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Recovery of Diverse Virulence Factors in Phages of Methicillin Resistance *Staphylococcus aureus* (*MRSAp*) Isolated from Mastitic Cows

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ABSTRACT

Staphylococcus aureus is an opportunistic human and animals' pathogen that is found passively colonizing a large percentage of the population primarily on the skin or mammary glands in cows. As the capacity of a given *S. aureus* strain to cause infection varies among strains and is determined by the presence or absence of a large number of virulence-associated genes, some of which are encoded by phages. In order to determine the abundance and characterize the diversity of *S. aureus* strains which caused mastitis in cows in various provinces in Baghdad city, Polymerase Chain Reaction (PCR) was used to detect the presence of *S. aureus* phage-type specific DNA which carry genes coding for diverse virulence factors such as Panton-valentine leukocidin, enterotoxins and exfoliative toxins. *S. aureus* used in this study was isolated from mastitic cow milk grew on blood agar and methicillin-resistant *Staphylococcus aureus* (*MRSA*) were selected as indicator strains to investigate the presences of phage segments. The isolates of wild lytic phages from the transient stocks were propagated with the corresponding host clinical *MRSA* isolates using the plate method. Plates were incubated overnight at 37˚C and plaque morphology and growth characteristics were recorded. Genomic DNA from isolated *S. aureus* phage was extracted and phage segments were investigated. Results showed that the phage content virulence factors in *S. aureus* isolates were, Enterotoxin A, enterotoxin P and Exfoliative toxin A, toxic shock syndrome toxin-1, lipase. However, Panton –valentine leukocidin gene failed to amplify among strains and the diversity of Baghdad city is relatively high. The current study concluded that the role of bacteriophages in changing ecology and virulence of *S. aureus* was evident.

Keywords: Molecular biology, Virulence factors, PCR, Methicillin Resistance *Staphylococcus aureus*, Phages, Mastitic Cows.

1. INTRODUCTION

Methicillin-resistant *Staphylococcus aureus* (*MRSA*) is a worldwide pathogen that is responsible for a variety of diseases ranging from soft tissue and skin infections to life threatening conditions such as pneumonia, bacteremia and sepsis as well as bovine mastitis (1-8). *MRSA* is one of the major human pathogen that may cause community and hospital acquired infections as well as zoonotic etymological livestock polymorph transmitted by food chain (1-9). *S. aureus* phages belong to the order *Caudovirales* (tailed phages), which are composed of an icosahedral capsid filled with double-stranded DNA and a thin filamentous tail. Based on the tail morphology, they can be further classified into three major families: *Podoviridae*, which have a very short tail; *Siphoviridae*, which have a long non-contractile tail; and *Myoviridae*, which have a long, contractile, double-sheathed tail (10-13).

S. aureus phages were characterized according to their lytic activity, morphology and serological properties. The evolution of phage lineages seems to be driven by the lateral gene transfer of interchangeable genetic elements (modules), which consist of functionally related genes. The *Siphoviridae* genomes are usually

organized into six functional modules: lysogeny, DNA replication, packaging, head, tail, and lysis (10-14). In many pathogens, phages provide the bacteria with additional genes that enable them to establish a new lifestyle. In *S. aureus,* several such phage-encoded virulence factors have been described, an observation originally described as phage conversion. Positive lysogenic conversion of different virulence factors was described for the genes coding for Panton-Valentine leukocidin (*lukSF)* (15), exfoliative toxin A (*eta*) (16), the cell-wall anchored protein SasX (17) and the immune evasion cluster (IEC) composed of enterotoxin S (*sea*), staphylokinase (*sak*), the chemotaxis inhibitory protein (*chp*) and the staphylococcal complement inhibitor (*scn*) (18). Additionally, the mutual interaction between phage- and bacterial-encoded factors is only partially understood but is most likely quite common. Importantly, methods to analyze and quantify the three-partner interaction between phage, bacteria and the mammalian host should be established. By such means, the emergence of new virulent or resistant strains might be rendered more predictable. Thus, the objective of this molecular approach designed to recovery of *MRSA* phages ($MRSA_p$) from milk samples of mastitic cows in some regions of Baghdad Province.

2. MATERIALS AND METHODS

2.1 Isolation of *Staphylococcus aureus* wild phages:

Fifty milk samples collected from cow suffered from mastitis were investigated for isolation of *Staphylococcus aureus*. Antibiotic sensitivity test was done to select Methicillin Resistant *Staphylococcus aureus* (*MRSA*). Phage was isolated according to method described by (**19**). Briefly, Approximately 50 ml of cow's milk from each was collected in a sterile sample collection tube (100 ml). Then 15 ml of the milk sample was centrifuged at 3500 rpm for 15 min, the supernatant was filtered through 0.22µm Millipore filter, the filtrate was assayed for plaque using the double agar layer technique. Ten milliliters of each supernatant were transferred into 80 ml of nutrient broth (NB; Hi-media, India) and subjected to vortex for 30 sec. Then 1ml of 8hr of each NB culture of the 8 *MRSA* mastitis clinical isolates were added and incubated at 37˚C. After 18 hrs. 10 ml of the mixture was with drawn into a sterile 15 ml test tube and centrifuged for 5 min at 5000 rpm at room temperature. The supernatant was aspirated into new sterile 15 ml test tubes. One ml of chloroform (Sigma, USA) was added to supernatant with a gentle shaking for 5 min then all tubes were incubated on crushed ice for 5 min. A milky solution appears due to bacterial proteins digestion by chloroform. Further centrifugation at 5000 rpm for 5 min at room temperature was carried-out. The top aqueous supernatant was collected into 15 ml sterile tubes and stored at 4˚C as a possible phage solution.

2.2 Optimization of the Phages Lytic Characteristics:

The isolates of wild lytic phages from the transient stocks were propagated with the corresponding host clinical MRSA isolates using the plate method as follows: Tenfold serial dilution (10^{-1} to 10^{-6}) were made with -buffer for the phage stock solutions by taking 100 µl of the phage solution into 900 µl of buffer. Next transferred 100 µl of each dilution for each phage stock solution into 15 ml volume sterile plastic container with 100 µl of 10^9 colony-forming units (CFU) mL^{-1} of 18hr NB culture of targeted bacteria and incubated at 37˚C. After 10 min incubation, added 2.5 ml of top layer agar cooled to 45˚C and poured over NA plates. Plates were incubated overnight at 37˚C and plaque morphology and growth characteristics were recorded (20).

2.3 Phage DNA preparation, quantification and concentration:

Genomic DNA from the two *S. aureus* phage was extracted according to method instructions described by Gene aid Kit II protocol (*www.geneaid.com*). DNA concentration was determined using nano drop (19).

The individual primer pair PCR was prepare according to (21). A 25 µl reaction mixture was made consisting of 50 ng of template DNA, PCR buffer (1X), 200 µM dNTP mixture, 2 mM MgCl2, 1.5 units Platinum Taq Polymerase, one of the following primers pairs: SGA1,SGA2/SGB1/SGB2/SGFa1,SGFa2, (Table 1), and enough nuclease-free water to reach the 25 µl volume. The primer sets were designed from conserved genomic sequences from phage type's ø3A, ø11, ø77). Each mixture was placed in a thermal cycler (iCycler™, BioRad, Hercules, CA) for initial denaturation (5 min, 94º C) and 30 cycles of amplification consisting of denaturation (1 min, 94º C), annealing (1 min, 58º C), and chain extension (1 min, 70º C). Amplification was followed by one final chain extension cycle (3 min, 70º C) and electrophoresis of 8 µl of PCR product and 2 µl of 5X loading buffer in a 2.0% agarose gel for 1hour at 90V in 0.5X TBE buffer.

Table 1. Primers used in PCR for *S. aureus* bacteriophage detection, their PCR product length and virulence factors

Primer	Primer sequence 5→3	PCR product length	Known associated virulence factors
SGA1	*TATCAGGCGAGAATTAAGGG*	*744bp*	*Panton –valentine leukocidin*
SGA2	*CTTTGACATGACATCCGCTTGAC*		
SGB1	*ACTTATCCAGGTGGYGTTATTG*	*405bp*	*Exfoliative toxin, toxic shock syndrome toxin-1,lipase*
SGB2	*TGTATTTAATTTCGCCGTTAGTG*		
SGFa1	*TACGGGAAAATATTCGGAAG*	*548bp*	*Enterotoxin A , enterotoxin p*
SGFa2	*ATAATCCGCACCTCATTCCT*		

2.4 Multiplex PCR:

A 25 µl reaction volume was mixed following the protocol set by Pantucek *et al.* (21) which consisted of 50 ng of template DNA, PCR buffer (1X), 200 µM dNTP mixture, primers, and, SGA1/SGA2 SGB1/SGB2, (0.6µM each), and SGFa1/SGFa2 (0.8 µM each), 2 mM MgCl2, 1.5 units Platinum Taq Polymerase, and enough nuclease-free water to reach the 25 µl volume. Each reaction mixture was then loaded into a thermal cycler for initial denaturation (5 min, 94º C) and 30 cycles of amplification consisting of denaturation (1 min, 94º C), annealing (1.5 min, 58º C), and chain extension (1.5 min, 70º C). Amplification was once again followed by a final chain extension cycle (3 min, 70º C) and electrophoresis of 8 µl of PCR product and 2 µl of 5X loading buffer in a 2.0% agarose gel for 1 hour at 90 V in 0.5X TBE buffer (22).

3. RESULTS AND DISCUSSION

3.1 Isolation and identification of *MRSA*:

API Staph system were used to identify *S. aureus* from 50 samples milk, we obtained 30 isolates of S. *aureus*. Only 8 isolates termed S1to S8 out of 30 of S. *aureus* were shown to be *MRSA*, they were highly resistant to antibiotic cefoxitin 30mg, methicillin 5mg. All 8 isolates were taken for phage isolation Two phages were isolated from 50 milk samples (*SA1*, *SA2*), were found highly lytic and forming clear plaques on host bacterial lawn of all *MRSA* isolates of clinical mastitis grown on NA plates incubated overnight at 37ºC as is shown in figure 1.

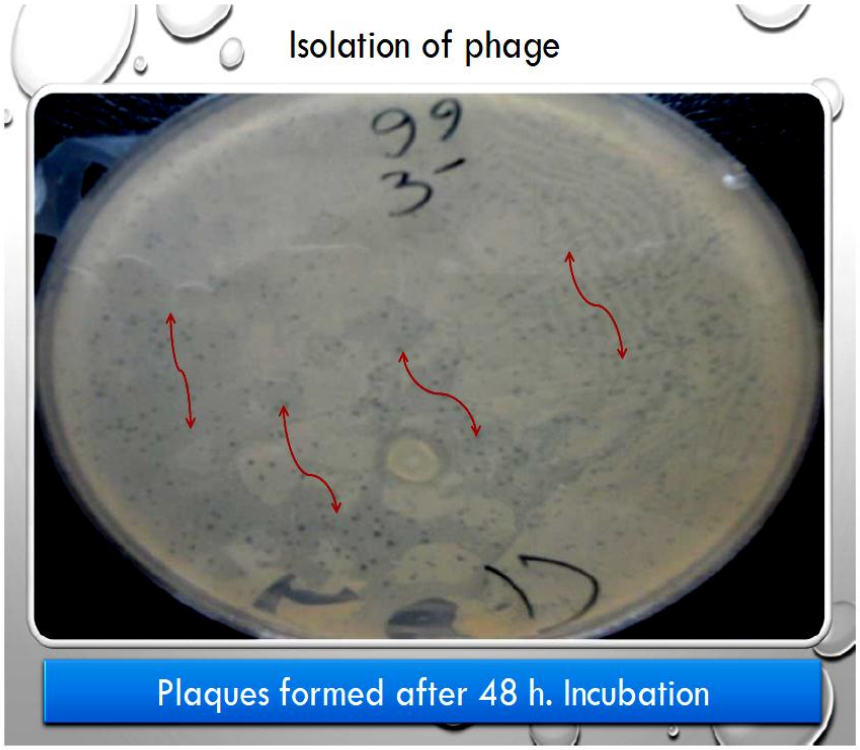

Figure 1. Found highly lytic and forming clear plaques on host bacterial lawn of all *MRSA* isolates of clinical mastitis grown on NA plates incubated overnight at 37 ˚C

3.2 Isolation, characterization and morphology of phage:

The *SEM* has revealed as it observed by (negative staining with uranyl acetate) the phage has a moderately elongated head 96.8 nm long and 79.5 nm wide and contractile tail 200.4nm long and is termed phage *SA1* while, the second is termed *SA2* (elongated head 97.3 nm long 78,2nm wide and contractile tail 202,3nm long. The two phages were found highly lytic and forming clear plaques when incubated with its host of all 8 *MRSA* isolates of clinical mastitis on NA plates over night at 37 ˚C (23).

3.3 Molecular detection of virulence factor by PCR:

The PCR technique was performed to detect the virulence factor gene for *S. aureus* phages using specific primers. The PCR results were interpreted by the presence or absence of specific bands of amplified gene on 2% agarose. Two of the targeted phages types were successfully amplified by the polymerase chain reaction when we used Exfoliative toxin A, toxic shock syndrome toxin-1, lipase 405bp, Enterotoxin A, enterotoxin p (548bp) primers. But Panton –valentine leukocidin 744bp primer failed to amplify as it shown in figure 2.

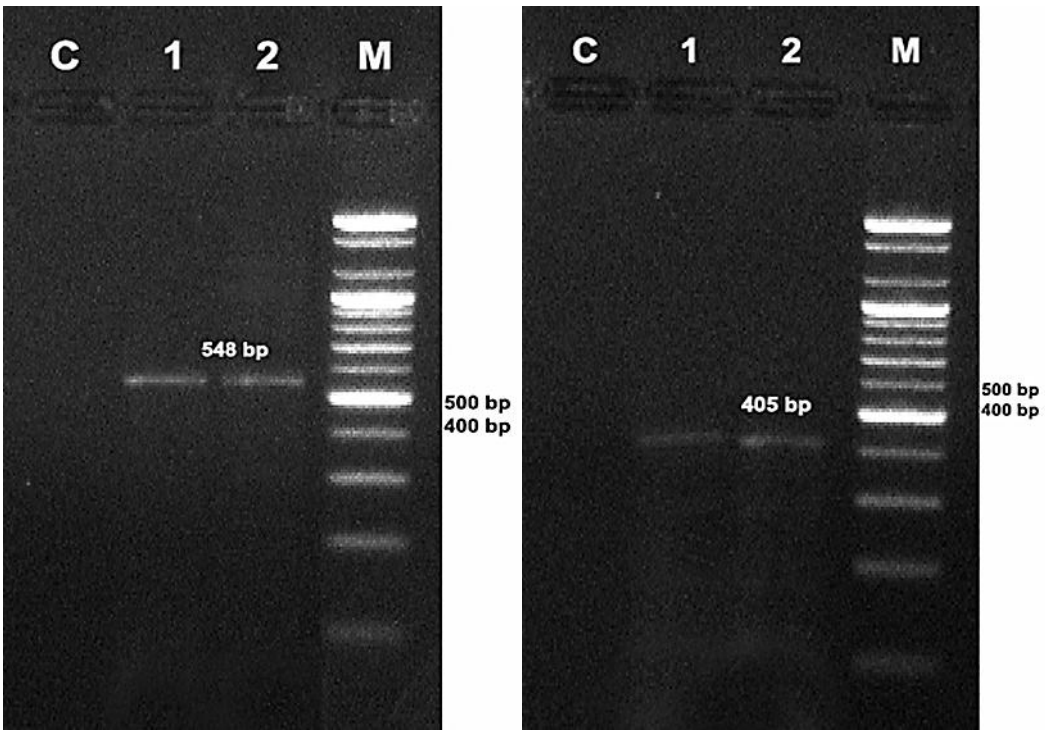

Figure 2. (**A**) analysis of genomic DNA of S. aureus phage showed a presence of gene Enterotoxin A, Enterotoxin p (548 bp), (**B**) analysis of genomic DNA of *S. aureus* phage showed a presence of gene Exfoliative toxin A, toxic shock syndrome toxin-1, and lipase (405 bp). Lane C, Negative control (had all PCR mixture including water instead of DNA template) Lane 1, 2, DNA of S. aureus phage toxin. Lane M, 100 bp DNA ladder

Most S*. aureus* strains carry several phages, some of which encode virulence genes. The mobilization of these phages is crucial for the short–term evolution of the bacterial species and for the emergence of new virulent *S. aureus* lineages (24). The current study investigated the abundance and characterize the diversity of *S. aureus* strains that causes mastitis in cows in Baghdad (Iraq) province. In order to approach the goal of study, Polymerase Chain Reaction (PCR) was used to detect the presence of phage-type specific DNA segments after the isolation of Staphylococcus aureus from mastitic cow milk, methicillin-resistant *Staphylococcus aureus* (*MRSA*) was used as an indicator strain. Results shows that there are two lytic phages namely *SA1* and *SA2*

In our study we diagnosed three virulence gene in mastitic cases and this were agree with previous researches (25 & 26), that there are now growing genome data available for *S. aureus* isolated from non-human mammals. Recently it was shown that livestock–associated *S. aureus* strain isolates originated in human (27) and some of the avian isolates carried a Sa3int-like phage with two putative avian –niche specific genes(25). When we made detection by PCR for these three factors in *S. aureus* phage we found that Enterotoxin A, Enterotoxin p (548 bp), and the presence of gene Exfoliative toxin A, toxic shock syndrome toxin-1, and lipase (405bp) and we didn't found the Panton –valentine leukocidin (744 bp) therefore ,the results could not be analyzed because no band could be detected, and for that the DNA product generally contributed to several reaction condition factors, such as the DNA concentration ,primer quality and concentrate the *Taq* polymerase and *dNTPs* concentration, a number of the cycles in the program and number of the runs (28). Therefore, many experiments were made to optimize empirically these factors. After the optimization of PCR reaction, the obtained results helped in the classification of the 3 different primers in detection the virulence factors. May be because this bacterium did not have this gene or this gene did not transfer between different *S. aureus* strains and mainly because of phage receptor, specificity did not present (29).

Thus, mobile genetic elements present in one strain will move to a strain of the same lineage at the higher frequency than to strains of other lineages, *S. aureus* lineages carry a unique combination of core variable genes suggesting only vertical transmission of these genes (29). The most important virulent gene which we detected in present study was the exfoliative toxins (ET) which are the most virulence factors of *S. aureus* causes staphylococcal scaled –skin syndrome (SSSS),the ETA gene (eta) is carried in the genomes of *Sa1*int phages (30). However these phages can be differentiated into at least six different types due to variation in different modules (31). These eta-phages were associated with out breaks of *MRSA* and *MSSA* strains of different countries in Japan and the Czech Republic (32). The clinical symptoms described for infection with strains harboring ETA-phages varied from blisters to multiple lesions complicated by

conjunctivitis and SSSS avian isolates carried a *Sa3*int-like phage with two putative avian specific genes (25).

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