Virus Research 240 (2017) 8–17

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Effects of single and combined use of bacteriophages and antibiotics to inactivate *Escherichia coli*

Nádia Valério, Cristiana Oliveira, Vânia Jesus, Tatiana Branco, Carla Pereira, Catarina Moreirinha, Adelaide Almeida*

Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193, Aveiro, Portugal

ARTICLE INFO

Keywords:
Bacterial-phage inactivation
Antibiotic resistance
Ciprofloxacin
Urinary tract infections

ABSTRACT

A major concern of phage therapy is the emergency of phage-resistant mutants. This limitation can be overcome by the combined use of phages and antibiotics. It has been shown that the combination of antibiotics and phages is an alternative that cannot only be effective at reducing bacterial numbers, but also to contribute to the management of resistance levels. However, this view has only been discussed with regard to antibiotic resistance and not to control phage-mutant emergence. In our study we compared not only the resistance of the bacteria to the four antibiotics tested with and without phages addition, but also the resistance to the phages in the presence and absence of antibiotics. The aim of this study was to evaluate the potential synergistic effect of phages and antibiotics in the inactivation of *Escherichia coli* in order to control infections, namely urinary tract infection (UTI), and to reduce the development of bacterial resistance to phages. Phage therapy combined with antibiotics (ampicillin, piperacillin, kamanycin, tetracycline, chloramphenicol and ciprofloxacin) was evaluated in the inactivation of *E. coli*, both in saline solution and urine samples. Phage and antibiotic combinations could result in high synergistic effects in the inactivation of bacteria. The combination of phage and ciprofloxacin at sublethal concentration decreased the bacterial counts in urine samples by 7.8 ± 0.1 log CFU/ml after 8 h, but when phages or the antibiotic were tested alone, the decrease was of 3.9 ± 0.3 log CFU/mL and 1.2 ± 0.1 log CFU/mL, respectively, after the same time. The efficacy of the combination of the two therapies depends on the antibiotic resistance status of the targeted bacteria to the employed antibiotic and of the antibiotic type (bactericide or bacteriostatic), causing the same or less bacterial resistance than phages and antibiotics applied alone ($1.2 \pm 1.0 \times 10^{-5}$ to $2.4 \pm 1.5 \times 10^{-7}$ CFU/mL for the combined treatment, $2.7 \pm 0.2 \times 10^{-4}$ CFU/mL for the antibiotics and $5.0 \pm 1.5 \times 10^{-6}$ CFU/mL for the phages). The addition of antibiotics, at subinhibitory concentration, during phage treatment can control the phage-mutant. The high bacterial inactivation efficiency of these combined techniques and the long periods of phage survival in urine, pave the way for depth studies to control UTI and to overcome the development of resistances by bacteria.

1. Introduction

Escherichia coli is one of the most frequent causes of common bacterial infections, including abdominal infections, urinary tract infections (UTI), enteric infections, pneumonia, bacteremia and meningitis (Tortora et al., 2012). This bacterium is the leading cause of both community-acquired and nosocomial UTI, being a problem of public health. Up to 50% of females eventually experience at least one episode of UTI. *E. coli* causes 12–50% of nosocomial infections and 4% of cases of diarrheal disease (Tabasi et al., 2015; Wanke and Sears, 2010).

Overuse of antibiotics has significantly increased the emergence of antimicrobial multidrug-resistant bacteria, over the years. Most of strains of *E. coli* developed resistance to most antibiotics available, including drugs with different mechanisms of action. It can be resistant to either 1) bacteriostatic, inhibiting cell growth without killing the cells, for example by inhibiting the protein synthesis (e.g. tetracycline, chloramphenicol); or 2) bactericidal, resulting in cell killing, also by inhibiting the cell wall syntesis (e.g. piperacillin, ampicillin), acid nucleic synthesis (e.g. ciprofloxacin) or protein synthesis (e.g. kanamycin) (Fair and Tor, 2014; WHO, 2014).

Phage therapy (use of lytic phages to inactivate bacteria) can be used as an alternative to control bacterial infections (Pereira et al., 2016a). Bacteriophages (or phages) are viruses that are capable to infect exclusively bacteria. Until the advent of antibiotics, phage therapy was widely used, especially in the Eastern Europe countries. It fell into disuse and now, due to the overuse of antibiotics and consequent

* Corresponding author.
E-mail address: aalmeida@ua.pt (A. Almeida).

http://dx.doi.org/10.1016/j.virusres.2017.07.015
Received 4 March 2017; Received in revised form 13 June 2017; Accepted 19 July 2017
Available online 23 July 2017
0168-1702/

appearances of resistant bacteria, a growing interest in this therapy approach can be noted (Brüssow, 2005; Pereira et al., 2016a; WHO, 2014). Several studies already shown that phages efficiently inactivate *E. coli*, even antibiotic resistant strains (Brüssow, 2005; Pererva et al., 2008; Rahmani et al., 2015).

A major concern regarding the use of phages to control infections is the emergency of phage-resistant mutants (Rahmani et al., 2015; Salman and Abdulamir, 2014; Viertel et al., 2014). Resistance may result from the alteration or loss of the bacterial cell surfacoe receptors; inhibition of phage DNA penetration; production of restriction endonucleases which degrade the phage DNA, clustered regularly interspaced short palindromic repeats (CRISPR) system, a widespread microbial response to by-pass the selective pressure exerted by phage infection, among others (Deveau et al., 2010). This limitation can be overcamed by the combined use of phages and antibiotics. Some studies reported a synergetic effect of the combined use of antibiotics and phages (Kirby, 2012; Verma et al., 2009; Zhang and Buckling, 2012). Zhang and Buckling (2012) showed that the combination of phage SBW25φ2 and kanamycin reduced the resistant evolution of a strain of *Pseudomonas fluorescens* SBW25 relatively to the antibiotic alone. It has been stated that the decrease in bacterial resistance to phages and/or antibiotics in dual therapy is due to the fact that a strain that is non susceptible to one antimicrobial agent can be eliminated by the second one (Viertel et al., 2014). Another study performed by Comeau et al. (2007), showed a synergism between the phage φMFP and several antibiotics, like aztreonam and cefixime against an uropathogenic strain of *E. coli*. The combination of the phage with those antibiotics showed an increase of the phage lysis plaques production by the host bacterium. Verma et al. (2009) reported that although the treatment with a lytic phage and with the conjugation of phage and antibiotic were equally effective against a biofilm of *Klebsiella pneumoniae* B5055, the combination of both therapies showed a decrease in the formation of resistant variants to the tested antibiotic. However, a recent work suggests an increase of resistant bacteria when the combination of phage and antibiotics was used (Cairns et al., 2016). According the authors, sub − minimum inhibitory concentrations (MIC) of streptomycin alter bacteria–phage interaction and resistance evolution, with ecological and evolutionary outcomes different from those expected under selection by antibiotic concentrations exceeding MIC (Cairns et al., 2016). Although it has been shown that the combination of antibiotics with phages is an alternative that cannot only be effective at reducing bacterial numbers, but also to contribute to managing resistance levels, this view has only been discussed with regard to antibiotic resistance and not to control phage-mutant emergence.

The aim of this study was to evaluate the potential synergistic effect of phages and antibiotics in the inactivation of *E. coli* in order to control infections, namely UTI, and to reduce the development of bacterial resistance to phages and antibiotics. In this study it was compared not only the resistance of the bacteria to four antibiotics with and without phages, but also the bacterial resistance to the phages in the presence and in the absence of the antibiotics.

2. Materials and methods

2.1. Bacterial strain and growth conditions

Escherichia coli strain ATCC 13706 was used in the study. Fresh plates of the bacterial strain *E. coli* were stored in Tryptic Soy Agar with 15% agar (TSA; Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was transferred to 30 mL of Tryptic Soy Broth (TSB; Liofilchem, Italy) and grown overnight at 37 °C. Then, 300 μL of fresh culture were transferred to 30 mL of TSB and incubated overnight to reach the optical density (O.D. 600 nm) of 0.8, which correspond about 10^9 cells per mL, according to a growth curve previously determined (Pereira, 2016).

2.2. Phage selection and quantification

The phage ECA2 was isolated in a previous work from sewage samples (station EEIS9 of SIMRIA Multi Sanitation System of Ria de Aveiro) (Pereira et al., 2017a). The phage suspension was prepared from the phage stock in SM buffer (0.1 M NaCl, 8 mM $MgSO_4$, 20 mM Tris-HCl, 2% (w/v) gelatin, pH 7.5). Five hundred microliters of the phage stock were added to 50 mL of *E. coli* culture in the exponential growth phase. Suspension was incubated at 37 °C for 6–8 h. All lysate was centrifuged at 13.000 × *g* (Heraeus Megafuge 16R Centrifuge; Thermo Scientific) for 10 min, to remove intact bacteria or bacterial debris. Phage suspensions were stored at 4 °C after the adition of 1% chloroform.

The quantification of phages was determined, in duplicate, by the agar double layer technique, using TSA medium (Adams, 1959). Successive dilutions of the phage suspension were performed in a phosphate buffered saline (PBS: 137 $mmol^{-1}$ NaCl (Sigma), 2.7 mmol L KCl (Sigma), 8.1 $mmol^{-1}$ $Na_2HPO_4{\cdot}2H_2O$, 1.76 $mmol^{-1}$ KH_2PO_4 (Sigma), pH 7.4) and 500 μL of each dilution together with 100 μL of the *E. coli* culture were mixed with 3 mL of TSB 0.6% top agar layer and placed over a TSA plate. The plates were incubated at 37 °C for 6–8 h, with the number of plaques being counted and the results expressed as plaque-forming units per mililitre (PFU/mL).

2.3. Antibiotic preparation

The antimicrobial agents evaluated in the study included ampicillin (Amp, Applichem Panreac ITW companies, Germany) and piperacillin (Pip, Sigma–Aldrich, St. Louis) as β-lactam, kamanycin (Kan, Applichem Panreac ITW companies, Germany) as aminoglycoside, tetracycline (Tetra, Sigma–Aldrich, St. Louis), chloramphenicol (Chl, Applichem Panreac ITW companies, Germany) and the fluoroquinolone ciprofloxacin (Cip, Sigma–Aldrich, St. Louis). Stock solutions were prepared following the manufacter instructions and used for preparation of the dilutions.

2.4. Minimum inhibitory concentration (MIC) selection

The minimum inhibitory concentrations (MIC) used for the six antibiotics under study were selected according to CLSI (Clinical Laboratory Standardization Institute) and (CLSI, 2013; EUCAST, 2015). The estimated MICs were: ampicillin (8 mg/L), kanamycin (32 mg/L), piperacillin (16 mg/L), tetracycline (4 mg/L), chloramphenicol (8 mg/L) and ciprofloxacin (0.5 mg/L).

2.5. Antimicrobial susceptibility tests

The antimicrobial susceptibility tests were done according the EUCAST standards (EUCAST, 2015). *E. coli* were tested for susceptibility to ampicillin (Oxoid, UK), piperacillin Oxoid, UK), kamanycin (Oxoid, UK), tetracycline (Oxoid, UK), chloramphenicol (Oxoid, UK), and ciprofloxacin (Oxoid, UK). The bacterial culture (O.D = 0.8) was diluted 1:100 in 0.85% saline solution to obtain a density of 0.5 MacFarland. After that, a sterile cotton swab was dipped into the suspension and spreaded the inoculum over the entire surface of the plate of Muller-Hinton (Oxoid, UK) by swabbing in three different directions. Then, the disks with antibiotic were placed at the plate and incubated at 37 °C during 16–20 h. The diameters of inhibition zones were measured and the results were interpreted according EUCAST (2015).

2.6. Kill curves with phage and antibiotics in phosphate buffered saline

E. coli inactivation was determined using phage ECA2 and antibiotics at MIC with different mechanisms of action, at a MOI of 100 in PBS. Ciprofloxacin was also tested at a subinhibitory concentration (1/10 of MIC = 0.05 mg/L, Cip0.05). In order to obtain a MOI of 100,