isolates No</th>
<th>Antibiotic discs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ak Ac Ca Ce Cf Cs Le Tb</td>
</tr>
<tr>
<td>DMEC-1</td>
<td>R    R  R  R  R  R  R  R</td>
</tr>
<tr>
<td>DMEC-2</td>
<td>R    R  S  R  R  R  R  R</td>
</tr>
<tr>
<td>DMEC-3</td>
<td>S    R  R  R  R  R  R  R</td>
</tr>
<tr>
<td>DMEC-4</td>
<td>R    S  R  S  S  R  S  S</td>
</tr>
</tbody>
</table>


Pattern : R- Resistant, S-Sensitive

16S rRNA gene sequencing and phylogenetic analysis:
The 16S rRNA gene 287 bp amplicons base sequences of the DMEC-1 were blasted and phylogenetic tree was constructed with closely matched aligned sequence. The phylogenetic tree reveals that isolate closely relates to E. coli.
strains—O157 H7 str S, strain 7, MREC33 and 2012K1. The phylogenetic tree of 16S rRNA gene of isolate is presented in Figure 1. The phylogenetic tree confirmed the isolate as *E. coli* (GenBank sequence accession number: KF408380).

**Figure 1. Phylogenetic tree of 16S rRNA gene of DMEC.1**

**Isolation and morphological features of ΦDMEC-1**

The cocktail of sewages originated from hospital, domestic, municipal waste and the water from water treatment plant were used as source to isolate the phage ΦDMEC-1. The samples were screened using the host strain multidrug resistant *E. coli* (DMEC.1) which has produced clear plaques of 2 mm diameter in the double-agar layer technique. Phage ΦDMEC-1 was purified by picking a single prominent plaque, negatively stained with 2% (w/v) potassium phosphotungstate (pH 7.0) and observed by transmission electron microscopy (Fig.2). Transmission electron microscopy has provided the basis for the recognition and establishment of bacteriophage families and is one of the essential criteria to classify novel viruses into families. It allows for instant diagnosis and is thus the fastest diagnostic technique in virology (Ackermann HW., 2012). The family *Siphoviridae*, whose members are characterized by long, noncontractile tails, is by far the largest family i.e over 3600 descriptions, or 57.3% (Ackermann and Prangishvili., 2012). The phage ΦDMEC-1 possesses an isometric icosahedral head of approximately 65 nm diameter connected to a long tail of 240 x 16 nm belongs to members of the family *Siphoviridae* of the order *Caudovirales* (Ackermann and Dubow., 1987; Ackermann., 2009). The morphological characteristics of phage was examined by Virologist, Hans-W Ackermann and confirmed as phage belonging to the family *Siphoviridae*.

**Figure 2. Electron micrograph of Φ DMEC-1**
Host range
Phage ΦDMEC-1 has an ability to lyse the host and also have broad host range on, among the E. coli isolates obtained from pyogenic skin infections. The lytic activity of the phage was assessed on E. coli isolates by spot test. 03 isolates (75%), which produced completely, cleared zones in spot tests; thus, these isolates were considered to be susceptible to infection with ΦDMEC-1. The remaining 01 E. coli (25%) isolate developed no plaques in spot test and the isolate was resistant to ΦDMEC-1 infection. The result shows that, ΦDMEC-1 has a wide host range among E. coli strains isolated from pyogenic skin infections.

Stability of the phage ΦDMEC-1
Stability of the phage was examined under different parameters by exposing at varied conditions. Phage lysates with a titre of 10⁰pfu/mL were stored at room temperature, -40°C, -20°C, 4°C, and 20°C and also in presence of 20% (v/v) glycerol at -40°C and -20°C. Phage titres maintained at various conditions were examined after 3, 6, 9 and 12 months of storage by double agar layer technique (Adams, 1959).

There was no significant decrease in the level of phage titre after 3 months storage at all of the tested temperatures, except at room temperature, where the titre was reduced by 30%. ΦDMEC-1 was highly sensitive to higher temperature (60°C) for 15 minutes, with a 100-fold decrease in titre. There was no decrease in the titre in chloroform treated sample after 4 and 24 h. Phage ΦDMEC-1 was completely lysed at pH 2 and 4 after 1 h of incubation at both temperatures. Incubation at pH 4 and 37°C caused a three and seven log decrease in phage titre after 1 and 5 h of incubation, respectively. The same acidity reduced the strength of the titre (PFU/ml) 10-fold at room temperature after 5 h. Phage ΦDMEC-1 was stable within a pH range 5–10.

DISCUSSION
The literature pertaining to skin infections with specific references in the introductory part of the article clearly explains E. coli is one among the various other bacteria to cause skin infections and the development of multidrug resistance of the organisms is essentially warranted. Phage therapy as an alternative approach to antibiotics, to combat drug resistant E. coli is shown to be quite promising in earlier studies.

Markoishvili et al., 2002 report on the use of PhagoBioDerm, the phage impregnated polymer, to treat infected venous stasis skin ulcers. Complete healing of ulcers was observed in 70% of nearly 100 patients. Slopek et al., 1987 report 95.0% positive cases for phage treatment of 162 “Diseases of the skin and subcutaneous tissue”, which includes furunculosis. A successful physician initiated FDA-approved phase I safety trial of phage therapy against skin ulcerations and other wounds was completed in 2008 at the Wound Care Center in Lubbock, Texas, following a series of positive results with Pyophage.

For the phase I trial (Rhoads et al., 2009) formulation of fully sequenced phages prepared by the company Intralytix, containing only two phages active against S. aureus, five against P. aeruginosa and one against E. coli, was applied to chronic infections without observation of significant side effects (Abedon et al., 2011). In the present study, ΦDMEC-1 isolated from the cocktail of sewage sample is an E.coli- specific partially characterized virulent lytic phage exhibiting bacteriolytic activity against 75% of MDR clinical isolates of E. coli and also has storage stability for three months at lower temperatures without significant decrease in phage titre. The phage lysate was stable within the pH range 5-10 at various temperatures and time and may be used for phage therapy after complete characterization.

ACKNOWLEDGEMENTS
We gratefully acknowledge Hans-W. Ackermann, Professor emeritus, Laval University, Quebec, Canada., former Vice-President of International Committee on Taxonomy of Viruses (ICTV) and chairman of ICTV bacterial virus sub-committee, for his technical support in electron microscopy and guidelines for bacteriophage classification. The corresponding author is grateful to Vision Group on Science and Technology (VGST), Govt. of Karnataka, India, for their financial support under Seed Money to Young Scientist for Research (SMYSR 2012-13 – Ref: VGST/SMYSR/2012-13/286 dated 19th March 2013)., Management of Bapuji Educational Association, Chairman, Director, Principal., and Head, Dept.of Biotechnology and Research Centre, Bapuji Institute of Engineering and Technology, Davangere, Karnataka for their ever encouraging support for the present research work.
REFERENCES


