BF-1443
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RESEARCH ARTICLE Indian Journal of Animal Research

Determination of Serogroup and Lytic Activities of Bacteriophages Isolated from Phage Plaques in *Staphylococcus aureus* Cultures Identified from Sheep Milk with Mastitis

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10.18805/IJAR.BF-1443

ABSTRACT

Backgorund: Bacteriophages are closely related to the evolution and virulence of some important bacterial pathogens. Due to their highly significant roles in pathogenesis and virulence, *S. aureus* bacteriophages are frequently studied. Bacteriophages are grouped into two main categories depending on their life cycles. There are highly consistently lytic phages (virulent) and temperate phages. This study aimed to isolate bacteriophages and determine their phage serogroups from phage plaques in *S. aureus* cultures in order to show if they are lytic or lysogenic, the latter plays a major role in horizontal gene transfer.

Methods: A total of 234 *S. aureus* isolates were recovered from milk samples from cases with gangrenous mastitis in sheep. Staphylococcal phages are determined based on the type and serogroup by PCR using specific primers.

Result: Our study allowed us to determine serogroups of the isolated bacteriophages. Two phage stock samples included only one serogroup while the others included more than one phage serotypes and needed further purification Fa, L and D serogroups were not determined in the study. Present work revealed that all the isolated phages were temperate phages, which play a highly significant role in horizontal gene transfer.

Key words: Bacteriophages, Sheep, *Staphyloccocus aureus*.

INTRODUCTION

Mastitis is an inflammation of the mammary gland which causes severe economic loss in milk producing animals. Mastitis can occur due to specific microorganisms, mainly *Staphylococcus aureus*, coagulase-negative Staphylococci, streptococci, *Escherichia coli, Mycoplasma agalactiae* and *Truperella pyogenes, etc*. (Radostits *et al*., 2007).

S. aureus is a type of bacteria that causes severe diseases in both humans and animals. The adaptation and evolution of *S. aureus* result from mainly the presence of mobile genetic elements (MGEs) that carry virulence and resistance genes. This type of MGEs includes bacteriophage, staphylococcal cassette chromosomes (SCCs), plasmids, *S. aureus* pathogenicity islands (SaPIs) and transposons and they play pivotal role in horizontal gene transfer (HGT) in bacteria. They are generally present everywhere where bacteria are present and have been continuing to evolve together with bacteria for 3 to 4 billion years of life on earth. Many bacteria carry bacteriophages as either extrachromosomal genetic elements or in the form of a prophage integrated into their DNA. Bacteria cells that carry a prophage are called lysogen (Fortier and Sekulovic, 2013; Keary *et al*., 2014; Maslanova *et al*., 2013; Moon *et al*., 2015; Xia and Wolz, 2014).

Bacteriophage transduction is probably the most common way of HGT mechanism for *S. aureus*. (Lindsay, 2010; McCharty *et al*., 2012). Bacteriophages were first used in typing of clinical *S. aureus* isolates. Due to their essential roles in pathogenesis and virulence, *S. aureus* phages were examined comprehensively. Most temperate phages encode and disseminate effective staphylococcus virulence factors. It has been found out that all *S. aureus* isolates carry at least one prophage and most of them carry more than four prophages. They encode a high number of staphylococcus toxins responsible for pathogenesis. So far, more than 250 phages have been identified in staphylococci (Goerke *et al*., 2009; Pantucek *et al*., 2004; Peton and Le Loir, 2014).

A large majority of all *S. aureus* phages known so far have double-stranded DNA and long-tail temperate phages in the *Siphoviridae* family in the order *Caudovirales*. The phages enter a lytic cycle in 80-90% of the host infections

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How to cite this article: Gürbilek, S.E., Arserim, N.B., Tel, O.Y., Sertkaya, Z.T. and Keskin, O. (2022). Determination of Serogroup and Lytic Activities of Bacteriophages Isolated from Phage Plaques in *Staphylococcus aureus* Cultures Identified from Sheep Milk with Mastitis. Indian Journal of Animal Research. DOI: 10.18805/IJAR.BF-1443.

Submitted: 23-09-2021 **Accepted:** 06-12-2021 **Online:** 14-01-2022

with temperate phages, while they enters the lysogenic cycle in 10-20% of the bacterial infections. In the initial studies, *S. aureus* phages were divided into 6 serogroups. Most of the moderate phages in the *Siphoviridae* family are in serogroups A, B and F. The bacteriophages in this family are divided into 6 phage types by their lytic activity, morphology and serological characteristics. The bacteriophages in serogroup D are in the *Myoviridae* family and those in serogroup G are in the *Podoviridae* family and non-lysogenic (Deghorain and Van Melderen, 2012; Xia and Wolz, 2014).

In this study, it was aimed to isolate bacteriophages from tiny phage plaques in *S. aureus* cultures from sheep with gangrenous mastitis. Our major aim was to isolate bacteriophages, to determine their serogroups and to reveal if they temperate or lytic phages. According to the results, all the isolated bacteriophages were temparete phages. Since these phages play important role in HGT (Fortier and Sekulovic, 2013; Maslanova *et al*., 2013; Moon *et al*. 2015; Xia and Wolz, 2014). Their isolation from directly clinical material like milk, might demonstrate different *S. aureus* strains with different virulence factors. It is important to continue to isolate *S. aureus* phages in order to increase our knowledge in virulence factors of *S. aureus*.

MATERIALS AND METHODS

Bacterial strains

S. aureus (n=234) isolates were obtained from Department of Microbiology, Faculty of Veterinary Medicine, Harran University, Turkey. All the isolates were recovered from gangrenous mastitis of sheep and organisms were stored in glycerol at -20°C.

S. aureus test phages

Bacteriophage samples isolated from phage plaques were used in the study. For this purpose, phage samples collected with a pipette tip from a single phage plaque were diluted in PBS, propagated in their hosts and purified. They were named MK1-MK6, phage stock solutions.

Positive and negative control DNA

S. aureus DNAs that were lysogenized with prophages specific to serogroups (NCTC 8325, MSSA476, COL, Newman, Mu50) and DNAs from *S. aureus* CCM 4890 strain, which is prophage free strain, were kindly provided as positive and negative controls from Dr. Pantucek, Department of Molecular Biology and Genetics, the Czech Republic, Masaryk University.

Phage DNA isolation

Phage supernatants were concentrated with centrifugal filter tubes (Amicon ultra 15, MWCO 10K) and then genomic DNA isolation was performed using a Phage DNA isolation kit (Norgen Biotek, Canada).

PCR

PCR was performed according to the method reported by Pantucek *et al*. (2004) to test and control DNA samples using 6 pairs of primers to identify the phages listed in Table 1. The amplicons were run in 2% agarose gel at 120 V for 60 minutes and visualized under UV.

RESULTS AND DISCUSSION

According to the PCR results, MK4Ø and MK5 Ø phage solutions were positive for only single serogroup, while MK2, MK3 and MK6 phage solutions were positive for 2 serogroups. On the other hand, MK1 phage sample was positive for 3 serogroups. Therefore except, MK4Ø and MK5 Ø phages, they were not purified and included more than one phage. None of the phage samples were positive for Fa, L and D serogroups (Fig 1, 2, 3) and (Table 2).

MK4 Ø solution and MK1 phages solution showed 88.9% and 27.8% lysis, respectively in 234 *S. aureus* test isolates. The reason of high lytic effect of MK 4 phage solution was that it contained 3 different phage serogroup (Table 3).

Bacteriophages are the most important actors in the evolution and virulence of some important bacterial pathogens (Fortier and Sekulovic, 2013). Due to their highly

Table 1: Primers used in the study (Pantucek *et al*., 2004).

Phage type and serogroup	Primer	Primer sequence	PCR product	Lysogenic *S. aureus* strains control DNAs	Phage samples
3 A Like	SGA1	TATCAGGCGAGAATTAAGGG	744 bp	COL	3A, 3C, 6, L54b Ø
A serogroup	SGA2	CTTTGACATGACATCCGCTTGAC			
11 Like	SGB1	ACTTATCCAGGTGGYGTTATTG	405 bp	Newman	11, 80, 85, 96 Ø
B serogroup	SGB2	TGTATTTAATTTCGCCGTTAGTG			
77 Like	SGFa1	TACGGGAAAATATTCGGAAG	548 bp	CCM 7079	77, 42 D, 84 Ø
F a sub serogroup	SGFa2	ATAATCCGCACCTCATTCCT			
Fb sub serogroup	SGFb1	AGACACATTAAGTCGCACGATAG	147 bp	NCTC 8325[53+]	13 Ø
	SGFb2	TCTTCTCTGGCACGGTCTCTT			
187 Like	SGL1	GCTTAAAACAGTAACGGTGACAGT	648 bp	187 Ø	187 Ø
L serogroup	SGL2	GCTACATCATCAAGAACACCTG			
Twort Like	SGD1	TGGGCTTCATTCTACGGTGA	331 bp	812 Ø	Twort Ø
D serogroup	SGD2	GTAATTTAATGAATCCACGAGAT			

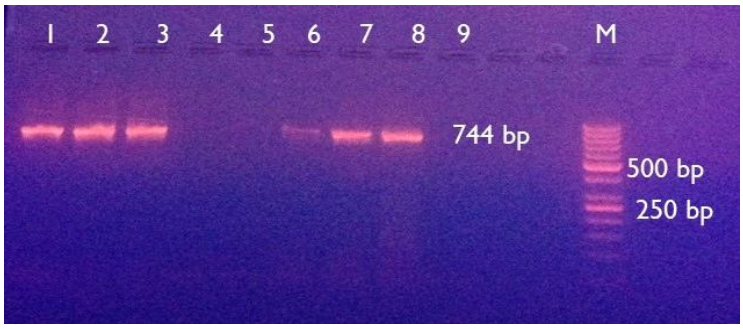

Fig 1: A phage serogroup PCR results 1. MK1; 2. MK2; 3. MK3; 4. MK4; 5. MK5; 6. MK6; 7. COL; 8. MSSA 476; 9. Negative control M. DNA marker.

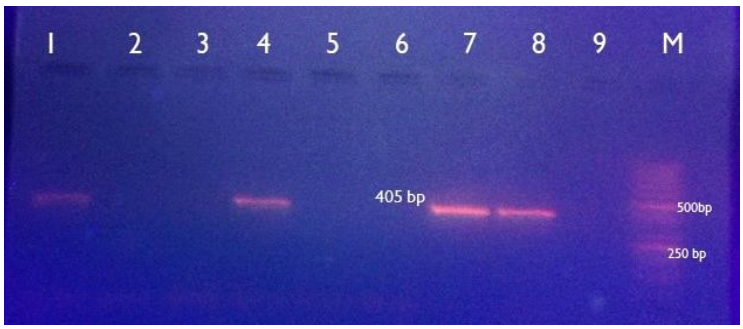

Fig 2: B phage serogroup PCR results 1. MK1; 2. MK2; 3. MK3; 4. MK4; 5. MK5; 6. MK6; 7. Newman; 8. Mu50; 9. Negative control M. DNA marker.

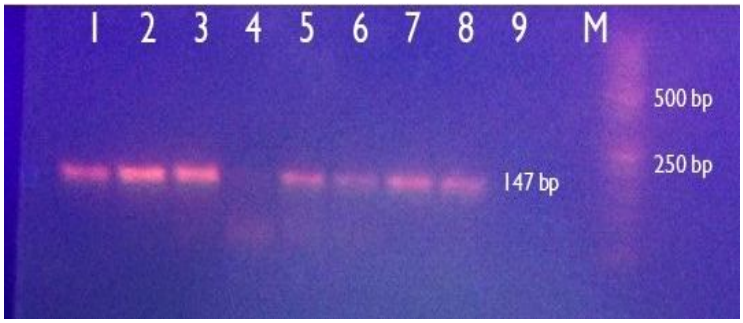

Fig 3: Fb phage serogroup PCR results 1. MK1; 2. MK2; 3. MK3; 4. MK4; 5. MK5; 6. MK6; 7. NCTC 8325; 8. MSSA476; 9. Negative control; M. DNA marker.

significant roles in pathogenesis and virulence, *S. aureus* bacteriophages are frequently studied. Bacteriophages are grouped into two main categories depending on their life cycles. There are highly consistently lytic phages (virulent) and temperate phages (Xia and Wolz, 2014).

Numerous virulence factors such as staphylococcal superantigens, proteases, leucotoxins, bacteriocins, antibiotic resistance genes are transferred by prophages settled among various human and animal *S. aureus* strains. This phenomenon called horizontal gene transfer (HGT). Moreover, it is also reported that phages play a significant role in the adaptation of *S. aureus* to very different and highly challenging conditions (Moon *et al*., 2015; Deghorian and Melderen, 2012; Keary *et al*., 2014).

S. aureus has been reported to have more than 250 phages. Large majority of *S. aureus* phages are reported to be temperate phages in the *Siphoviridae* family and those in serogroups A, B and F are the most common (Pantuchek *et al*. 2004; Xia and Wolz, 2014). In this study, in parallel to this data, only temperate phages from serogroups A, B and F were identified.

In this study, serogroups were identified for the phages isolated using specific primers for 6 serogroups. Accordingly, it was found that the MK1 phage sample carried prophages from serogroups A, B and Fb; MK2, MK3 and MK6 phage samples carried prophages from serogroups A and Fb and MK4 only carried prophages from serogroup B. Therefore, it was concluded that MK1, 2, 3, 5, 6 phages solutions included more than one prophage and needed to be purified.

A part of temperate phages can become lysogenic, especially in laboratory conditions, by DNA damaging, UV, mitomycin C and some antibiotics, mainly quinolones. It is assumed that the test phages isolated in this study transitioned from silent prophage status to lytic status for

Table 2: Serogroups of the isolated phages by PCR.

Phage serogroup	MK1	MK2	MK3	MK4	MK5	MK6
A	+	+	+	-	-	+
B	+	-	-	+	-	-
Fa	-	-	-	-	-	-
Fb	+	+	+	-	+	+
L	-	-	-	-	-	-
D	-	-	-	-	-	-

Table 3: Lysis results of isolated phages.

Phage	Number of lysed isolates	Number of non-lysed isolates	Total
MK1	65	169	234
MK2	-	234	234
MK3	-	234	234
MK4	208	26	234
MK5	-	234	234
MK6	-	234	234

these reasons and they most probably came from the host strain. These findings are also in compliance with the previous workers (Deghorian and Melderen, 2012; Lindsay, 2010).

In this study, 234 *S. aureus* isolates were used to evaluate the lytic effect of phages. Most was caused by phages from B serogroup. Researchers (Xia and Wolz, 2014) reported that serogroup B phages were the most studied phages and this group of phages has very high transduction efficiency. For this reason, this phage is believed to be capable of a very high lytic effect. That MK1 and MK4 phage solution include serogroup B phages strengthens this assumption.

In our next study will be to determine lysogenic level of our *S. aureus* isolates. Temperate phages in clinical *S. aureus* isolates can be identified with multiplex PCR. Goerke *et al.* (2009) determined one or more prophages in most of the tested isolates in their study on the diversity of prophages on dominant *S. aureus* lineages. Maslanova *et al.* (2013) reported that multiple lysogens were observed in most *S. aureus* strains in the natural environment. To detect prophages in our local *S. aureus* strains will increase our knowledge virulence mechanisms of *S. aureus*.

CONCLUSION

In conclusion, it was demonstrated that bacteriophages isolated through purification in the study were temperate phages that played a significant role in horizontal gene transfer. Continuing studies on the isolation of temperate phages that play such an important role in horizontal gene transfer are of utmost importance in demonstrating some phages yet to be discovered. It is planned to identify the lysogenic e *S. aureus* test strains and then study the existence of significant *S. aureus* virulence factors in them for the next step of the study.

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