P- ISSN 1991-8941 E-ISSN 2706-6703 *Journal of University of Anbar for Pure Science (JUAPS)* Open Access
2017,11 (3) :08-16

Molecular Detection of *fnbA*, *fnbB* and *nuc* Genes and Phenotypic Detection of some Virulence Factors in Local *Staphylococcus aureus* Isolates.

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ARTICLE INFO

Received: 10 / 9 /2017
Accepted: 5 / 11 /2017
Available online: 22/6/2018
DOI: 10.37652/juaps.2017.145252

Keywords:
fnbA, *fnbB* and *nuc* Genes , Phenotypic , Virulence Factors *Staphylococcus aureus*
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ABSTRACT

In this study, *Staphylococcus aureus* (*S. aureus*) isolates (32) were collected from different hospitals in Baghdad and were diagnosed by conventional methods. Antimicrobial susceptibility test was performed by disc diffusion method for ten antibiotics. Hemolytic production activity and Deoxy ribonuclease (DNase) production ability were detected. The biofilm production ability was detected by microtiter plate method. The diagnosis was confirmed using PCR to detect the thermostable nuclease gene (*nuc*). Then, PCR technique was used to detect the fibronectin binding protein A and fibronectin binding protein B genes (*fnbA* and *fnbB* respectively).

The results showed a high prevalence (78%) of Methicillin-resistant *Staphylococcus aureus* (MRSA) isolates, Imipenem and vancomycin were the most affective antibacterial agents tested. All isolates produced DNase enzyme . On blood agar plate, 65.6% of isolates produced β-hemolysis zones while (34.4%) of isolates did not produce any hemolytic zone. The results of biofilm production assay showed that 41% of isolates gave a weak positive results. All isolates (100%) that had been previously diagnosed as *S. aureus* by routinely used methods harbored the *nuc* gene. *fnbA* and *fnbB* were found in (100%) and (25%) of isolates respectively.

Conclusion: Molecular detection of *nuc* gene by PCR has high specificity for identifying *S. aureus* with a low cost which requires less time as compared with biochemical methods. *fnbA* and *fnbB* genes are important virulence factors of *S. aureus*. *fnbA* gene is the highest prevalence of toxinogenic and can be used as a genetic marker for diagnosis of local *S. aureus* isolates while *fnbB* gene does not play an important role in infection of *S. aureus*

Introduction

S. aureus is the most repeatedly segregated bacterium among both community acquired and nosocomial infections. It causes the infection of skin, soft tissue, respiratory tract, bone, joint, and endovascular disorders (1,2). This microbe has the unique capability to permeate deeper of host, generating lesions in all organ systems (3).

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S. aureus strains requires an over plus of virulence factors for an infection promotion, it secretes more than fifty polypeptides that enable bacterial escape from innate immune responses, tissue and cellular damage and impair connective tissues (4).

Fibronectin binding protein A (FnBPA) and Fibronectin binding protein B (FnBPB) are important virulence factors that belong to surface adhesive protein (microbial surface component recognizing adhesive matrix molecules "MSCRAMMs"); Both proteins are responsible for initiation of infection by *S. aureus* (5). FnBPA and FnBPB are encoded by *fnbA* and *fnbB* genes, they are multi-function adhesive

proteins that mediates binding with fibronectin (glycoprotein that found in fluids, on the surfaces and extra cellular matrix (ECM) of human and animals cells), fibrinogen and elastin (6,7). The multi-function binding ability of these proteins is very necessary not only for establishment of colonization but also for spreading of pathogen in host tissues, where the binding with fibrinogen helps in valve colonization and binding with fibronectin helps in spreading of infection (8). In addition to attack endothelial and epithelial cells (9), they have been observed to mediate to colonization in surgical infections (10). Also they can arrest the plasma plasminogen (11). They recognize the integrin (α5β1) of host cells and extend a bridge from bacterial cell to it (12). that facilitate *S. aureus* up take by endocytosis (13). Moreover, these proteins helps in an accumulation of bacterial cell forming biofilm, that occur because the cells will interact with other by FnBPs in addition to the secreted polysaccharide (14). On the other hand, the variations that occur in these proteins can play as a mechanism of immune evasion (15).

The functional region of these proteins is typically divided into A and B regions. The N-terminal A region is responsible for ligand binding and specificity; the C-terminal B is responsible for anchorage with bacterial cell wall. Variations on this organization occur, including the presence of the R structural region instead of or in addition to a B region (16,17). FnBPs has many functional region where the N1, N2 and N3 folded subdomains "which forming of an A region" are specialized in binding to elastin and fibrinogen; followed by unfolded (tandemly) repeats that specialized in binding to fibronectin. FnBPB harbors 10 rather than 11 tandemly repeats in FnBPA , but it contains the same number of high affinity fibronectin-binding sites as fibronectin-binding protein A (18). Binding of FnBPs to fibrinogen and elastin occurs using dock lock latch mechanism (19), while the binding to fibronectin occurs using tandem beta-zipper mechanism (20).

A thermostable nuclease is an important virulence factor. It is used as unique diagnostic marker for *S. aureus* (21). It is encoded by *nuc* gene with a total gene size 682 bp (Gene Bank-NCBI). This enzyme activity can resist high temperature (130 ˚C for 16.6 minutes), its production is inhibited by anaerobic incubation and enhanced by aeration (22). It degrades single and double stranded DNA and RNA. In one hand, this process resulting in destruction of tissue and dissemination of *S. aureus*. on the other hand, the destroyed DNA and RNA are used by bacteria as protection coating against defense mechanisms of host and programmed cell death (23).

Staphylococcal biofilm formation are functionally resistant to the immune system components and antibiotics (24,25). Development of antimicrobial resistance reduces responsive to treatment of this bacterium, these often life-threatening infections (26).

For all above, this study was designed to detect *nuc*, *fnbA* and *fnbB* genes that encod thermostable nuclease enzyme "specific to *S. aureus*", Fibronectin binding protein A and fibronectin binding protein B by using PCR technique and detection of some virulence factors of local *S. aureus* isolates.

Materials And Methods

Bacterial isolates collection and biochemical diagnostic

A total of 32 *S. aureus* clinical isolates were isolated from different hospitals in Iraq-Baghdad including (Ibn Al Bitar, Al Wasity, Al Yarmouk, Al-Nu'man, Baghdad and Educational laboratories in medical city). The specimens were collected from different infection sources including: wounds inflammations, burns, osteomyelitis, endocarditis, pneumonia, abscesses, and nose from July 2016 to November 2016. The bacterial isolates were identified based on colony morphology, Gram stain and biochemical tests, such as catalase, oxidase, mannitol fermentation on Mannitol Salt Agar (MSA) medium, slide and tube coagulase tests (27) and API Staph system (biomerieux-France).

Antibiotics susceptibility test

The disk diffusion assay was used according to Kirby-Bauer's method and Clinical and Laboratory Standard Institute (CLSI) (28,29). Ten antibiotic agents were tested against *S. aureus*. they were amikacin, cefotaxime, ciprofloxacin, clindamycin, erythromycin, imipenem, oxacillin, penicillin, tetracycline and vancomycin. The bacterial inoculum was diluted and adjusted to 0.5 McFarland standard (1.5×10^{8} CFU.ml^{-1}), then it was spread on Muller Hinton agar by swab that was slanted at 45 º with the plates surface. After incubation at 35 ºC for 24 hours, inhibition zone diameter in millimeters was recorded.

Finally, these data were compared with the standard inhibition zone of CLSI (29).

Detection of hemolytic activity

It was done by culturing of overnight incubation cultures on 5% blood agar plates and incubation at 37 ºC for 24 hours. After that, the hemolytic activity was recorded by observing of the clear zone around the grown colonies.

Detection of DNase production activity

The DNase agar was used for this purpose. The pure colonies were inoculated with heavy spots and lines. After incubation at 37 ºC, the overnight heavily growth was flooded with HCl (0.1N). Then, the DNase activity was detected by observing clear zones around the bacterial growth as a positive result (30).

Detection of biofilm production activity

The micro titer plate of biofilm production assay was used according to (31,32), a follows: the bacterial inoculum was diluted in broth and adjusted to 0.5 McFarland standard. 200 µl of inoculum were added in to well "three replicates of each isolate" and incubated at 37 ºC for 18 hours for negative control, 200 µl of sterile broth medium were added. All wells were washed by PBS solution and dried, then the adhered cells were fixed by adding 200 µl of methanol in each well for 10 minutes. It was removed and the washing process was repeated with carefully. After that, the crystal violet stain (0.1 % w/v) was added (200 µl in to each well); after incubation for 15 minutes at room temperature, the plate was washed by PBS for 3 times to remove of execs stain and was perfect dried. Finally 200 µl of ethanol (95 % v/v) was added for (10) minutes and microtiter-plate reader (with optical density (OD) at 630 nm) was used to detect formed biofilm. Then, the mean of control and each sample was calculated and classified as non, weak, moderate and strong biofilm production according to table 1:

Extraction of genomic DNA

Each bacterial isolate was grown in brain heart infusion broth for 20 hours in aerobically condition with shaking (150rpm) at 37 ºC. Growth of bacterial cultures were precipitated in 1.5 ml tubes by micro centrifuge at 14500 rpm for 2 minutes. Finally the pellets were used for genomic DNA extraction according to a method that was described in the used kit (Geneaid "presto", Korea).

Table 1: Classification of biofilm formation abilities, Ab: mean of sample absorption, Abc: mean of control absorption .

Absorption	Biofilm Formation abilities
Ab > (4×Abc)	Strong
(2×Abc) < **Ab** ≤ (4×Abc)	Moderate
(Abc) < **Ab** ≤ (2×Abc)	Weak
Ab ≤ (Abc)	None

Primers design

Genes sequences were obtained from Gen Bank/NCBI. MP Primer program was used for primers design and the outputs of this program were conformed with previous study "which were mentioned by Tristan *et al.*, 2003 and Karmakar *et al.*, 2016" (33,34). The primers oligonucleotides were synthesized by (Eurofins Genomic, Germany), table.

Table 2: primers sequences used in this study.

Gene	Primers' Sequences (5'→3')	Tm (ºC)	Product size (bp)	Accession no.	Gene size (bp)	Reference
fnbA	F:CACAACCAGCAAATATAG	49.1	1362	NC007795	2973	33
	R:CTGTGTGGTAATCAATGTC	52.4				
fnbB	F:GTAACAGCTAATGGTCGA ATTGATACT	60.4	524	X62992	2891	33
	R:CAAGTTCGATAGGAGTAC TATGTTC	59.7				
nuc	F:GCGATTGATGGTGATACG GTT	57.9	270	DQ507382	682	34
	R:AGCCAAGCCTTGACGAAC TAAAGC	62.7				

Estimation of DNA purity and concentration

The Nano-drop device was used to measure the DNA concentration and purity (absorbance at 260nm / absorption at 280nm).

Molecular diagnosis of *S. aureus* by PCR amplification of *nuc* gene

The thermal cycling conditions included an initial denaturation step (5 min at 94°C) followed by 25 cycles of amplification (denaturation for 1 min at 94

°C, annealing for 1 min at 55°C, and extension for 30 seconds at 72 °C). The reaction was terminated with a 10-min incubation step at 72 °C. The amplification was performed in a Thermal Cycler (Bioneer, Korea). The protocol was used according to master-mix provide company (Bioneer "PCR premix", Korea). Primers were described in table 2.

Detection of fnbA and fnbB genes by simplex-PCR

The thermal cycling conditions for *fnbA* gene amplification included an initial denaturation step (5 min at 94 C̊) followed by 30 cycles of amplification (denaturation for 1 min at 94 C̊, annealing for 1 min at 50 C̊ and extension for 2 min at 72 C̊) . for completion (10 min incubation at 72°C). The amplification was performed in a Thermal Cycler (Bioneer, Korea). The condition for *fnbA* gene amplification included an initial denaturation step (5 min at 94 C̊) followed by 25 cycles of amplification (denaturation for 1 min at 94 C̊, annealing for 1 min at 55 C̊ and extension for 1 min at 72 C̊). The reaction was finished after a 10 min incubation at 72 C̊.

The protocol was used according to master-mix provide company (Bioneer "PCR premix", Korea). Primers were described in table 2.

Detection of DNA bands by electrophoresis

For genomic DNA bands, agarose gel (1%) was used in electrophoresis for 1 hour with 80 volt. For PCR production bands, agarose gel (1.5%) was used for 1.5-2 hours with 70 volt. Finally, the gels were photographed with UV light by gel documentation device.

Results and Discussion

Culture, microscopic examination and biochemical tests

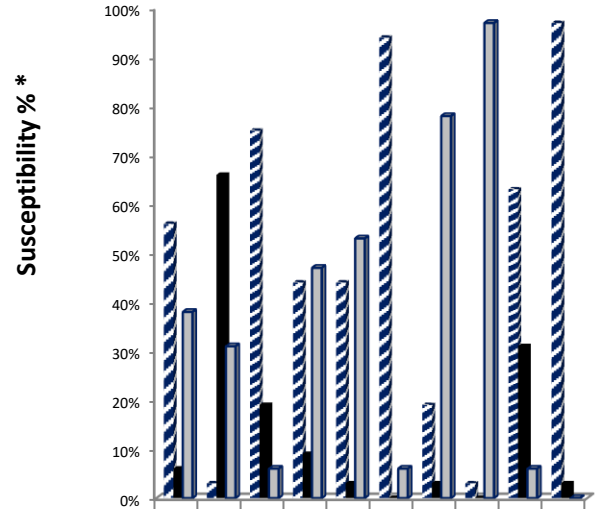
All selected isolates didn't grow on MacConkey agar , they grew on blood agar (5%) with opaque, round, smooth, raised, 1 to 3 mm in diameter and glistening Colonies. When isolated cells were stained by Gram stain, they appeared as Gram positive cocci. All isolates [32(100%)] gave positive results for mannitol fermentation, slid and tube coagulase tests; while all these isolates gave negative results for oxidase test. Thirty two isolates (100%) were detected as *S. aureus* by API Staph system.

Antibiotics susceptibility test

Results of antibiotic susceptibility test were explained in figure 1.

A variant susceptibility for antimicrobial agents was detected. Twenty five isolates (78%) were obtained as a methicillin resistant *S. aureus* (MRSA) depending on obtained of oxacillin resistant *S. aureus* (ORSA) by oxacillin disk diffusion.

Vancomycin and Imipenem "both antibiotics effect on cell wall of bacterial cells" are the strongest anti *S. aureus* agents were 31 (97%) tested isolates were sensitive to vancomycin, 30 isolates (94%) were sensitive to Imipenem and 1 isolates (3.1%) appeared as vancomicin intermediate resistant *S. aureus* (VISA). On the other hand, penicillin and cefotaxime had the lowest affect (3%). Others antibiotic had variant influences among different isolates but from interest that 21 isolates (66%) expressed intermediate resistance to cefotaxime.



	AK	CTX	CI	CD	E	IPM	OX	P	TE	VA
Sensitive	56%	3%	75%	44%	44%	94%	19%	3%	63%	97%
Intermediate R.	6%	66%	19%	9%	3%	0%	3%	0%	31%	3%
Resistant	38%	31%	6%	47%	53%	6%	78%	97%	6%	0%

Figure 1: Antibiotics susceptibility test; AK: amikacin, CTX: cefotaxime, CI: ciprofloxacin, CD: clindamycin, E: erythromycin, IPM: imipenem, OX: oxacillin, P: penicillin, TE: tetracycline and VA: vancomycin. *: the percentage values were approximated to the nearest integer.

The high oxacillin resistance obtaining in this study is considered risky indicator to spread MRSA strains in local Iraqi isolates. Many studies reported that the results of oxacillin susceptibility test are sufficient to investigation of the *mecA* presence, therefore all ORSA is MRSA in the same time . PBP2a is a recent penicillin binding protein that is encoded by This gene. By this property, the MRSA strain can resist of all beta

lactam (35,36). That is the same cause to high resistance to penicillin (97%) where all MRSA isolates in this study were penicillin resistant. In addition to β-lactam, MRSA isolates resist to many of the tested antibiotic and appeared as a Multi-drug Resistant. That leads to complicate the treatment of this infections. MRSA strains can have resistance genes on plasmids and in other genome spot in addition to those in the SCC*mec*, that explains the cause of multi antibiotic resistant in these strains (37) Resistance of these antibiotic occurs by different mechanisms such as chemical modification, ribosomal protection, and efflux pump (38,39).

Detection of hemolysis activity

On 5% blood agar, twenty one (65.6%) isolates produced β-hemolysis zones. Eleven (34.4%) isolates didn't produce any zone (gamma- hemolysis) while there is no alpha hemolysis activity was detected, table 3.

Detection of DNAse production activity

All 32 selected isolates produced DNase by appearance of a clear zone around the colonies on DNase agar after flooding by (0.1N) HCl, table 3.

Detection of biofilm production ability

Thirteen isolates (41%) gave a weak positive results for biofilm production test (table 3 and figure 2). There is no strong ability to form biofilm was recorded. The biofilm formation ability (weak) high related with MRSA strains.

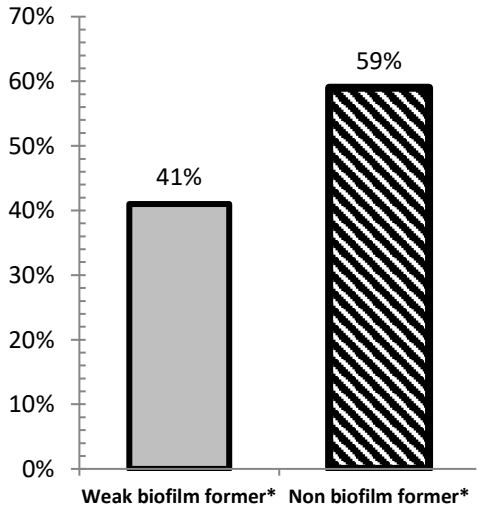


Figure 2: Biofilm production test, *: the percentage values were approximated to the nearest integer.

In addition to the multi resistance that was recorded, The biofilm former *S. aureus* isolates are the most difficulty isolates in treatment because the many resting cells are agglomerated within the matrix of biofilm. Extracellular matrix provides protective barrier against antimicrobial agent, reducing of their penetration in to the biofilm (40). A gathering of multi-drugs resistance and ability of biofilm production increases the problem.

Table 3: virulence factors and molecular detections.

No. of isolates	Source of infection	Virulence factors			Molecular detections			No. of isolates	Source of infection	Virulence factors			Molecular detections		
		Hemolysis (β)	DNase	Biofilm (weak)	*nuc*	*fnbA*	*fnbB*			Hemolysis (β)	DNase	Biofilm (weak)	*nuc*	*fnbA*	*fnbB*
1	Carditis	-	+	-	+	+	-	17	Nose	+	+	-	+	+	-
2	Carditis	-	+	+	+	+	-	18	Wound	+	+	-	+	+	+
3	Carditis	-	+	-	+	+	-	19	Wound	+	+	+	+	+	-
4	Carditis	-	+	+	+	+	-	20	Wound	-	+	+	+	+	-
5	Carditis	-	+	+	+	+	+	21	Wound	+	+	+	+	+	-
6	Sputum	+	+	-	+	+	-	22	Wound	+	+	+	+	+	-
7	Sputum	+	+	-	+	+	+	23	Nose	-	+	+	+	+	-
8	Sputum	+	+	+	+	+	-	24	Nose	+	+	-	+	+	-
9	Sputum	+	+	-	+	+	-	25	Abscesses	+	+	-	+	+	+
10	Bone	+	+	-	+	+	+	26	Abscesses	-	+	+	+	+	+

11	Bone	-	+	+	+	+	-	27	Abscesses	+	+	-	+	+	-
12	Bone	+	+	-	+	+	-	28	Abscesses	+	+	-	+	+	-
13	Bone	+	+	-	+	+	-	29	Burn	+	+	+	+	+	-
14	Bone	+	+	+	+	+	-	30	Burn	+	+	-	+	+	+
15	Nose	+	+	-	+	+	-	31	Burn	-	+	-	+	+	-
16	Nose	-	+	-	+	+	+	32	Burn	+	+	-	+	+	-

Extraction of genomic DNA and estimation of its purity and concentration:

DNA bands appeared in the extraction outputs of all samples with high concentration and typical purity (figure 3). for all isolates, Nano-drop data ranged (1.81-2.0) for DNA purity and (60.1-250) ng/µl for DNA concentration.

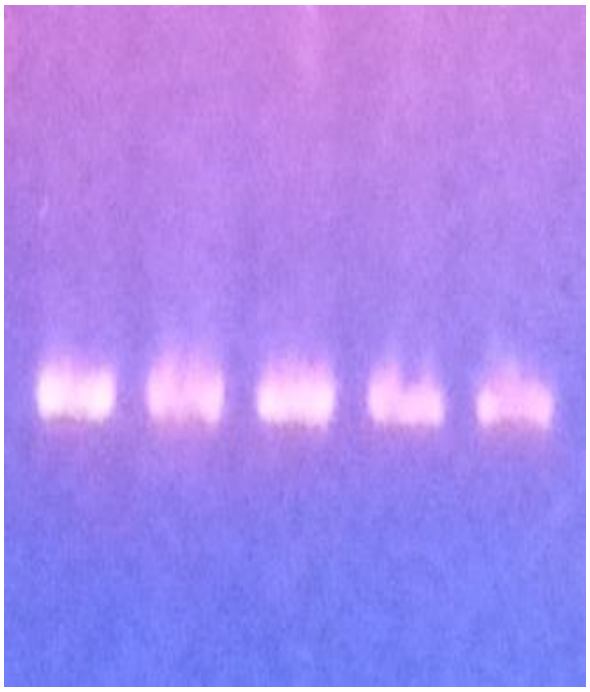


Figure 3: 1 % agarose gel electrophoresis of *S. aureus* genomic DNA.

Molecular diagnostic by PCR Amplification of *nuc* gene:

The *nuc* gene was found in all isolates (100%) with a PCR product size "270 bp" (table 3 and figure 4). This gene "which expresses thermostable nuclease" is a specific gene for *S. aureus* (41). Therefore, the detection of *nuc* gene in all isolates provide high accuracy in isolation of *S. aureus* and confirm its diagnosis in this study. In 2016, Al-Shammary also reported that diagnostic of *S. aureus* by detection of *nuc* gene is more accurate than biochemical and cultural ways (42); but in our study, we did not notice any differences between the genetic diagnosis and the diagnosis by conventional methods. that may occurred because we neglected all isolates that are negative for coagulase test, thermo nuclease test and API Staph system. Therefore, all isolates that expressed the thermo nuclease enzyme have *nuc* gene.

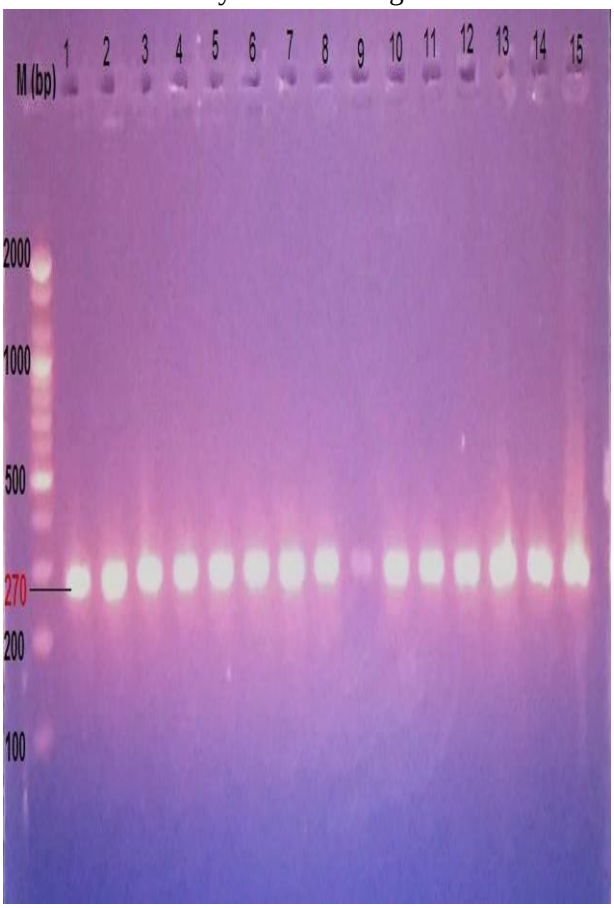


Figure 4: 1.5 % agarose gel electrophoresis for *nuc* gene, M: DNA marker.

Detection of fibronectin binding protein A gene (*fnbA*) and fibronectin binding protein B gene (*fnbB*)

All isolates (100%) contained the *fnbA* gene with final PCR product size (1362) bp (table 3 and figure 5), but eight isolates (25%) harbored the *fnbB* with end product equal to (524) bp (table 3 and figure 6), these 8 *fnbB* gene did not associated with specific isolates source, they distributed in all sources where only one isolate from each source contained *fnbB* gene except two isolates from abscess contained this gene.

In 2013, Bnyan also mentioned that fibronectin binding protein A gene was found in 100% of *S. aureus* isolated from Hilla – Iraq (43). The FnBPs (A and B) are very important protein for *S. aureus* colonization. They have similar function role, they mediate binding with fibronictin, fibrinogen and elastin (44). These proteins help the colonization of this microbe to epithelial and endothelial cells (45, 46). Farthermore, the FnBPs bind to platelet and contact with α5β1 integrin by bridge formation, that facilates the evasion from immune response by sneaking of microbe from blood stream in to organs (47). In 2011, Shinji *et al*., reported that FnBPA is more necessary than FnBPB for invivo and invitro infection in *S. aureus* (48).

From above previous studies, the multifunction of Fnbps interprets the high distribution of *fnbA* in all isolates without exception of any infection source, that increases the pathogenicity of tested isolates in this study. In spite of the high prevalence of *fnbA*, the low prevalence of *fnbB* is considered positivly results.

On the other hand, the prevalence of *fnbA* and *fnbB* can increase the bacterial resistance to antibioticc agents, where McCourt *et al.*, 2014 mentioned that the FnBPs are required for cell-cell binding and biofilm formation in *S. aureus* (49).

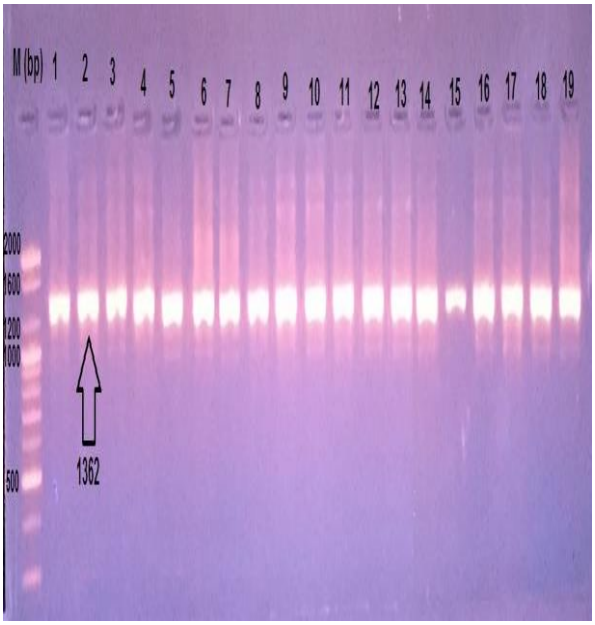


Figure 5: 1.5 % agarose gel electrophoresis for fnbA gene with end product size (1362)bp, M: DNA marker.

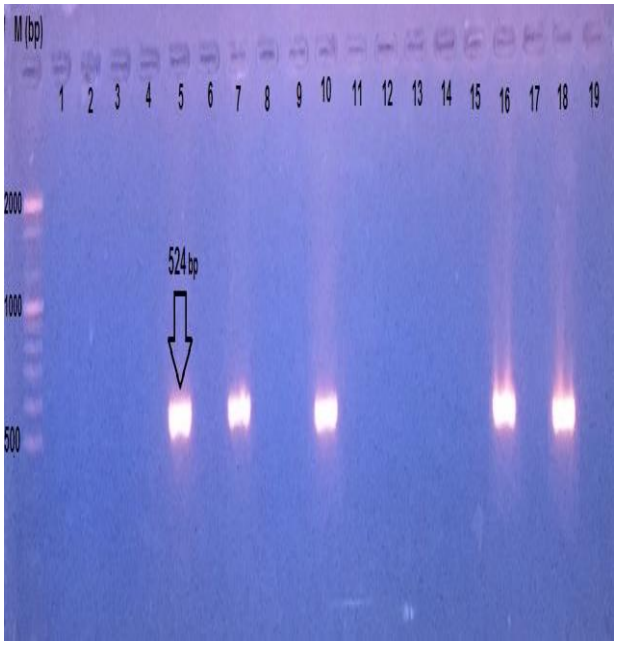


Figure 6: 1.5 % agarose gel electrophoresis for *fnbB* gene with end product size (524)bp, M: DNA marker.

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