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*Microbiology*

# New Bacteriophage Cocktail against Antibiotic Resistant *Escherichia Coli*

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**ABSTRACT. Pathogenic *Escherichia coli* are one of the most important groups of bacteria causing different infections in humans and animal. E.coli isolates were collected at three geographically distinct private farms in Georgia. From investigated 350 calves with symptoms of diarrhea pathogenic bacteria infections were identified in 120. Investigation of isolated bacteria indicated that the major infectious cause of diarrhea in calves were strains of Enterotoxigenic *E.coli*. All of the isolates showed high resistance to antibiotics used in the test. Six, newly isolated bacteriophages were selected from 16 samples for creation of effective phage cocktail which showed lytic activity against 95.8% out of 120 isolates of *E.coli* strains. Phage growth parameters, particularly rate of adsorption, rise period, latent period, and burst size were also determined. Based on the morphology, phages were classified into *Myoviridae* or *Siphoviridae* family. It was created a phage preparation with high lytic activity and broad spectrum that might be significant alternative for prevention and treatment of colibacillosis in calves caused by multidrug resistant *E.coli* pathogens.** *© 2018 Bull. Georg. Natl. Acad. Sci.*

**Key words:** *Escherichia coli*, bacteriophage, morphology, biological properties

Pathogenic *Escherichia coli* are one of the most important groups of bacteria causing different infections in humans and animal. The occurrence of toxigenic *E.coli* strains in humans and calves with diarrhea is well documented and cattle have been considered an important reservoir of *E.coli* strains involved in human disease. Infection caused by enterotoxigenic *Escherichia coli* is an infectious bacterial disease of calves that occurs during the first few days of life. These enterotoxigenic *Escherichia coli* are shed into the environment by infected animals in the herd and are ingested by newborn calves soon after birth. There is some natural immunity to enterotoxigenic *Escherichia coli*; however, it often fails to protect calves born and raised under modern husbandry conditions.

The severity of NCD infections may be associated with a single or various risk factors acting simultaneously; however intensive animal breeding systems have recently increased the

transmission of infectious diseases such as NCD [1]. Enterotoxigenic (ETEC) and enteropathogenic *E.coli* (EPEC) are two of the most common pathotypes associated with NCD, and are responsible for high morbidity and mortality rates [2, 3]. Additionally, enterohemorrhagic (EHEC)/Shiga toxin producer *E.coli* (STEC) are relevant because they are important zoonotic pathogens, and bovines have been identified as their natural reservoir [4, 5].

The intestinal microbiota, as well as pathogenic microorganisms, intimately interact with the eukaryotic host. This interaction depends on the microorganism and host, as well as the stage of infection in the case of pathogens. The ability of pathogenic *E.coli* to adhere to epithelial cells and persist in biofilms has important implications in the early stages of bacterial infection [2]. Additionally, the expression of toxins is a crucial step in the infection process. The toxicity of Shiga toxins 1 and 2 toxins of EHEC/STEC have been the focus of numerous studies, mainly because of the lethal effects of human infections [6].

Monitoring and control of these foodborn pathogens will reduce their occurrence in the environment and food line. Consequently, it could help prevent the occurrence of food pathogens in humans. Early detection of epizootic pathogens and identification of appropriate molecular targets can be used to conduct epidemiological monitoring.

Antibiotic therapy is frequently used to treat different infectious diseases in animals, including NCD. While this therapy may be associated with a mortality rate reduction in some cases, the indiscriminate use of antibiotics has been accompanied by an increase in bacterial resistance, generating important public health issues and economic losses in production industries in recent years [7]. The most commonly used antibiotics in animals include β-lactams, aminoglycosides, fluoroquinolones, and tetracyclines [8]. The first 3 groups are also widely used in the treatment of various infectious diseases in humans; therefore, selection pressure exerted by an inadequate use may have a direct impact on public health.

It is also known that the causative agents of E.coli-infections are characterized by weak sensitivity to antibiotics and sulphanilamide preparations which is why, obtaining of new biological preparations, in particular, specific *E.coli* bacteriophages, becomes necessary [9].

Targeted use of bacteriophages against *E.coli* could reduce this problem. Bacteriophages are ubiquitous, naturally abundant viruses, which invade and kill specific bacteria. They are recognized as gastrointestinal (GI) commensal microorganisms and have been isolated from various sources [10, 11].

Bacteriophages have a high specificity in their host-cell recognition that leads to little disturbance of commensal bacteria through the oral consumption of phages targeted at pathogens, and while bacteria develop specialized phage-defence mechanisms, phages also continuously adapt to these changed host systems [12]. Also, to address this issue, it is highly recommended to use cocktails of several phages to obtain sufficient breadth of host range and to reduce the probability of phage resistance [13].

In a study by O'Flynn et al., a cocktail of *E.coli* O157:H7-specific bacteriophages completely reduced pathogen counts on the surface of beef steaks [14]. Additionally, studies have shown that phages can effectively eliminate *E.coli* O157:H7 in biofilms on various food-processing surfaces such as stainless steel and high-density polyethylene [15,16].

Existing in nature abundance of bacteriophage clones with different morpho-biological properties and molecular organization, differing from one another both in mechanism of interaction with host cells and reproductive capacity, requires a clear differentiation and in-depth study of principal and some subsidiary taxonomic characters for their purposeful use in treatment and prevention of *E.coli*-infections.

The aim of the present work was monitoring of prevalence of pathogenic *E.coli* isolates in calves, characterization of these pathogens by serotyping, antimicrobial-susceptibility testing. Isolation, identification and characterization of lytic bacteriophages capable of infecting *Escherichia coli* serotypes and on the base of these isolated phages creation of high-effective phage preparation, which will be used in dairy farms for treatment and prophylaxis of different diseases induced by pathogenic strains of *E.coli* in calves.

## Materials and Methods

Isolation and identification of *E.coli* pathogens. Fecal samples were collected from different farms in Georgia and kept in sterile sampling box. The samples were brought to the laboratory in a sampling box maintaining low temperature (≤4°C) using ice pads.

The samples were first transferred to the sterile flask containing sterile Tryptic Soy Broth (TSB). The samples were then homogenized with and incubated at 37°C for 16-24 h. Following the incubation, for isolation of *E.coli* strains, a loopful from the diluted specimens was inoculated into MacConkey's agar and incubated at 37°C for 18–24 hours. Bacterial colonies were identified based on gross morphology, a number of colonies and the hemolytic pattern. Appropriate biochemical and serological tests were performed on the colonies isolated to identify the pathogens.

**Serogrouping of *E.coli* isolates.** *E.coli* serogroups were identified serologically by slide agglutination test using standard polyvalent and monovalent *E.coli* antisera according to Edwards and Ewing (1972).

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**Antimicrobial susceptibility testing.** All the E.coli isolates were exposed to different antibiotics for its antimicrobial susceptibility and drug resistance pattern determination using disk diffusion assay following the guidelines of clinical and laboratory standard institute [17]. Pre-incubated 24 h cultures of *E.coli* was swabbed over Brain Heart agar. After placing the antibiotic discs aseptically, the plates were incubated at 37°C for 18-24 h and zone of inhibition were measured subsequently. 15 different antimicrobial agents, most widely used in clinics and also used in this study were: Penicillin, Ampicillin, Carbenicillin, Oxacillin, Chloramphenicol, Streptomycin, Tetracycline, Gentamicin, Kanamycin, Erythromycin, Methicillin, Cephamycin, Ceftazidime, Ketotifen, Cefotaxime.

**Bacteriophage isolation.** The bacteriophages were isolated from wastewater by filtration, followed by the addition of broth concentrate and 18-h cultures of different strains (test strains) to the filtrate. After 24-h incubation in a thermostat, the mixture was filtered through filters with a pore diameter of 0.45 μm (Millipore, United States), and the filtrates were spot-tested for the presence of phages by application of the filtrate (0.1 mL) on the lawn of the test strain on a solid nutrient medium. The result was considered positive if there was a lysis zone on the lawn in 18–24 h of cultivation at 37°C [18]. The highly specific bacteriophage strains were screened and selected by the plate method according to Gracia [19].

**Bacteriophage cloning by repetitive single plaque isolation**. The phages isolated from the initial material are not uniform. Mixtures of various phage types are frequent, as judged by plaque morphology. Plaques are of various sizes determined by virion size, speed of phage reproduction, etc. Plaques have other characteristic features, such as the sharpness of their edges, the presence of a halo and the completeness of the clearing. A single plaque may contain $10^7$-$10^9$ virions. A number of theoretical and practical considerations require the use of "pure" phage lines (clones) during therapy. For this purpose an isolated phage plaque is touched with the fine end of a Pasteur pipette and small specimen is collected, subjected to serial dilution, mixed with plating

bacteria and molten soft agar and spread on a plate. Isolation of a reliably pure bacteriophage clone requires about 3-5 such steps.

**Electron microscopy.** The morphology group membership of bacteriophages was investigated by the method of electron microscopy. Phages with a high titer of $>10^{10}$ CFU/mL were negatively stained with 1% uranyl acetate. Pictures were obtained by transmission electron microscopy (JEM 100 SX, JEOL, Japan) with negative contrasting of the preparations by uranyl acetate.

Antibiotic sensitivity test was performed for all 120 Enterotoxigenic *E.coli* strains isolated from calves to ascertain an antibiotic resistance level. Results are presented in Diagram 1.

All of the isolates were resistant to penicillin, high resistance was shown to erythromycin and methicillin (97.8%). Certain resistance level was observed to carbenicillin, oxacillin, chloramphenicol, streptomycin. Stains showed sensitivity to ceftazidime, cephamycin and ketotifen.

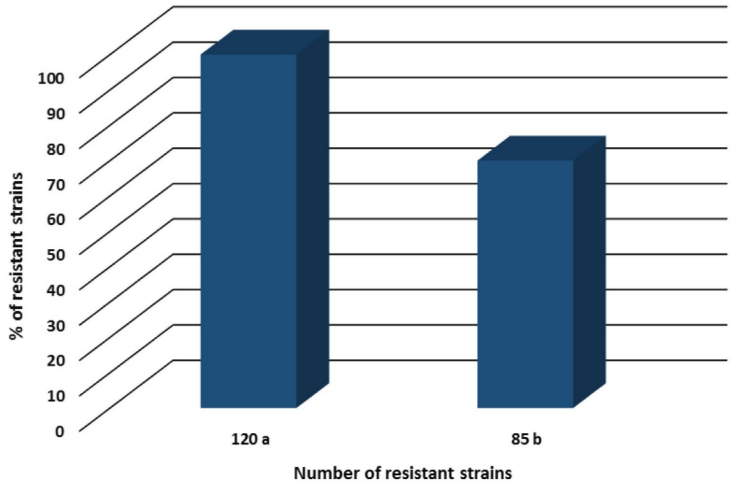


**Fig. 1.** Incidence of antimicrobial resistance in Pathogenic *E.coli* isolates
[a]Isolates resistant to one or more antimicrobials
[b]Isolates resistant to three or more antimicrobials

## Results and Discussion

*E.coli* isolates were collected at three geographically distinct private farms in Krtsanisi, Gardabani and Senaki. We have studied cases of neonatal calf diarrhea in calves of one week old or less which were not treated. From investigated 350 calves with symptoms of diarrhea pathogenic bacteria infections were identified in 120. Investigation of morphological, cultural properties and their antigenic structure of the isolated bacteria indicated that the major infectious cause of diarrhea in calves were strains of Enterotoxigenic *E.coli* serotypes, such as serotype 08 (29.1%), 015 (20.8%), 078 (15%), 020 (15%), 055 (12.5%), 0119 (4.1%) and 086 (3.5%).

As we can see from Fig. 1, pathogenic E.coli isolates were characterized with high level of antibiotic resistance. 70% of Isolates were resistant to three of more antibiotics.

To isolate bacteriophages effective against new isolated pathogenic E.coli strains 40 samples of urban sewage water, sewage of farms, feces of calves, river water and other environmental sources were investigated. From them 16 samples contained phages lysed strains of pathogenic E.coli. For formulating polyvalent phage preparation with widest spectrum of action and high litic activity all 16 phages were characterized according to host range. These phages were tested against all 120 new isolated pathogenic E.coli strains from our collection. 6 phages conditionally named TG-1,

TG-2, TG-3, TG-4, TG-5 and TG-6, with wide, complementary, non-overlapping host ranges were selected for further characterization (Fig. 4).

It has been established that E.coli phages belong to the morphological types of Myoviridae and Siphoviridae.

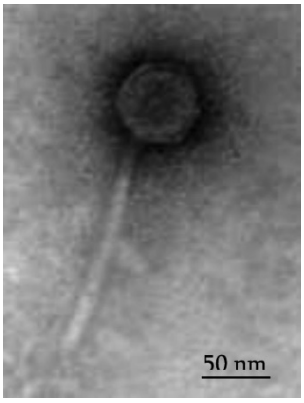


**Fig. 2.** Electron micrograph of *E.coli* phage TG-6 (strain *E.coli* 15)
Description: Siphoviridae. Size: head: 50 nm X 50 nm, tail: 125 nm X 10 nm

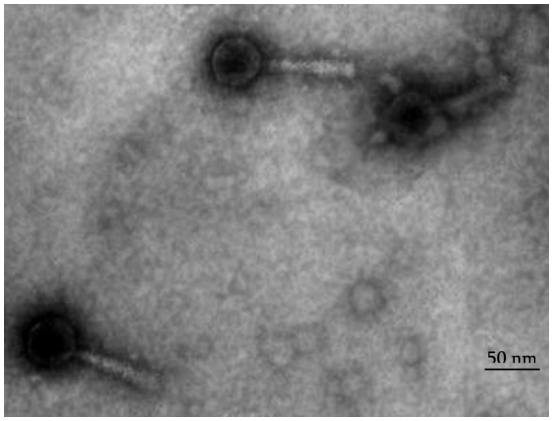


**Fig. 3.** Electron micrograph of E.coli phage TG-5 (strain E.coli 12)
Description: Myoviridae. Size: head: 50 nm X 50 nm, tail: 120 nm X 15 nm

All six selected phages were characterized based on their morphological and biological properties such as morphology of negative colonies, some biological features of these phage clones, such as adsorption rate, duration of latent period and medium harvest per cell.

The adsorption time of the studied phages varied within 10-12 min, the latent period – 20-24 min, and the yield – 100-110 phage particles per one virion.

All of the 6 characterized bacteriophages and polyphage created with these phages were tested against all 120 strains. Results are presented in Fig. 4.

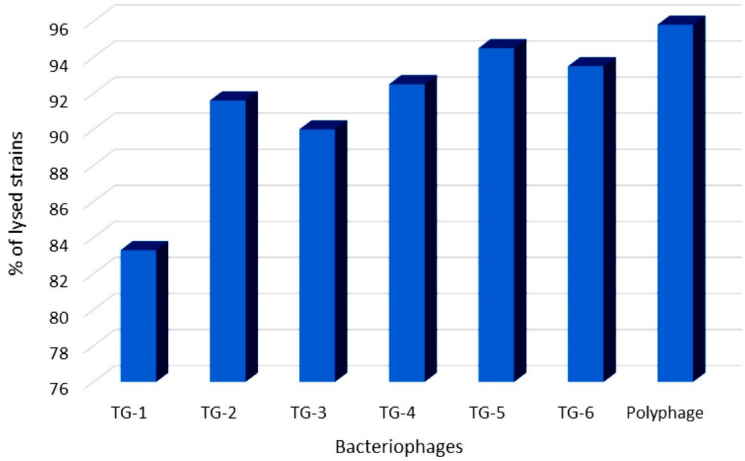


**Fig. 4.** Host ranges of candidate bacteriophages and polyvalent phage preparation

The continuing emergence of food borne pathogens that are resistant to antimicrobials is a cause of increasing concern. Following the discovery that antibiotics promote growth as well as prevent diseases in farm animals, the use of antibiotics in farms increased considerably. Wide and often groundless application of antibiotics and

chemical preparations in veterinary medicine, especially those with a wide range of action, have promoted the general spread of bacteria with natural and acquired drug resistance. In many cases multiresistant bacteria infecting humans are directly linked to resistant organisms in animals. Existence of such pathogens is problematic not only for animal health, but also because of possible transmission of antibiotic resistant bacteria from animals to humans through the food supply.

Antibiotic-resistant bacteria from farms contribute to the spread of antibiotic-resistant bacteria in the air, water, and soil so antibiotic-resistant bacteria from food production can contribute to rising rates of antibiotic resistance. The development of alternative antimicrobial remedies has become one of the highest priorities of modern medicine and biotechnology. Interest in the application of bacteriophage as an alternative antimicrobial chemotherapy in various fields including human infections, food safety, agriculture, and veterinary applications has been increased recently. The use of bacteriophages may be a safe and effective alternative to antibiotics for the treatment and prevention.

Our investigation reveals prevalence of pathogenic E.coli associated with infections in calves in Georgia. Most of these strains are characterized with high level of antibiotic resistance. A phage preparation on the base of 6 newly isolated bacteriophages with a high range of activity against different strains of pathogenic E.coli was created. This preparation was effective against 95.8% of investigated strains. Considering high lytic activity and broad spectrum of this polyvalent phage preparation, it will be significant alternative for prevention and treatment of colibacillosis in calves caused by multidrug resistant E.coli pathogens. Control of these food-borne pathogens at the firm level will reduce their occurrence in the environment and food line. Consequently, it could help to prevent the occurrence of food pathogens in cattle, food, humans and environment.

*მიკრობიოლოგია*

# ახალი ფაგური კოქტეილი ანტიბიოტიკორეზისტენტული *E. coli* საწინააღმდეგოდ

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**გ. ელიავას ბაქტერიოფაგიის, მიკრობიოლოგიისა და ვირუსოლოგიის ინსტიტუტი, თბილისი, საქართველო

(წარმოდგენილია აკადემიის წევრის თ. სადუნიშვილის მიერ)

საქართველოს სხვადასხვა რეგიონის მეცხოველეობის ფერმებიდან აღებული სინჯების კვლევის შედეგად გამოვლინდა, რომ ხბოებში დიარეით მიმდინარე ინფექციური დაავადებების ძირითადი გამომწვევია ენტეროტოქსიკური ***E. coli***. გამოყოფილი შტამების უმრავლესობა ხასიათდებოდა მაღალი რეზისტენტობით ანტიბიოტიკების მიმართ. შერჩეულ იქნა 6, ახლად გამოყოფილი, ბაქტერიოფაგი ეფექტური ფაგური კოქტეილის შესაქმნელად. აღნიშნულმა ფაგურმა პრეპარატმა შესწავლილი შტამების მიმართ გამოავლინა მაღალი ლითიური აქტივობა - 95,8%. ფაგური პრეპარატი, მაღალი ლითური აქტივობისა და ფართო სპექტრის გათვალისწინებით, შესაძლებელია ჩაითვალოს როგორც ალტერნატიული საშუალება ხბოებში კოლიბაქტერიოზის მკურნალობისა და პროფილაქტიკისათვის.

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