

Nasal Carriage of *Staphylococcus aureus* in Healthy Children and its Possible Bacteriophage Isolates in Basrah, Iraq

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There is an evidence of high infections with community acquired *Staphylococcus aureus* among the healthy children. Anterior nasal colonization with *S. aureus* plays important role of spreading such infections with this organism. To evaluate the prevalence of *S. aureus* among healthy children, nasal swabs were collected from 119 children within the age from 1 month to 5 years. Parents or/and guardians combined the children were also interviewed for a questionnaire associated with the organism risk factors. *Staphylococcus aureus* was isolated in percentage of 14.28% out of 119 samples processed; among these 41.2% were Methicillin resistant *S. aureus*. The carriage of the organism was significantly noticed between the age groups that were live with big family size and were not attending preschool. In this study, the prevalence of MRSA was relatively high. Three bacteriophages specific for *s. aureus* were isolated as a candidate for biocontrol or treatment of the nasal carriages.

Keywords: Bacteriophages; Basrah; Nasal Carriage; *Staphylococcus aureus*.

Staphylococcus aureus has been recognized as the main causative agent and the most common microorganism in community-acquired and hospital-acquired infections. (Askarian *et al.*, 2009). It asymptotically colonizes mainly the anterior nares of a human; It is also a clinically important factor in the pathogenesis and spread of *S. aureus* infection (Wertheim *et al.*, 2005). The methicillin-resistant *Staphylococcus aureus* (MRSA) bacteria discovered in the 1960s are very serious health problems that have become more serious due to the increased occurrence of this organism that encodes the *mecA* gene for antibiotic resistance (Hartman and Tomasz, 1984; Sousa-Junior *et al.*, 2009). The anterior nares in both adults and children can carry *S. aureus* bacteria through contaminated hands and from surfaces where these organisms can live for

months (Kluytmans *et al.*, 1997). In addition, the spread of this organism occurs particularly in close contact areas such as schools, nurseries or daycare, households that may be via contaminated hands and surfaces (Peacock *et al.*, 2003). The incidence of community-associated MRSA infections in children, including children with no identified risk factors, has increased worldwide in the past decade. This indicates that healthy children may constitute a reservoir of MRSA in the community (Hussain *et al.*, 2001). One possible approach to reduce methicillin-resistant *Staphylococcus aureus* in nasal carriage is phage biocontrol program, which is described as the application of phage to reduce or selectively eliminate pathogen-susceptible organisms from certain environments (Kutter *et al.*, 2010). Phage therapy may be considered as an alternative to antibiotic therapy or biological microorganism control due

to its high phage specificity and effectiveness against multidrug-resistant bacteria (Rakhuba *et al.*, 2010; Golkar *et al.*, 2014). Therefore, this study aimed to investigate the prevalence of a nasal carriage of *Staphylococcus aureus* in healthy children in the nursery in Basrah, Iraq. Isolation of a group of phages effective against a wide range of MRSA isolates, characterization of these phages according to their morphological characteristics, and detection of the presence of genes encoding unwanted toxins in these isolated phages.

MATERIALS AND METHODS

Bacteriologic Media and chemicals

Blood agar base, Mannitol Salt Agar (M.S.A) and Mueller-Hinton agar were purchased from HiMedia Laboratories (Mumbai, India) and prepared according to the manufacturer's instructions. Biochemical tests to determine the identity of the Staphylococci as well as Gram stain, Catalase and oxidase were also purchased from HiMedia Laboratories. Brain heart infusion agar (Salucea, Netherlands), Brain heart infusion broth (HIMEDIA, India), Agarose (Bio Basic, Canada), Peptone (DIFCO, USA), Beef extract (OXOID, England), Yeast extract (Sigma-Aldrich, Switzerland), Sodium chloride (Sigma-Aldrich, Switzerland), Sodium hydroxide (Sigma-Aldrich, Switzerland), potassium dihydrogen phosphate (Sigma-Aldrich, Switzerland), Gelatin (BDH, England), Barium chloride (Hopkins & Williams Limited, England), Gram stain (HIMEDIA, India), Catalase (HIMEDIA, India), Coagulase plasma (HIMEDIA, India), Sulfuric acid (BDH, England), Hydrochloric acid (BDH, England), tris-(hydroxymethyl)-aminomethane (PH7.5) (Riedel-deHaën, Germany), Chloroform (Sigma-Aldrich, Switzerland), Glycerol (Chem-supply, Australia), Absolute ethanol (BDH, England), Nuclease free water (Promega, USA), Ethidium bromide (Fisher, USA), Bromophenol blue (Fisher, USA), TBE buffer (Promega, USA), TE buffer (Promega, USA), (Bioanalyse, USA), Cefoxitin disc (30µg) (Bioanalyse, USA), and DNA ladder marker (100-10000) bp (KAPA, USA).

Study Design

The study was conducted in pediatric outpatient clinics of two hospitals (Ibn-Gazwan and Teaching Hospitals, Basrah, Iraq), between

the period of March till December, 2019. Healthy children between the ages of 1 month and 5 years who visit these hospitals for a routine vaccination program were included in the study to determine nasal carriage of *S. aureus*. Only the healthy children were selected in this study. Ethical approval was obtained from the Board of the Iraqi Health and Higher Education Committee before sample collection begins. A questionnaire was filled-in for a total of 119 patients and the parents were interviewed for this purpose. The questionnaire contained patient information associated with their ages and other questions might assist in sampling process are shown in (Table 1).

Swabs previously moistened with sterile saline solution were obtained from all participating children. The swab was rotated in the visible areas of both anterior nares. The swabs were immediately transferred to the Microbiology Laboratory at University of Basrah, College of Medicine for processing. Swabs were cultured on blood agar and mannitol salt agar (MSA) and incubated at 37 C for 24-48 hours. Colonies growing on blood agar and MSA were identified as *S. aureus* by their typical colony morphology, gram's staining, catalase production and tube coagulase test. The isolates were screened for methicillin resistance by using cefoxitin disk screening test as mentioned by Baily and Scott (2013). The zone of inhibition diameter was measured and the results were interpreted according to CLSI criteria (CLSI., 2014).

Isolation of bacteriophage

Bacteriophages were isolated from raw sewage samples obtained from the treatment plant of the hospitals according to methods described by (Sambrook and Russell., 2001; Jamalludeen *et al.*, 2007). 200ml of fresh sewage was mixed with 20 ml of bacteriophage broth (Peptone (100g/L), Beef extract (30g/L), Yeast extract (50g/L), Sodium chloride (25g/L), Potassium dihydrogen phosphate (80g/L)), 20 ml of mixture of *S. aureus* isolates in broth culture [optical density at 600 nm ($OD_{600} = 1.4$)] and 20 ml of BHI broth were aseptically added to the 1 liter size flask and incubated at 37 °C for 24 h with gently shaking. After incubation, the mixture was centrifuged at 4500xg (MSE, England) for 15 minutes and the supernatant was transferred to a clean flask and then filtered through a sterile (0.45µm) membrane

filter(chm, Spain). The phage titer was determined by serial dilution in which 100 micro liter volumes of the filtrate was mixed with 100 microliter of broth culture containing *S. aureus* in a test tube and incubated at 37°C for 20 min then 3 ml of top agarose (7.0g/L) was added, the tube contents was then mixed and poured on to the surface of a BHI agarose plate and allowed to harden for few minutes and then incubated at 37°C for 16 h. Next day, the plates were examined for the presence of plaques. A control tube containing bacteria and 3 ml of top agarose without filtrate was also cultured on BHI agarose plate. A sterile Pasteur pipette with a rubber bulb was used to gently suction a well-isolated plaque. The pipette contents were transferred into a tube containing 1 ml of SM buffer (5.8g/L NaCl, 2g/L MgSO₄·7H₂O, 50ml/L of 1M Tris pH 7.5, 5ml/L of 2% gelatine) and 1 drop of chloroform was added to each tube. The tube was held at room temperature for 1–2 h to allow the bacteriophage particles to diffuse out of the agar. The phage titre was determined by the soft agarose overlay method and finally the phages were stored at 4°C until stocks were prepared.

Electron microscopy

Phage particles were negatively stained with 2% uranyl acetate (Sigma-Aldrich, Switzerland) on carbon-coated copper grid with standard procedure. TEM images was captured in a LEO 912AB energy filtered transmission electron microscope operated at 100KV (Guelph Regional STEM Facility, University of Guelph, Guelph, Ontario). All three phages were classified according to the International Committee on Taxonomy of Viruses ICTV (ICTV., 2005).

Bacteriophages DNA extraction

The bacteriophage DNA was extracted using the QIAprep Spin M13 kit (QIAGEN, Germany) and according to the manufacture instruction. The presences of DNA were ensured by using the Nano drop (Optizen, Korea) and were visualized by agarose gel electrophoresis (Sambrook and Russell., 2001).

Detection of possible toxins by polymerase chain reaction (PCR)

Undesirable genes including staphylococcal enterotoxins and exfoliating toxins as well as toxic shock syndrome toxin of the isolated phages were searched by using QIAGEN multiplex PCR kit (QIAGEN, Germany) using

a thermo cycler from Eppendorf (Germany) and according to the manufacture instructions. The primers were designed for this study by Eurogentec (Belgium). 100 µm stock of each primer was prepared according to the technical data sheet of each primer and kept in TE buffer at (-70) °C. *Staphylococcus aureus* specific genes, primers and their exact sequence as well as the size of the amplified product (bp) are listed in (table 2) (Mehrotra *et al.*, 2000).

Statistical analysis

The data was entered to the SPSS V.21 software for statistical analysis. Chi-square tests were used to test for statistical significance (0.5) between the variables.

RESULTS

One hundred nineteen children were involved; 59 were boys (49.6%) and 60 (50.4%) girls. Twenty nine percent of them were between 1 to 6 months of age, 28% were between 7 to 12 months, 32% were 13 to 24 months, and 12% were between 25 to 60 months (Table 1). A total percent of (53%) were having no breastfeeding and almost (47%) were feed with breastfeeding status. Around (94%) of the participated children were not attending school, whereas, only (6%) were attending preschool programs. Table 1 shows that (70%) of the participated children were belong to a family size composed from 5-10 family members and almost (46%) were having mother with education status for up-to secondary but most of them were not employed (89%). It was also reported that (82%) of the children were

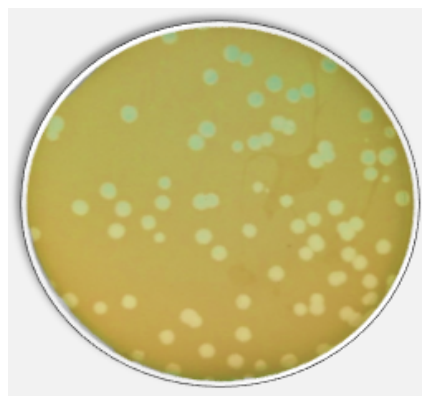


Fig. 1. Bacteriophage ϕ NC plaques on BHI agarose

using antibiotics within the last 2 weeks and almost (86%) were visiting hospital for a purpose other than immunization programs. The prevalence of *Staphylococcus aureus* was 17 (14.28%) among the total of 119 samples processed for isolation of this organism (Table 1), of these isolates 7 (41.2%) were identified as methicillin resistant *Staphylococcus aureus* (MRSA). Those isolates were significantly present at the children with no breastfeeding and were not attending school in a significant P-value of 0.009 and 0.26 respectively.

Bacteriophage isolation

Bacteriophage was successfully isolated from the sewage that was collected from the

hospitals. Three bacteriophages (xNC#1, xNC#2, and xNC#3) were selected and a stock of each of the bacteriophage sample was prepared and purified. All three phages produced clear large to medium sized plaques (4-6 mm in diameter) when propagated in *S. aureus* isolate (Figure 1). The appearance of the three phages under the transmission electron microscopy is shown in (figure 2). All three phages have icosahedral head and long thin non-contractile flexible tail with tail fibers. Based on their morphology all three phages belong to the family *Siphoviridae* (order *Caudovirales*).

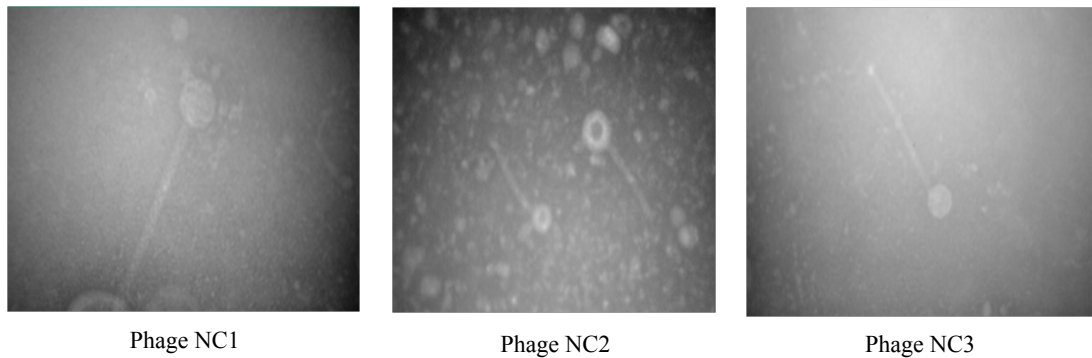


Fig. 2. Electron microscopy images of the three phages (x NC 1, 2 & 3). All phages have long non contractile flexible tail. Bar= 50nm

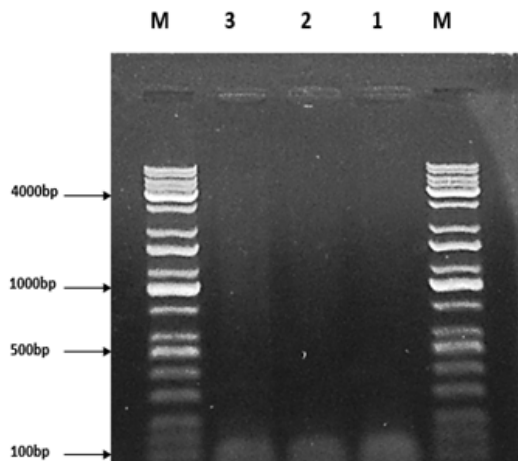


Fig. 3. Multiplex PCR for detection of Staphylococcal toxins: Sea (102 bp), Seb (164 bp), Sec (451 bp), Sed (278 bp), See (209 bp), Eta (93 bp), Etb (226 bp) and tst (326 bp). Using 10 Kb KAPA Universal Ladder as marker (M)

Multiplex PCR for detection of possible Staphylococcal toxins

The PCR results showed that all three phages doesn't encode for any of the staphylococcal toxins which includes staphylococcal enterotoxins (sea, seb, sec and see), exfoliating toxins (eta and etb) and the toxic shock syndrome toxin (tst) (figure 3).

DISCUSSION

This study showed that (14.28%) of healthy children attending two hospitals clinic were carried *Staphylococcus aureus*; out of which 41.2% were methicillin resistant *S. aureus* (MRSA). A research study was shown a prevalence of 16% for *S. aureus* and 19% of which were MRSA among school going children in India (Ramana *et al.*, 2009). As well, 36.4% from children study in Nashville, Tennessee were colonized with *S.*

aureus, and 9.2% were colonized with MRSA (Creech *et al.*, 2005). From Taiwan, Lo *et al.* (2007) were reported 9(13.2%) of 68 children had MRSA, and 17(25%) had *S. aureus* carriage. Hussain *et al.*, (2001) were also found that 24.4% of children tested were colonized with *S. aureus*, and 2.5% of the participated children had MRSA. This study finding have a higher rate of MRSA at the participated children, this may because of the age group studied, however, Creech *et al.*, (2005) was studied a group of age ranged from 5-14

years. Lowest ratio was come from a study done in Turkey (Ciftci *et al.*, 2007), the researchers found that 28.4% of *S. aureus* were colonized children and only 0.3% of which were MRSA. They also reported that the education levels of the mother and father were found to be associated with the rate of nasal carriage.

Factors have been shown association with carriage of *Staphylococcus aureus*. Previously, Peacock *et al.* (2003) and Kluytmans *et al.* (1997) had shown age dependent with the prevalence of

Table 1. *Staphylococcus aureus* associated with nasal carriage from children between 1 month to 5 years old in Basrah, Iraq and factors analysis

Factor	Totals (%)	<i>Staphylococcus aureus</i>		P value
		Positive (%)	Negative (%)	
Sex:				
Boy	59 (49.6%)	9 (15.3%)	50 (84.7%)	0.765
Girl	60 (50.4%)	8 (13.3%)	52 (86.7%)	
Age group:				
1-6 months	34 (28.6%)	6 (17.6%)	28 (82.4%)	0.009
7-12 months	33 (27.7%)	4 (12.1%)	29 (87.9%)	
13-24 months	38 (31.9%)	2 (5.3%)	36 (94.7%)	
25-60 months	14 (11.7%)	5 (35.7%)	9 (64.3%)	
Breastfeeding status:				
Yes	56 (47.1%)	3 (5.4%)	53 (94.6%)	0.009
No	63 (52.9%)	14 (22.2%)	49 (77.8%)	
Child attends:				
No school	112 (94.1%)	14 (12.5%)	98 (87.5%)	0.026
preschool	7 (5.9%)	3 (42.9%)	4 (57.1%)	
Family size:				
≤4	35 (29.4%)	6 (17.1%)	29 (82.9%)	0.337
5-10	83 (69.7%)	10 (12.0%)	73 (88.0%)	
Å10	1 (0.8%)	1 (100%)	0 (0%)	
Education of mother:				
Illiterate	20 (16.8%)	3 (15.0%)	17 (85.0%)	0.499
Up-to primary	32 (26.9%)	7 (21.9%)	25 (78.1%)	
Up-to secondary	55 (46.2%)	6 (10.9%)	49 (89.1%)	
Graduate or post graduate	12 (10.1%)	1 (8.3%)	11 (91.7%)	
Occupation:				
Yes	13 (10.9%)	1 (7.7%)	12 (92.3%)	0.426
No	106 (89.1%)	16 (15.0%)	90 (85.0%)	
Antibiotic usage(last2 wks.):				
Yes	98 (82.4%)	11 (11.2%)	87 (88.8%)	0.039
No	21 (17.6%)	6 (28.6%)	15 (71.4%)	
Hospitalization (last 2 wks.):				
Yes	2 (1.7%)	0 (0.0%)	2 (100%)	0.56
No	117 (98.3%)	17 (14.5%)	100 (85.5%)	
Hospital visit:				
Yes	102 (85.7%)	15 (14.7%)	87 (85.3%)	0.748
No	17 (14.3%)	2 (11.8%)	15 (88.3%)	

Table 2. *Staphylococcus aureus* specific genes, primers and their exact sequence as well as the predicted size of the amplified product (bp)

Gene	Primer	Oligonucleotide sequence (5'-3')	Size of amplified product (bp)
<i>Sea</i>	GSEAR-1	GGTTATCAATGTGCGGGTGG	102
	GSEAR-2	CGGCACTTTTTTCTCTTCGG	
<i>Seb</i>	GSEBR-1	GTATGGTGGTGTAAGTACGAGC	164
	GSEBR-2	CCAAATAGTGACGAGTTAGG	
<i>Sec</i>	GSECR-1	AGATGAAGTAGTTGATGTGTATGG	451
	GSECR-2	CACACTTTTAGAATCAACCG	
<i>Sed</i>	GSEDR-1	CCAATAATAGGAGAAAATAAAAG	278
	GSEDR-2	ATTGGTATTTTTTTTCGTTT	
<i>See</i>	GSEER-1	AGGTTTTTTCACAGGTCATCC	209
	GSEER-2	CTTTTTTTCTTCGGTCAATC	
<i>Eta</i>	GETAR-1	GCAGGTGTTGATTTAGCATT	93
	GETAR-2	AGATGTCCCTATTTTGCTG	
<i>Etb</i>	GETBR-1	ACAAGCAAAAAGAATACAGCG	226
	GETBR-2	GTTTTTGGCTGCTTCTCTTG	
<i>Tst</i>	GTSSTR-1	ACCCCTGTTCCCTTATCATC	326
	GTSSTR-2	TTTTCAGTATTTGTAACGCC	

colonization with *S. aureus*. Although, Pathak *et al.* (2010) found no statistically significant differences in the prevalence of *S. aureus* with age group and only a lower prevalence was observed in the first six months of life. This current study provided a similar result. A consistent result which come in agreement with the stated criteria for clarity. There was a lower prevalence of carriage at the first months of life. Whereas, the peak of colonization has been seen at the age between 2-3 years of age. This may be due to competing colonization with other bacteria in the anterior nares by which colonization by one bacterial strain prevents colonization by another strain (Bogaert *et al.*, 2004; Sivaraman *et al.*, 2009). Our study showed that children who do not go to school carry *Staphylococcus aureus* bacteria significantly more than children who go to school, especially with a family of 5-10 members. This might be due to poor hygienic measures and the overcrowded. Close contact may also play an important role in carriage of such organisms (Miller *et al.*, 2009; Regev-Yochay *et al.*, 2009). In this study, it was found that antibiotic use during the past two weeks showed a significant difference. Antibiotic use is one of the most important determinants of antibiotic resistance (Costelloe *et al.*, 2010). Most of the MRSA isolated from children who received antibiotics as well as other *S. aureus* isolates. Although, Pathak *et al.*, (2010)

have found the exposure to antibiotic was not an important factor associated with *S. aureus* carriage. However, they found that two of the children who carry the MDR MRSA were received antibiotics in the last two weeks before taking the samples. On the other hand, it has been observed that simple hygiene measures may reduce the spread of resistant organisms in the community (Lennell *et al.*, 2008).

An attempt was made to isolate a group of phages that have lytic activity against methicillin-resistant *Staphylococcus aureus* that were isolated from this study. However, phages are known to be the most abundant virus in the environment (Sandaa., 2012). They are widely spread and can appear in many different environments depending on the presence of their bacterial host such as marine and soil environments (Międzybrodzki *et al.*, 2007; Mathur., 2011). Waste-water treatment plant was considered as the main source of phages in the current study. The isolation of *Siphoviridae* phages was successful from the collected samples with high phage titer $e^{10}10^{10}$ since these phages are known for their ability to withstand adverse conditions due to their morphology (Lasobras *et al.*, 1997; Muniesa *et al.*, 1999).

Based on the morphological features of the phages observed by the electron microscopy all three phages belong to the *Siphoviridae* family

with icosahedral head and long non-contractile flexible tail. The name *Siphoviridae* comes from the Greek word *Siphon* which means tube referring to the long tail (ICTV., 2005). These isolated phages are virulent against a broad range of *Staphylococcus aureus* isolates as well as *Staphylococcus epidermidis* isolates (Deghorain and Van Melderen., 2012; Xia and Wolz., 2014).

In order to ensure that these phages are unable to encode virulence factors when suggesting their use in therapies or biological control; a PCR test were done to discover their empty from encoding genes. Some staphylococci bacteria are known to be able to encode some virulence factors such as staphylococcal toxin that includes staphylokinase (sak), enterotoxin A (sea), enterotoxin E (see), enterotoxin P (sep), exfoliative toxin A (eta), Panton-Valentine leukocidin (PVL), toxic shock syndrome toxin (tst), the innate immune modulators SCIN and CHIPS (Brüssow *et al.*, 2004; Boyed., 2012). These toxins are responsible for a wide range of life-threatening illnesses such as scalded-skin syndrome, food poisoning and toxic shock syndrome (Boyed., 2012). The inability of these phages to encode any of the staphylococcal toxins makes them a good candidate for application as a therapeutic agent against methicillin-resistant *Staphylococcus aureus* as an alternative to antibiotics.

CONCLUSION

This study demonstrated that *Staphylococcus aureus* carriage was present among healthy children and methicillin-resistant *S. aureus* (MRSA) was present in a high proportion among *Staphylococcus* isolates. It shows that the children lived in a large family and did not go to any school that was carrying *S. aureus* more than the other groups. More surveillance studies are needed to aid the spread and to accurately assess the epidemiology of *S. aureus* of nasal transport in different geographic regions. In addition, the three phages isolated with no toxin-coding genes indicate that *Siphoviridae* may be a potential alternative to antibiotics and may be used in the biological control of these organisms, but certainly more studies are needed to evaluate their activity in clinical trials.

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Conflicts of interest

There are no conflicts of interest.

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