Original Research Article

Evaluation of three isolation methods for Campylobacter bacteriophages from chicken skin: a comparative study

ABSTRACT

Background: Campylobacter strains are of the leading pathogens causing bacterial gastroenteritis, whose infections are generally considered to be one of the most common foodborne illnesses of animal origin. The etiology of this infection often goes back to eating contaminated raw meat or infected poultry. The bacteria are present in abundance in chicken skin. The use of appropriate bacteriophages is one of the most effective methods in eliminating Campylobacter strains. Phage therapy refers to the use of bacteriophages to treat bacterial infections. **Aim**: Accordingly, the present study aimed to compare three methods of bacteriophage isolation in ~~the~~ chicken skin. **Methods**: Thus, 15 samples of chicken skin were collected from five different fresh chicken suppliers in Ghaemshahr, Iran. The samples were transported to the laboratory aseptically in the vicinity of ice, and then cultured in a blood agar medium, and the isolates were identified by various tests including gram staining, catalase, and oxidase tests. **Results**: The results were compared before and after three bacteriophage isolation methods. Out of 15 chicken skin samples tested in all three methods, 6 (40%) strains were identified in the first method, 8 (53.4%) strains in the second method and 12 (20%) strains in the third method after bacteriophage therapy. **Conclusion**: The bacteriophage isolation methods alone or in combination with other intervention strategies are recommended as promising tools for greater food safety. These methods can be useful to increase food safety and reduce the risk of infection in humans through the consumption of potentially infected edible parts of the chicken. According to the results of this study, among the three proposed methods, the method of chicken skin enrichment in Bolton selective media containing target isolates was the most efficient approach, which showed a high limit of detection at low concentrations and the highest rate of phage recovery. This can be a more reliable way to isolate the Campylobacter bacteriophages and eliminate the Campylobacter strains.

Keywords: *Campylobacter, Campylobacter Bacteriophage, Chicken skin, Food Contamination*.

1- INTRODUCTION

Campylobacter strains have been the most common cause of human bacterial diarrhea in many developed countries over the past two decades. In general, infection of chicken carcasses with Campylobacter strains is common and plays an important role in human infection. The EU reports have confirmed the rate of human Campylobacteriosis outbreak at around 50 cases per 100,000 people in more than 17 countries. It is estimated that approximately 9 million people experience human Campylobacteriosis annually in the EU regions [1,2]. For health, economic and nutritional reasons, ~~the~~ chicken skin and meat are the main sources of animal protein needed by communities today, so that more than 50% of this need is met through these products. Despite its low incidence, Campylobacter is a significant infectious agent due to the high volume of poultry meat consumption and the potential risk of this pathogen. Despite sanitary measures in poultry farms, almost all of the bacterial infections studied are found in samples of processed poultry meat in industrial slaughterhouses. The prevalence of Campylobacter strains in raw poultry products is in the range of 0 to 100% and on average of 62% [3]. Various mitigation strategies, such as competitive exclusion, the use of chemical or antibiotic additives, and strict health protocols, have been implemented with relative success in the EU [4]. There has been a worrying elevation in antibiotic resistance in farm animals in recent years [5]. The World Health Organization (WHO) has included fluoroquinolone-resistant Campylobacter strains in its list of global antibiotic-resistant bacteria that pose the greatest risk to humans [6]. An important challenge for global public health is the search for new alternative ways to control Campylobacter infection by reducing the use of antibiotics in food production [7]. A promising candidate for reducing Campylobacter outbreak in the farm to fork process is the use of bacteriophages (phages) as biological control agents. Phages are viruses specifically capable of infecting and killing bacteria widely distributed in the environment and often exist as normal microbiota in the diet, including poultry products [8]. Bacteriophages have properties that seem attractive to those looking for new solutions to control foodborne pathogens and spoilage microorganisms. These phages have a history of safe use and can be host-specific and replicated in the host. Campylobacter, Salmonella, *Listeria monocytogenes* and various spoilage microorganisms have responded to phage control in some food products. However, the employment of

Comment [MH1]: Delete "the" before chicken

Comment [MH2]: Add "the" before chicken

Comment [MH3]: Add "a" before blood

Comment [MH4]: Delete "the" before the chicken

Comment [MH5]: Add "the" after potential

phages as microbial biological control agents can be complicated by factors such as the apparent need for the host threshold level before further replication and with sub-optimal performance, at best, under sub-optimal temperatures for the host. Razei et al. (2017) dealt with the rapid detection of *Campylobacter jejuni* based on PCR technique and assessment of its sensitivity and specificity. Their aim was to design a specific PCR process to identify *C. jejuni* [9]. Numerous studies have been conducted on the use of bacteriophages to strengthen various food products. In this study, bacteriophages have been used successfully to control the growth of pathogens in food. They are supposed to play an important role in food safety in the future. However, many foods and particulate matter processes in the intestines of animals inactivate phages and reduce their virulence capacity. Encapsulation technologies have been successfully used to protect phages against extreme environments and have been shown to maintain their activity and release in targeted environments [10]. The use of Campylobacter-specific bacteriophages seems to be a promising tool in ~~the~~ food safety for the biological control of this pathogen in the poultry meat production chain. However, the isolation of bacteriophages is a complex challenge because they appear to be low on chicken skin or meat. Isolation of Campylobacter bacteriophages is the first challenge in developing a bacteriophage-based product to control Campylobacter. They are isolated wherever their hosts are present and also from environmental samples and poultry products [11]. However, the presence of Campylobacter bacteriophages is very low even in these samples [12]. On the other hand, the isolation rate of Campylobacter bacteriophages varies in published articles, probably due to differences in isolation methods or the type and origin of the sample [7]. Various isolation methods have been proposed to date, but no standard methods for the isolation of Campylobacter bacteriophages have yet been developed. To optimize existing methods and suggest the best method, the present study selected three different methods introduced in several previous articles as appropriate protocols in terms of phage recovery rate, with the aim of comparing the three isolation methods of Campylobacter bacteriophages from chicken skin. In this study, in addition to determining the effectiveness of Campylobacter bacteriophages on the bacteria separately, finally, three methods of isolating Campylobacter bacteriophages from chicken skin were compared and the best method was introduced.

Comment [MH6]: foods

Comment [MH7]: delete "the"

Comment [MH8]: Add "the" before biological

2- MATERIALS AND METHODS

In this study, 15 samples of fresh chicken skin, thighs or wings or neck were randomly collected from five different chicken suppliers in Ghaemshahr (Iran). The samples were transferred to Rai Azma Food Hygiene and Health Laboratory aseptically in the vicinity of ice, immediately followed by performing the necessary tests. First, the chicken skin pieces inside the Falcon tubes were completely vortexed with normal saline. The skin pieces were removed by forceps under sterile conditions, and the remaining fluid was transferred to sterile Falcon tubes. The Falcon tubes were centrifuged at 4000 rpm for 5 minutes. The supernatant was discarded, and the remaining precipitate was added with 30 ml of Preston enrichment broth containing antibiotics. The Falcon tubes were incubated in a special jar under micro aeration conditions at 42 ± 1°C for 24 hours. It should be noted that the micro aeration conditions were created by lighting a candle inside the jar. All steps were examined with standard strains to ensure the accuracy of the isolation process. After 24 hours, the samples were taken out of the incubator and immediately cultured in four regions onto blood agar media containing antibiotics. Re incubation was performed at 42 ± 1°C for 48 h under micro aeration conditions. Finally, the plates were examined macroscopically. According to the morphology of the grown colonies and biochemical tests (including gram staining, oxidase, catalase, nitrate reduction, and nalidixic acid resistance tests), suspicious Campylobacter colonies were isolated and their purification was performed for all three subsequent isolation methods [13]. Campylobacter isolates were used as host bacteria in this study. For isolation, chicken skin samples were diluted at a ratio of 1: 4 (w/v) in SM buffer [50 mM tris-xcl (pH = 7.5), 0.1 M NaCl, 8 mM $MgSO_4$, 0.01% (w/v) gelatin], and were cultured by Rapid method of Campylobacter Detection. The plates were dried at ambient temperature and stored at 37°C for 72 h under micro aeration conditions (5% oxygen, 10% carbon dioxide, and 85% nitrogen). The Campylobacter isolates were stored at -80°C in Brain Heart Infusion (BHI) Broth with 10% Glycerol. To prepare the final phase cultures, the frozen-thawed samples (200 µi) were cultured onto Columbia Blood Agar (oxoid) with Defibrinated Sheep Blood (5%v/r, oxoid) under micro aeration conditions at 37°C. After overnight incubation, the cells were harvested up to 0.6 (10^8 CFU/ml) in BHI Broth until reaching OD_{600}, and kept again at 37°C for 4 hours [7].

Three different isolation methods for Campylobacter bacteriophages were evaluated and applied to all 15 chicken skin samples.

Method 1: The chicken skin samples were placed in sterilized filter bags and enriched in 15 ml of BHI broth by culturing the final phase of host Campylobacter strains to reach a final concentration of 10^6 CFU/ml. After enrichment, the mixtures were kept at 37°C for 48 h under micro aeration conditions.

Method 2: 10 g of chicken skin samples were added to SM buffer (50 Mmol/1 Tris-HCl [pH = 7.5], 0.1 mol/1 ~~Nacl~~, NaCl 0.008 mol/1 $MgSO_4$) and stored for 4 hours at 4°C [8]. The suspension was centrifuged at 8600 gr for 10 min and the resulting aqueous phase was treated with chloroform (4: 1, v/v) and re-centrifuged at 8600 gr for 10 min [14].

Comment [MH9]: Replace Nacl with NaCl

Method 3: The chicken skin samples were placed in sterile-filtered bags containing 10ml of Campylobacter Selective Bolton Broth (oxoid), selective antibiotics (oxoid) and 5% lysed horse blood, and 10 ml of fresh Bolton broth supplemented with 400 Mg/ml of $CaCl_2$ and 400Mg/ml of $MgSO_4$. The mixture was vortexed and stored at 42°C for 18 h under micro aeration conditions and treated with chloroform. The prepared mixture was enriched by the host Campylobacter strains within the log phase until a final concentration of 6 CFU/10ml. The mixture was kept at 42°C for 48 hours under micro aeration conditions [7].

Comment [MH10]: Add "the" before log

Phage identification: One drop of each phage sample (10 µl) was added to each of the Campylobacter strains and the plates were incubated. The lysis area was scratched and suspended in 100 µl of SM band, and re-plate on the third level of Campylobacter. Different dilutions were prepared and individual phage plaques were obtained and this test was performed in triplicate to ensure purity. Fresh phage lysates were stored in sterile tubes at 4°C and finally at -80°C in SM buffers with 20% glycerol [7]. All tests were performed in triplicate.

3- RESULTS AND DISCUSSION

Since the data are quantitative (with a sample size of n <30), the three dependent groups were compared by pairwise comparisons (pre/post) through the statistical method of mean comparison to show the difference and one-factor repeated-measures analysis of variance (ANOVA) using SPSS software. The results are shown as tables and line graphs for each method.

Comment [MH11]: Add "a" before sample

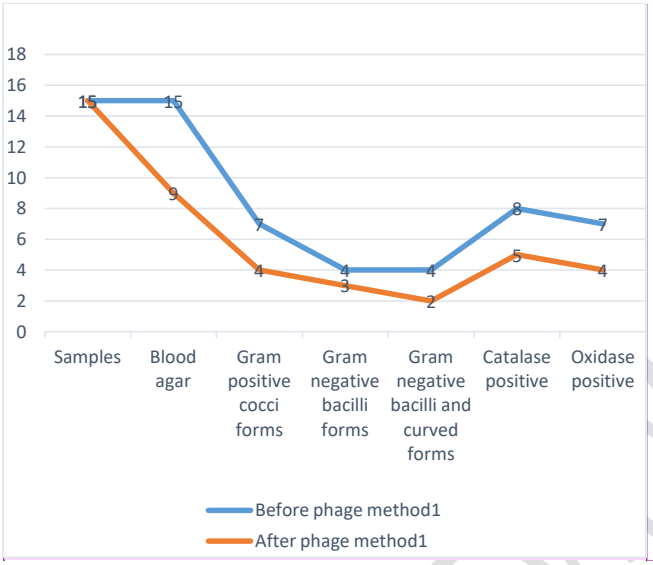
Table 1- Frequency and percentage of samples with Campylobacter strains observed before and after performing the first method in blood agar medium, gram staining, and diagnostic tests

Comment [MH12]: Add "the" before first

Samples	Positive growth in blood agar medium (%)	Gram staining			Diagnostic tests	
		Gram-positive cocciform bacteria (%)	Gram-negative bacilliform bacteria (%)	Gram-negative curved and bacilliform bacteria (%)	Catalase positive result (%)	Oxidase positive result (%)
Before phage therapy in the first method (frequency)	15	7	4	4	8	7
After phage therapy in the first method (percentage)	60.0±4.60	26.60±0.82	20.0±0.56	13.30±1.16	33.30±0.79	26.60±0.56

According to the table above, after phage therapy in the first method, no Campylobacter strains were observed in 40% of the samples.

First method



Comment [MH13]: It is good to write what is in the y-axis

Figure 1- Line graph of Campylobacter strains observed before and after the first method in blood agar medium, gram staining, and diagnostic tests

According to the above diagram, a decreasing trend is observed in the number of Campylobacter strains after phage therapy in the first method.

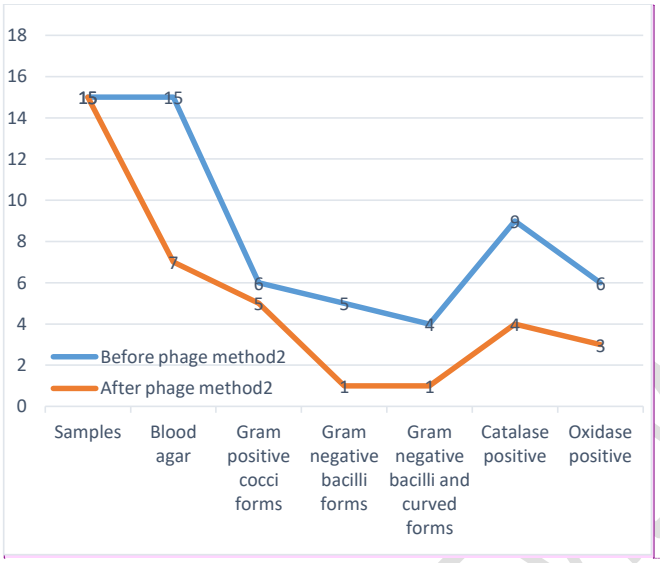
Table 2- Frequency and percentage of samples with Campylobacter strains observed before and after performing the second method in blood agar medium, gram staining, and diagnostic tests

Comment [MH14]: Add "the" before second

Samples	Positive growth in blood agar medium (%)	Gram staining			Diagnostic tests	
		Gram-positive cocciform bacteria (%)	Gram-negative bacilliform bacteria (%)	Gram-negative curved and bacilliform bacteria (%)	Catalase positive result (%)	Oxidase positive result (%)
Before phage therapy in the second method (frequency)	15	6	5	4	9	6
After phage therapy in the second method (percentage)	46.60±0.68	33.30±0.79	6.60±0.15	6.60±0.15	26.60±0.56	20.0±0.52

According to the table above, after phage therapy in the second method, no Campylobacter strains were observed in 53.4% of the samples.

Second method



Comment [MH15]: It is good to write what is in the y-axis

Figure 2- Line graph of Campylobacter strains observed before and after the second method in blood agar medium, gram staining and diagnostic tests

According to the above diagram, a decreasing trend is observed in the number of Campylobacter strains after phage therapy in the second method.

Table 3- Frequency and percentage of samples with Campylobacter strains observed before and after performing third method in blood agar medium, gram staining and diagnostic tests

Samples	Positive growth in blood agar medium (%)	Gram staining			Diagnostic tests	
		Gram-positive cocci form bacteria (%)	Gram-negative bacilliform bacteria (%)	Gram-negative curved and bacilliform bacteria (%)	Catalase positive result (%)	Oxidase positive result (%)
Before phage therapy in the third method (frequency)	15	7	3	5	7	8
After phage therapy in the third method (percentage)	80.0±1.20	13.30±0.22	6.60±0.15	-	6.60±0.15	13.30±0.22

According to the table above, after phage therapy in the third method, no Campylobacter strains were observed in 80% of the samples.

Third method

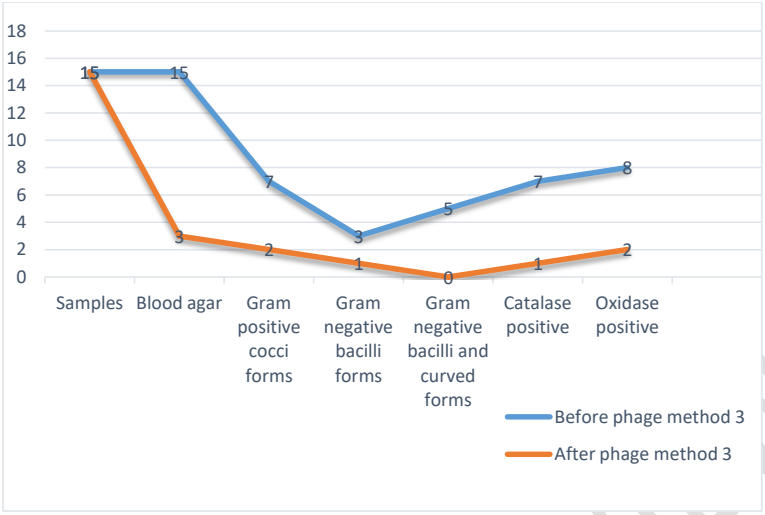


Figure 3- Line graph of Campylobacter strains observed before and after the third method in blood agar medium, gram staining, and diagnostic tests

According to the above diagram, a decreasing trend is observed in the number of Campylobacter strains after phage therapy in the third method.

Failure to treat these infections with antibiotics has led researchers and scientists to use more efficient and alternative methods to eliminate and control these bacteria. One of these successful alternatives or supplements is the use of bacteriophages (phages) to treat infections in many refractory infections [15]. This phenomenon, or phage therapy, means the use of bacteriophages to treat bacterial infections, and especially the combination of two or more phage types called phage cocktails has been used to increase the host spectrum of a particular genus against various bacterial infections [16,17]. Unlike most antibiotics, phages are smart weapons that act specifically and thus exert little harm to beneficial bacteria in the body, such as gut bacteria, while antibiotics eliminate them. Phages, on the other hand, act in a limited way, entering their inactive life cycle after destroying harmful bacteria, and show virtually no activity against non-host bacteria [7]. The use of phages is an attractive strategy for producing safe food because they specifically affect the pathogen. They are harmless to humans, animals, and plants and have no negative effect on normal microbiota or other beneficial properties of food. Campylobacter-specific bacteriophages can be applied in poultry farms to prevent or reduce Campylobacter contamination of birds [18]. According to the analysis of the results obtained from the present study, the first method showed bactericidal effects but was not very satisfactory (40%). This method appears to reduce the growth of other bacteria present in chicken skin samples, resulting in reduced growth of Campylobacter strains. The results of this study are consistent with studies by Nafarrate et al. in 2020 [7] and Hungaro et al. in 2013. This method can exhibit the effect of bacteriophages as an alternative factor to reduce the contamination of poultry carcasses in industrial conditions [19]. The present study demonstrated that the use of the second method can also affect the bactericidal rate (53%). This method was performed by Atterbury et al. in 2005 on chicken fecal samples [8] and then by Janez et al. in 2014 on fresh chicken meat samples [15]. The results of their study also showed a decrease in bacterial density after phage inoculation, so that Atterbury et al. (2005) reported a 56% bactericidal rate [8]. Comparison of the results from the first and second methods indicated that since Campylobacter strains have inactive metabolism at temperatures below 4°C, storage of samples at this temperature increases the efficiency of the method [7]. However, the results obtained in the third method revealed that 80% of the samples had no Campylobacter strains and in a way, it can be said that bactericidal activity was much more effective in this method. The highest isolation rate of Campylobacter bacteriophages was observed in the third method, compared to the lower isolation rates through the first and second methods. This higher rate appears to be related to the proliferation of strains during storage of the samples in Bolton selective broth medium, which has led to the growth of Campylobacter strains on chicken skin. Increased growth of Campylobacter strains enhances the likelihood of phage attachment to host cells. In general, the third method was the most efficient in phage isolation and showed the best phage recovery rate from the sample surface and the lowest presence of Campylobacter strains in the samples. Apart from the fact that the difference in results between repetitions was minimal, this method was introduced as a reliable and repeatable approach. The results of this study are completely in line with the findings reported by Nafarrate in 2020, which considers

Comment [MH16]: Add “was” before not

Comment [MH17]: Add “a” before 56%

the above method as the most effective method of bacteriophage isolation among the existing methods and also introduces this method as a reliable approach compared to others. The findings of this study confirm the fact that poultry products, especially chicken skin, are a rich source of Campylobacter strains, as previously reported by other researchers [3,10].

4- CONCLUSION

Phages are normally present in food products and may be consumed in our diet. This is very important for food safety because reducing the density of Campylobacter strains in food-producing animals or disinfecting carcasses and other raw products during food processing through the use of bacteriophage does not mean adding a foreign element to our diet. On the other hand, phages can be recruited as biological control tools for Campylobacter strains. These bacteriophages can be utilized alone or in combination with other intervention strategies as a promising tool for food safety applications. Diversity in Campylobacter phage treatment methods can be effective in developing new approaches to promote food safety. These methods can be useful to increase food safety and reduce the risk of infection in humans through the consumption of potentially infected edible parts of the chicken. Given that most people use packaged chicken, which contains the skin and other parts of the chicken and can lead to contamination of the chicken's food and various parts of the kitchen, the third method, among the three proposed methods, could be a more reliable approach to eradicate Campylobacter strains.

Comment [MH18]: Add "the" before chicken

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

5- REFERENCES

1. European Food Safety Authority Journal. Analysis of the baseline survey on the prevalence of Campylobacter in broiler batches and of Campylobacter and Salmonella on broiler carcasses in the EU. 2008. Part A: Campylobacter and Salmonella prevalence estimates. EFSA Journal 2010; 8(03):1503). [99 pp.]. doi:10.2903/j.efsa.2010.1503. Available online: www.efsa.europa.eu.
2. European Food Safety Authority Journal. (EFSA). Panel on Biological Hazards (BIOHAZ); Scientific Opinion on Campylobacter in broiler meat production: control options and performance objectives and/or targets at different stages of the food chain. EFSA Journal; 2011. 9(4):2105.
3. Arritt, F. M., J. D. Eifert, M. D. Pierson, and S. S. Sumner. 2002. Efficacy of Anti microbials against *Campylobacter jejuni* on Chicken Breast Skin. J. Appl. Poult. Res. 11: 358-366.
4. Hwang, S., J. Yun, K.P. Kim, S. Heu, S. Lee and S. Ryu. 2009. Isolation and characterization of bacteriophages specific for *Campylobacter jejuni*. Microbiol. Immun. 53:559–566.
5. Van Boeckel, T.P., J. Pires, R. Silvester, C. Zhao, J. Song, N.G. Criscuolo, M. Gilbert, S. Bonhoeffer and R. Laxminarayan. 2019. Global trends in antimicrobial resistance in animals in low- and middle-income countries. Sci. 365, eaaw1944.
6. WHO, 2017. Global Priority List of Antibiotic Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics.
7. Nafarrate, I., E. Mateo, F. Amárita, I. M. de Marañón and A. Lasagabaster. 2020. Efficient isolation of Campylobacter bacteriophages from chicken skin, analysis of several isolation protocols. Food microbial. 90: 103486.
8. Atterbury, R. J., E. Dillon, C. Swift, P. L. Connerton, J. A. Frost, C. E. Dodd and I. F. Connerton. 2005. Correlation of Campylobacter bacteriophage with reduced presence of hosts in broiler chicken ceca. Applied and Environmental Microbiology, 71(8), 4885-4887.

9. Razei, A., R. A. H. I. M. Sorouri, H. O. S. S. I. N. Aghamollaei and S. L. Mousavi. 2017. Rapid Detection of Campylobacter jejuni by Polymerase Chain Reaction and Evaluation of its Sensitivity and Specificity. Avicenna Journal of Clinical Medicine, 24(1):56-62.
10. Hussein, Y. S., K. A. El-Dougdoug and B. A. Othman. 2018. Application of Salmonella phage cocktail to control *Salmonella tiphimurium* in vitro. Arab Universities J. Agri. Sci. 26(2): 679-688.
11. Aprea, G., D. D'Angelantonio, A. Boni, P. Connerton, I. Connerton, S. Scattolini, F. Marotta, F. Pomilio, G. Migliorati, N. D'Alterio and E. Di Giannatale. 2018. Isolation and morphological characterization of new bacteriophages active against Campylobacter jejuni. Am. J. Clin. Microbiol. Antimicrob. 1:1004.
12. Nowaczek, A., R. Urban-Chmiel, M. Dec, A. Puchalski, D. Stępień-Pyśniak, A. Marek, E. Pyzik. 2019. Campylobacter spp. and bacteriophages from broiler chickens: characterization of antibiotic susceptibility profiles and lytic bacteriophages. Microbiol. 8.
13. Elbrissi, A., Y.A. Sabeil, K.A. Khalifa, K. Enan, O.M. Khair and A.M. El Hussein. 2017. Isolation, identification and differentiation of Campylobacter spp. using multiplex PCR assay from goats in Khartoum State, Sudan. Trop. Anim. Health Prod. 49: 575–581.
14. Carvalho, C., M. Susano, E. Fernandes, S. Santos, B. Gannon, A. Nicolau, P. Gibbs, P. Teixeira and J. Azeredo. 2010. Method for bacteriophage isolation against target Campylobacter strains. Lett. Appl. Microbiol. 50: 192–197.
15. Janez, N., A. Kokošin, E. Zaletel, T. Vranac, J. Kovač, and M. Peterka. 2014. Identification and characterisation of new Campylobacter group III phages of animal origin. FEMS microbial. Let. 359(1): 64-71. Kol
16. Inal, J. M. 2003. Phage therapy: a reappraisal of bacteriophages as antibiotics. Archivum immonulogiae et therapiae experimentalis-English edition- 51(4): 237-244.
17. Ryan, E. M., S. P. Gorman, R. F. Donnelly and B. F. Gilmore. 2011. Recent advances in bacteriophage therapy: how delivery routes, formulation, concentration
18. Atterbury, R.J. 2009. Bacteriophage biocontrol in animals and meat products. Microbiol. Biotech. 2: 601–612.
19. Hungaro, H. M., R. C. S. Mendonça, D. M. Gouvêa, M. C. D. Vanetti and C. L. de Oliveira Pinto. 2013. Use of bacteriophages to reduce Salmonella in chicken skin in comparison with chemical agents. Food Res. Inter. 52(1):75-81.