JRVM
Journal of Research in Veterinary Medicine

RESEARCH ARTICLE

J Res Vet Med. 2021: 40 (2) 88-92
DOI:10.30782/jrvm.904155

Sensitizing And Control Of Colistin-Resistant *E. Coli* O157:H7 with Bacteriophage Application

Gizem CUFAOGLU[1]*, Naim Deniz AYAZ[1]

[1]Kirikkale University, Faculty of Veterinary Medicine, Department of Food Hygiene and Technology, Kirikkale-Turkey

Received 26-03-2021 Accepted 04-08-2021

Abstract

In these days that we are drifting into the post-antibiotic era, antibiotics called "last-resort" are begun to be used more frequently. Colistin is one of the last-resort antibiotics that act on Gram-negative bacteria. The aim of the study was to investigate antibiotic re-sensitization effect of lytic bacteriophages on colistin resistant E. coli O157:H7 in-vitro. In the study, four E. coli O157:H7 isolates (encoded 25KA, 44RA, 120RA, and 168KA) were included. These isolates had different features such as harboring some of the mcr genes but not showing resistance to colistin, or demonstrating resistance to colistin without carrying any mcr genes. A lytic bacteriophage cocktail was prepared with three Myoviridae family member phages. In order to determine the effect of lytic bacteriophage application on the colistin resistance of E. coli O157:H7 strains before, during and after bacteriophage treatment, minimum inhibitory concentrations (MIC) of the isolates were determined by broth microdilution method. The results were interpreted according to EUCAST. According to the results, up to 3.6 log cfu/ml reductions in colistin-resistant E. coli O157:H7 were detected within 6h incubation at 23°C. Colistin and phage combination showed synergistic effect. While strains 25KA and 168KA became susceptible to colistin, 44GA and 120RA were totally eliminated. The survivors of the phage treatment also became sensitive to colistin. Phage-resistant mutants of 25KA and 168KA showed susceptibility to colistin (1 µg/ml and 0.5 µg/ml, respectively). In addition, 44GA and 120GA remained susceptible. The findings of this study highlight that in addition to taking advantage of the lytic activity of phages in biocontrol area, phages also play a major role in re-sensitization to a last-resort antibiotic like colistin. The results show the synergy between phage–antibiotic combination treatment and give the promising idea that this approach has the potential to extend the effective lifetime of antibiotics.

Key words: Bacteriophage, colistin, E. coli O157:H7, re-sensitization, MIC

Introduction

Antibiotics have played a significant role in health care since their first discovery. These antibacterial chemotherapeutics have served for many years in improving the quality of life and expanding the life span. However, today human health is threatened by the emergence of antibiotic-resistant bacteria due to the unsuitable and misuse of antibiotics. WHO estimates that the deaths caused by antibiotic-resistant bacteria will result in 10 million deaths per year by 2050.[1] In these years that we are drifting into the post-antibiotic era, antibiotics called "last-resort" are begun to be used more frequently in the fight against bacteria that show resistance to multiple antibiotics. Colistin is one of such antibiotics that act on Gram-negative bacteria by altering cell membrane permeability.[2] Colistin, which was not among the first choice antibiotics because of its toxic effect on the kidney, has been used in the last two decades due to the increase in infections caused by multi-drug resistant bacteria.[3] Unfortunately, bacteria rapidly developed resistance against this last-resort antibiotic, and since 2015

* Corresponding author: Gizem Cufaoğlu Department of Food Hygiene and Technology Faculty of Veterinary Medicine, Kirikkale University 71450, Yahsihan - Kirikkale/Turkey E-mail: gizemcufaoglu@kku.edu.tr Tel: +90 318 357 4242

ten plasmid-mediated colistin genes have been reported in various species belonging to the *Enterobactericea*.[4,5]

As the number of antibiotic-resistant bacteria increases day by day, it has become inevitable to seek alternative methods in combating pathogens. Bacteriophages, which were discovered at the beginning of the 19th century but lost their popularity with the discovery of antibiotics, have been gaining importance again.[6] These natural killers of bacteria have been used in many fields including biocontrol of foodborne pathogens, phage therapy, biosanitation and biopreservation.[7] The use of phages in combination with antibiotics is a practice that has come to the fore in recent years and has applied in various case studies.[8] In this context, the combination of antibiotics and phage is expected to create a synergistic effect and show a detrimental effect on antibiotic-resistant bacteria. Herein, we aimed to investigate antibiotic re-sensitization effect of lytic bacteriophages on colistin-resistant *E. coli* O157:H7 in-vitro.

Materials and Method

Isolates

Four *E. coli* O157:H7 isolates (coded as 25KA, 44RA, 120RA and 168KA) were included in the study. The isolates were part of a collection obtained from cattle carcass (K) and recto-anal mucosal swap (R) samples.[9] Among these isolates: i) *E. coli* O157:H7 25KA is sorbitol fermentative, harboring none of the *mcr-1, mcr-2, mcr-3, mcr-4*, and *mcr-5* genes but carrying 62 different antimicrobial resistance genes including β-Lactams, aminoglycosides, fluoroquinolones, tetracyclines, macrolides, phenicols, penams (individual data sequence is available at GenBank under BioProject accession number ID PRJNA503568. Review link to the data: https://www.ncbi.nlm.nih.gov/nuccore/CP033605.1). At the same time, 25Ka is a colistin resistant isolate with a MIC value of 128 µg/ml. ii) *E. coli* O157:H7 168KA is a sorbitol non-fermentative isolate that, harboring both of the *mcr-2* and *mcr-3* genes, and resistant to colistin with a MIC value of 128 µg/ml. iii) *E. coli* O157:H7 44GA is a non-sorbitol fermentative, carrier of both mcr-2 and *mcr*-3 genes, and colistin susceptible with a MIC value of 0.25 µg/ml. iv) *E. coli* O157:H7 120GA is a non-sorbitol fermentative isolate, harboring none of the *mcr-1, mcr-2, mcr-3, mcr-4, mcr-5 genes*, and colistin susceptible isolate with a MIC value of 0.25 µg/ml.[10]

A lytic phage cocktail was prepared with Myoviridae family members (M8AEC16, M11AEC16, and M12AEC50) in order to use in the in-vitro analysis. The lytic activities of these phages were previously described.[11]

In-vitro bacteriophage treatment to *E. coli* O157:H7 strains

E. coli O157:H7 strains were diluted in Tryptic Soy Broth (TSB, Oxoid CM0129, UK) for the expected concentration of 102 cfu/ml according to previously determined OD600 values and infected with a final titer of 108 pfu/ml phage, then incubated at 37°C for 24 h. Colony counts of *E. coli* O157:H7 strains were detected at various times (0., 1., 3., 6. and 24. h) on Chrom-EHEC agar (CHROMagarTM STEC, LF-EXT-033, France) after incubation at 37°C for 24 h.11 Up to 5 colonies that survived and showed resistance to lytic bacteriophage application were re-suspended in caution-adjusted Mueller Hinton broth (caMHB, Thermo Scientific, UK) for minimum inhibitory concentrations (MIC) test.

Minimum inhibitory concentrations (MIC) profiles of E. coli O157:H7 strains

In order to determine the effect of lytic bacteriophage application on the colistin resistance of *E. coli* O157:H7 strains before, during, and after bacteriophage treatment, minimum inhibitory concentrations (MIC) of the isolates were determined by broth microdilution method using the FRCOL kit (Thermo Scientific, Sensititre FRCOL, West Sussex, UK) following the manufacturer's instructions. For this purpose, firstly *E. coli* O157:H7 strains were subjected to MIC test before bacteriophage treatment. In parallel, bacteria and phage combination with 103 MoI were subjected to MIC test in order to compare the MIC values of the strains with and without bacteriophage application. On the same day, *E. coli* O157:H7 strains that were survived and showed resistance to lytic bacteriophage after 6 hours were also subjected to MIC test with the same conditions to find out the changes of phenotypic colistin resistance after bacteriophage application. The plate was incubated at 35-37°C for 18-24 h.

The range of colistin in the test was between 0.12-128 µg/ml. The results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Table 2) and MIC values higher than 2 µg/ml were defined as resistant. The MIC test was repeated three times.

Results

In the study, bacteriophage cocktail (M8AEC16, M11AEC16 and M12AEC50) was detected as highly effective for the biocontrol of colistin-resistant *E. coli* O157:H7 strains. According to the in-vitro analyses, up to 3.6 log cfu/ml reductions were detected within a 6 h incubation period at 23°C. The reductions were shown in Table 1.

After 24 h incubation at 23°C, an increase in *E. coli* O157:H7 counts was determined for phage treated inoculums. Bacteriophage-resistant *E. coli* O157:H7 strains were inoculated to Chrom-EHEC agar overnight at 37°C and then these resistant colonies were subjected to microdilution test to found out the MIC values to colistin.

Table 1. Mean reductions of bacteriophage applied colistin-resistant E. coli O157:H7 strains.

Initial MoI (log[pfu/cfu]/ml)	Initial colony count (log cfu/ml)	Storage temperature (°C)	Mean reduction (log cfu/g)			
			1 h	3 h	6 h	24 h
5.6	2.8	23	1,6	3,2	3,6	1,9

MoI: Multiplicity of Infection
cfu: colony forming unit
pfu: plaque forming unit

In our previous study, isolates were subjected to PCR, and it was determined that two of the *E. coli* O157 isolates (44GA and 168KA) were both *mcr-2* and *mcr-3* genes carriers. However, in the other two *E. coli* O157:H7 isolates (25KA and 120RA) none of the mcr genes (from mcr-1 to mcr-5) were detected.

MIC test results of the isolates before the bacteriophage treatment showed that despite the absence of mcr genes, 25KA showed resistance to colistin with a high MIC value of 128 µg/ml. Besides, *mcr-2* and *mcr-3* genes harbored 168KA were also found to be resistant to colistin with a MIC value of 128 µg/ml. In contrast, *mcr-2* and *mcr-3* carriers 44GA and 168KA were found to be susceptible to colistin with a MIC value of 0.25 µg/ml and 0.5 µg/ml respectively, before bacteriophage treatment (Table 2).
MIC test results of colistin and phage combination showed a different profile compared to untreated strains. Strains 25KA and 168KA became susceptible to colistin with a MIC value of 0.5 µg/ml and 0.25 µg/ml, respectively. On the other hand, MIC values of 44GA and 120RA could not be detected because of the lytic activity of the bacteriophage cocktail.

Table 2. Mcr genes characteristics of the strains, and their MIC values before, during and after in-vitro analysis.

Strains	*mcr* genes	MIC values of the strains (µg/ml)		
		Untreated	Phage + colistin	Phage-resistant mutants
25KA*	-	128	0.5	1
168KA	*mcr-2*, *mcr-3*	128	0.25	0.5
44RA	*mcr-2*, *mcr-3*	0.25	nd	1
120RA	-	0.5	nd	1

MIC: minimal inhibitory concentration
* sorbitol fermentative *E. coli* O157:H7
- not harboring any of the *mcr* genes
nd: non-detected due to the phage lysis

The survivals of the phage treatment became susceptible to colistin. According to the microbroth dilution test results, phenotypically resistant 25KA and 168KA showed susceptibility to colistin with a MIC value of 1 µg/ml and 0.5 µg/ml, respectively. In contrast, MIC values of the susceptible strains 44GA and 120GA were increased to 1 µg/ml, but remained under 2 µg/ml (breakpoint of susceptibility) (Table 2).

Discussion and Conclusion

In the study, four *E. coli* O157:H7 isolates were chosen to display the bacteriophage effect on colistin resistance. These isolates had different features such as harboring some of the *mcr* genes but not showing resistance to colistin, or demonstrating resistance to colistin without carrying any mcr genes. Among them, 25KA has a distinct place because it does not harbor any mcr genes, but showing a high resistance to colistin (128 µg/ml), also unlike other *E. coli* O157 isolates it ferments sorbitol as an energy source. The whole-genome sequence (WGS) analysis of 25KA previously revealed that this isolate had chromosomal-mediated colistin resistance associated two-component systems, pmrAB and phoPQ, and the regulator mgrB.[12,13] In addition, 25KA has harbored the eptA gene, which plays a significant role in polymyxin resistance. Consequently, this isolate can acquire colistin resistance. On the other hand, WGS analysis also showed that 25KA has shiga toxin subunit A and B, HlyD family type I secretion periplasmic adaptor subunit, and also eaeA gene encoding gamma intimin which are all signs of 25KA is a typical enterohemorrhagic *E. coli* (EHEC) causing hemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS).10 In the light of this knowledge, it is a very promising situation that an isolate, which can have very devastating effects when it infects humans, can be sensitized to an antibiotic used as "last-resort". It should also be emphasized that 25KA was isolated from the bovine carcass. In cases where the conditions necessary for the eradication of the pathogen are not provided, it is very likely that such strains are passed to humans via food.

25KA and 168KA became susceptible to colistin when treated with phage. The synergistic effect was seen in both of the situations where antibiotic and phage were co-incubated, and in phage-resistant mutants. In both circumstances, it was obvious that bacteria became sensitive to colistin as they tried to evade phage attacks. This situation can be attributed to "trade-off" which simply means that the bacteria lose one quality or trait in return for gaining one aspect.[14] In different pathogens various mechanisms of trade-offs were reported such as impaired production of capsules [15,16], deprivation of virulence[17], and increased sensitivity to antibiotics.[18,19] Chan et al (2016) used a lytic phage against Pseudomonas aeruginosa which utilize an outer membrane porin (oprM) of the multidrug efflux system as

a receptor. They revealed the development of resistance to phage resulted in evolution of the efflux pump mechanism; consequently resulting in susceptibility to most preferred antibiotics in the infection caused by multi-drug resistant P. aeruginosa. Another good example for a "trade-off" in terms of antibiotic sensitivity was displayed by Altamirano et al (2021). The WGS analysis of phage-resistant mutants of Acinetobacter baumannii showed the loss-of-function mutation in genes, which play role in the biosynthesis of the bacterial capsule. As a result the pathogen became more vulnerable to the human immune system, unable to form biofilms, and became sensitive to beta-lactam antibiotics as well as to additional phages. In our study, while increased colistin sensitivity was observed in resistant isolates, susceptible isolates remained susceptible. The reason for this can be explained by one of the aforementioned mechanisms. Further studies such as WGS analysis of the phage-resistant mutants should be performed in order to fully understand the underlying cause of this antibiotic sensitivity.

Discordance in genotypic and phenotypic antibiotic resistance is a common occurrence.[10,20,21] Despite the existence of resistance genes bacteria may not show phenotypic resistance to antimicrobials or vice versa. *Mcr-2* and *mcr-3*-carrier 44RA was found susceptible to colistin in MIC test (0,25 µg/ml). This isolate was also unable to survive when co-incubated with phage cocktail and colistin. Interestingly, phage-resistant mutants emerged after six hours of incubation in the antibiotic-free environment. However, susceptibility to colistin remained for this. The same is valid for non-mcr harboring and colistin susceptible isolate 120RA. Thus, while the synergistic effect of colistin and phage in resistant strains increased sensitivity, no antagonist effect was observed in susceptible strains since they did not become resistant.

The ultimate goal of our study was to reveal whether the use of phages could increase antibiotic susceptibility in antimicrobial-resistant bacteria. The findings of this study highlight that in addition to taking advantage of the lytic activity of phages in the biocontrol area, phages also play a major role in re-sensitization to a last-resort antibiotic like colistin. Consequently, the results show the synergy between phage–antibiotic combination treatment and give the promising idea that this approach has the potential to extend the effective lifetime of antibiotics.

References

1. de Kraker ME, Stewardson AJ, Harbarth S. Will 10 million people die a year due to antimicrobial resistance by 2050? PLoS Med. 2016; 13(11): e1002184.
2. Mohamed YF, Abou-Shleib HM, Khalil AM, et al. Membrane permeabilization of colistin toward pandrug resistant Gram-negative isolates. Braz J Microbiol. 2016; 47(2): 381–388.
3. Zavascki AP, Goldani LZ, Li J, Nation RL. Polymyxin B for the treatment of multidrug-resistant pathogens: a critical review. J Antimicrob Chemother. 2007; 60: 1206–1215.
4. Aris P, Robatjazi S, Nikkhahi F, Marashi SMA. Molecular mechanisms and prevalence of colistin resistance of Klebsiella pneumoniae in the Middle East region: a review over the last 5 years. J Glob Antimicrob Resist. 2020; 22: 625-630.
5. Wang C, Feng Y, Liu L, et al. Identification of novel mobile colistin resistance gene mcr-10. Emerg Microbes Infect. 2020; 9: 508–516.
6. Tagliaferri TL, Jansen M, Horz HP. Fighting pathogenic bacteria on two fronts: phages and antibiotics as combined strategy. Front Cell Infect Microbiol. 2019; 9: 22.
7. Endersen L, Coffey A. The use of bacteriophages for food safety, Curr Opin Food Sci. 2020; 36: 1-8.
8. Abedon ST. Phage-antibiotic combination treatments: antagonistic impacts of antibiotics on the pharmacodynamics of phage therapy?. Antibiotics (Basel). 2019; 8(4): 182.
9. Ayaz ND, Gencay YE, Erol I. Prevalence and molecular characterization of sorbitol fermenting and non-fermenting Escherichia coli O157:H7+/H7– isolated from cattle at slaughterhouse and slaughterhouse wastewater. Int J Food Microbiol. 2014; 174: 31-38.
10. Ayaz ND, Cufaoglu G, Yonsul Y, et al. Plasmid-mediated colistin resistance in Escherichia coli O157:H7 cattle and sheep isolates and whole-genome sequence of a colistin-resistant sorbitol fermentative Escherichia coli O157:H7. Microb Drug Resist. 2019; 25(10): 1497-1506.
11. Gencay YE, Ayaz ND, Copuroglu G, et al. Biocontrol of Shiga toxigenic Escherichia coli O157:H7 in turkish raw meatball by bacteriophage. J Food Saf. 2016; 36: 120-131.
12. Gunn JS. The Salmonella PmrAB regulon: lipopolysaccharide modifications, antimicrobial peptide resistance and more. Trends Microbiol, 2008; 16; 284-290.
13. Cannatelli A, D'Andrea MM, Giani T, et al. In vivo emergence of colistin resistance in Klebsiella pneumo-

niae producing KPC-type carbapenemases mediated by insertional inactivation of the PhoQ/PhoP mgrB regulator. Antimicrob Agents Chemother. 2013; 57: 5521-5526.
14. Torres-Barcelo C, Hochberg ME. Evolutionary rationale for phages as complements of antibiotics. Trends Microbiol. 2016; 24: 249-256.
15. Capparelli R, Nocerino N, Iannaccone M, et al. Bacteriophage therapy of Salmonella enterica: a fresh appraisal of bacteriophage therapy. J Infect Dis. 2010; 201; 52-61.
16. Altamirano FG, Forsyth JH, Patwa R, et al. Bacteriophages targeting Acinetobacter baumannii capsule induce antimicrobial resensitization. Nat Microbiol. 2021; 6: 157–161.
17. Capparelli R, Nocerino N, Lanzetta R, et al. Bacteriophage-resistant Staphylococcus aureus mutant confers broad immunity against staphylococcal infection in mice. PLoS One. 2010; 5: e11720.
18. Chan BK, Sistrom M, Wertz JE, et al. Phage selection restores antibiotic sensitivity in MDR Pseudomonas aeruginosa. Sci Rep. 2016; 6: 26717.
19. Engeman E, Freyberger HR, Corey BW, et al. Synergistic killing and re-sensitization of Pseudomonas aeruginosa to antibiotics by phage-antibiotic combination treatment. Pharmaceuticals. 2021; 14(3): 184.
20. Mazurek J, Pusz P, Bok E, Stosik M, et al. The phenotypic and genotypic characteristics of antibiotic resistance in Escherichia coli populations isolated from farm animals with different exposure to antimicrobial agents. Pol J Microbiol. 2013; 62(2): 173-179.
21. Davis MA, Besser TE, Orfe LH, et al. Genotypic-phenotypic discrepancies between antibiotic resistance characteristics of Escherichia coli isolates from calves in management settings with high and low antibiotic use. Appl Environ Microbiol. 2011; 77(10): 3293–3299.