Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.47750/jptcp.2023.30.03.021

Distribution of hla, hlb, hlgC, hld & cylA hemolysin genes and their alleles in different bacterial species isolated from the variant clinical sources in Basrah

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Submitted: 16 November 2022; Accepted: 11 December 2022; Published: 05 January 2023

ABSTRACT

Hemolysin is the most important virulence factor responsible for defecting in the plasma membranes of various types of host cells. One hundred and twenty-eight samples were collected from different clinical sources showing 111 (86.72%) bacterial isolates including 24 different bacterial species identified by 16S rRNA sequencing. Most of bacterial isolates have the ability to lyse the blood cells, delta hemolysin showed 50 (45.05 %) of isolates were positive on blood agar (BA), while 97 (87.39%) of isolates had the hld gene, followed by gamma 47(42.43%) on BA and 98 (88.28%) have of hlgC and 39 (35.14%) of isolates as beta on BA and 75 (67.57%) of hlb followed by alpha 37(33.33%) on BA and 46 (41.4%) of hla. Finally, 68 (61.26%) had the cytolysin A gene (cylA). The sequence of hemolysins revealed different number of alleles for hla, hlb, hlg and hld except for the cylA gene containing one allele only. The hemolysins were detected in 24 different bacterial species in addition to S. aureus. hla showed a new spontaneous mutation containing a new allele recorded in the NCBI.

Keywords: *Hemolysis, Alleles , Alpha, Beta , Delta, Gamma, Mutation*

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INTRODUCTION

Hemolysis is the degradation of red blood cells before their lifespans expire due to inherited disease or infection with bacteria that have hemolysins, particularly Staphylococcus giving to it the ability to colonize and replicate at the site of infections (Mooney et al., 2019). Hemolysin is one of the most important virulence factor causing defect in the plasma membrane of RBC and other types of cells such as epithelial cells, endothelial cells, T cells, monocytes and macrophages to provide nutritional requirements especially iron having catalytic structural and regulatory role for bacterial virulence factor (Bullen et al., 2005 ; Zughaier and Cornelis, 2018). The family of hemolysins was described for the first time in the late of 19th century when the hemolytic activity showed by the culture of Staphylococci.

Alpha toxin is a toxic, hemolytic, and lethal protein encoded by the hla gene on the chromosome that plays a role in many bacterial infections such as pneumonia, skin and soft tissue infections, sepsis, septic arthritis, and brain abscesses. Beta hemolysin is a virulence factor in S. aureus (Glenny and Stevens, 1935). It is sphingomyelinase encoded by the hlb and breakdown many types of cells as lymphocytes, keratinocytes, and neutrophils based on the content of sphingomyelin (Rees and Barr , 2017; Miruka et al., 2022). Gamma hemolysin is a pore-forming toxin produced by S. aureus that lyse the white and red blood cells. Furthermore, γ-toxin caused toxic effects against phagocytic cells at the site of infection to evade the immune system. (Blake et al., 2018; Tarenzi et al., 2022). The toxin consists of two subunits including the F and S unit. In spite of the similarity of the sequence of the two units being estimated at 30%, the structure of proteins shows high similarity (Spaan et al.,2017). Delta hemolysin is a peptide toxin consisting of 26 amino acids produced by some strains of Staphylococcus. It is encoded by the hld gene a part of RNAIII, a part of accessory gene regulator related to phenol soluble modulins (PSMs) which is the family of peptide toxin (Recsei et al., 1986 ; Zhou et al.,2021). The toxin has several functions including the lysis of erythrocytes, post-phagocytic neutrophils and other mammalian cells, the toxic effect on other bacterial cells (Otto, 2014). Cytolysin A is a pore forming toxin consist of two-subunit encoded by an operon consist of six genes located on chromosome or plasmid in Enterococcus faecalis (Clewell, 1993; Shankar et al.,2002). The coding genes for hemolysins showed genetic variation and became a target for researchers since S. aureus is the agent of a deadly infection making important virulence factors a target for vaccines. Indeed, the hla gene displayed 14 genotypes as a result of nucleotide sequence diversity leading to amino acid substitution, which can impact its use as a vaccine target. The site of mutation or genetic variation determines whether the toxin becomes non-hemolytic, less active or more lethal (Zhang et al., 2018).

As a result of the increase in the rate of accidental death that is associated with bacterial infections and to determine the most important toxin produced by bacteria, the study aims to determine the frequency of hemolysin types and their genetic differences (alleles) in each type of hemolysin gene for different bacterial isolates from different sources.

MATERIAL AND METHODS

Bacterial isolation

One hundred and twenty-eight samples were collected from patients with different infections including blood (32), burn swabs (29), nasal swabs (22), stools (17), urine (12), wound swabs (5) and others (11) including CSF, sputum, body fluid, ear swab, eye swab and GP-throat. The positive samples were sub-cultured on blood agar until they had a pure colony then cultured again on Nutrient agar (TM, India) for testing.

Molecular identification

Bacterial species identification

DNA extraction begins after the bacterial isolate is activated in 5 ml of sterilized BHIB (TM, India) and incubated at 37 °C for 24 h then DNA was extracted according to the procedure of the Presto TM Mini gDNA bacteria Kit (Geneaid, Taiwan).

The bacterial species were identified by 16 S rDNA sequencing, the gene was amplified with the following primers 27 F 5-AGAGTTTGATCCTGGCTCAG-3 and 1492 R 5-GGTTACCTTGTTACGACTT-3 (Alpha, USA) according to Miyoshi et al.,(2005).

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The mixture was contained the following: 25 µl of Go Taq green master mix (Promega, USA), 2 µl for each forward and reverse primers, 19 µl of nuclease free water (Bioneer, Korea) and 2 µl of DNA template. The thermocycler condition including 35 cycles for denaturation 95° C for 30 sec annealing 55° C for 30 sec and extension 72° C for 1 min. Finally, extension was 72° C for 2min. (Abd Al Wahid and Abd Al-Abbas, 2019). Agarose gel electrophoresis was performed to detect the bands (1500bp) using 1.5% of agarose gel (Bioneer, Korea). Fifteen µl of PCR product for 111isolates were sent to Macrogen company for purification and sequencing. Bacterial species were identified by Basic Local Alignment search tool (BLAST) followed by National Center for Biotechnology Information (Kerbauy et al.,2011), the phylogenetic tree for 16S rRNA was drawn by MAFFT after concatenation at 1166 bp by adding all the corrected nucleotide sequences of the bacterial species together in the Clustal Omega program that's including 22 bacterial species with their reference strain.

Identification of hemolysins

The bacterial isolates were cultured on blood agar (5%) at 37° C for 24h. to detect the bacterial hemolysis types (Buxton, 2005) as follows: Alpha was a greenish zone around bacterial growth, beta a clear zone around bacterial growth and gamma appeared as no change on the BA plates. Furthermore, Gamma hemolysis was detected on blood agarose plate prepared using 1% agarose as solidifying agent added to nutrient broth and autoclaved for 20 min, when the prepared media cooled down to 45° C, the rabbit blood (5%) was added, the medium was poured in a petri dish, after solidifying, the activated bacteria (100 µl) in BHIB for 18-24 h at 37◦C was added to the well with 2mm in diameter that was formed by sterilized corkborer and incubated at 37◦C for 24h. Gamma hemolysis was detected as clear zone around the well. Delta hemolysis was detected by synergistic hemolysis method when beta hemolytic S. aureus cultured as a vertical line on 5% blood agar plates and the bacteria to be tested was cultured horizontally with 1 cm space from S. aureus. The plates incubated at 37o C for 18-20 h and left at room temperature for 4-6 h, the positive result appeared as increasing in hemolysis at the area closely to the S. aureus (Coia et al., 1992). The hemolysins were detected by PCR using the primer listed in the Table (1), the amplifying mixture contained 12 µl of Go Taq green master mix, 2 µl of the template DNA, 1 µl of both forward and reverse primers and 7 µl of nuclease free water. The following program was used to amplify the hemolysins: 35 cycles for denaturation at 94° C, annealing at 58° C and extension at 72° C, each step was run for 35 sec. The result was detected using of 1.5% agarose gel.

TABLE 1: Primers used to amplify hemolysin genes

No.	Primers	Primer sequence	Length (bp)	Product size (bp)
1	F- hla	5-CTGATTACTATCCAAGAAATTCGATTG-3	27	209[1]
	R- hla	5-CTTTCCAGCCTACTTTTTTATCAGT-3	25	
2	F- hlb	5-GTGCACTTACTGACAATAGTGC-3	22	309[1]
	R- hlb	5-GTTGATGAGTAGCTACCTTCAGT-3	23	
3	F- hlg	5-GACATAGAGTCCATAATGCATTYGT-3	25	390[1]
	R- hlg	5-ATAGTCATTAGGATTAGGTTTCACAAA-3	28	
4	F- hld	5-AAGAATTTTTATCTTAATTAAGGAAG-3	26	111[1]
	R- hld	5-TTAGTGAATTTGTTCACTGTGTCGA-3	25	
5	F- hld2	5-ATGGCAGCAGATATCATTTC-3	20	444[2]
	R- hld2	5-CGTGAGCTTGGGAGAGAC-3	18	
6	F- cylA	5-ACTCGGGGATTGATAGGC-3	18	688[3]
	R- cylA	5-GCTGCTAAAGCTGCGCTT-3	18	

1: Jarraud et al., (2002), 2:Marconi et al., (2005), 3:Vankerckhoven et al.,(2004)

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The samples were sent to Macrogen company for sequencing and the data analyzed using BLAST and Multiple sequence alignment (MSA) to compare the sequences of hemolysin genes for different bacterial isolates from different sources for determining the hemolysins alleles. The tree was drawn using MAFFT program to detect the relation among them.

Spontaneous mutation by gradient plate technique

Spontaneous mutation detected by gradient plate method (Frank et al., 2007). Fifteen ml of BHIA was poured into a petri dish and left it to solidify in a slant shape, after 20 minutes, approximately 15 ml of BHIA containing Amikacin (200 mg/ml) was poured on the slant agar and left to solidify as a flat surface (Figure 1). Two isolates of the sensitive S. aureus were tested by spreading each on the gradient plate and incubated at 37oC for 24 h, the positive result detected by the growth of colonies at the high concentration region of amikacin. The resistance S. aureus was seeded on a blood agar to detect hemolysis type and amplified by PCR to sequence it.

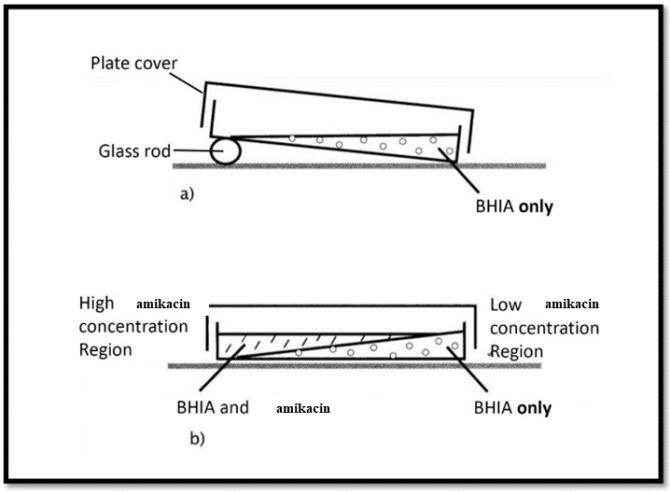

FIGURE 1: Gradient plate technique. a) BHIA only into a petri dish and b) BHIA with Amikacin.

RESULTS

One hundred and eleven bacterial isolates were obtained from 128 (86.72 %) samples of different sources (Table 2).

TABLE 2: Number of samples and isolates according to their sources

Sample sources	no. of samples	no. of Isolates n (100%)
Blood	32	16 (50)
Burn swab	29	28 (97)
Nasal swab	22	22 (100)
Stool	17	17 (100)
Urine	12	12 (100)
Wound swab	5	5 (100)
Others*	11	11 (100)
Total	128	111 (86.72)

16S rRNA gene amplification

The 16S rRNA of 111 bacterial isolates were shown on agarose gel electrophoresis and its position was approximately 1500 bp comparing with a molecular DNA ladder (Figure 2).

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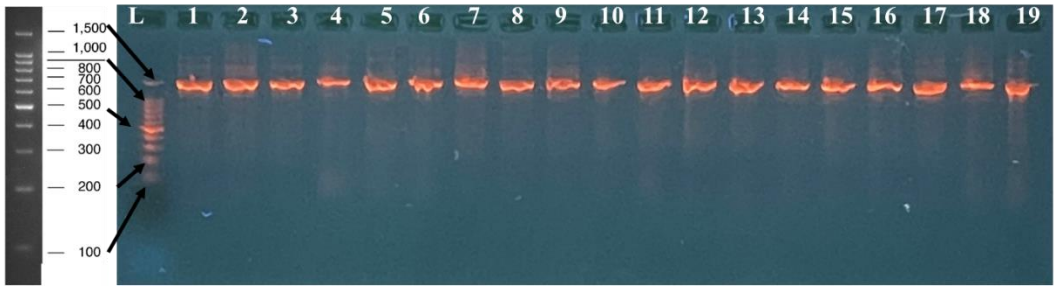

FIGURE 2 : Agarose gel electrophoresis (1.5%) showed a model of amplified 16S rRNA gene (1500bp). Lane L: 100 bp Marker, Lane 1-19: 16S rRNA gene bands for bacterial isolates.

Identification of bacterial species by 16 S rRNA sequencing

All amplicons of the 16 S rRNA gene were subjected to sequence and identified. The species were: Staphylococcus epidermidis (n=23/20.7%), Pseudomonas aeruginosa (n=15/13.5%), Escherichia coli (n=13 /11.7%), Staphylococcus aureus and Enterobacter hormaechei (n=8/7.2% for both), Enterococcus faecalis (n=7 /6.3%), Staphylococcus haemolyticus and Klebsiella pneumonia (n=5/4.5% for both), Staphylococcus hominis and Acinetobacter baumannii (n=4/ 3.6% for both), Proteus mirabilis and Bacillus safensis (n=3 / 2.7% for both), Escherichia fergusonii (n=2 /1.8%), Pseudocitrobacter anthropi, Enterobacter ludwigii, Bacillus cereus, Staphylococcus saprophyticus, Enterobacter mori, Pseudomonas mosselii, Escherichia marmotae, Bacillus paramycoides, Bacillus haynesii, Enterobacter bugandensis and Lacticaseibacillus rhamnosus (n=1 /0.9% for each).

Phylogenetic tree of 16S rRNA gene of bacterial species

The rooted phylogenetic tree for 16S rRNA sequences of studied bacterial species was constructed (Figure 3). Tree shows the distribution and phylogenetic relationships of 22 different bacterial species isolated from different clinical sources with the identity of their reference strains.

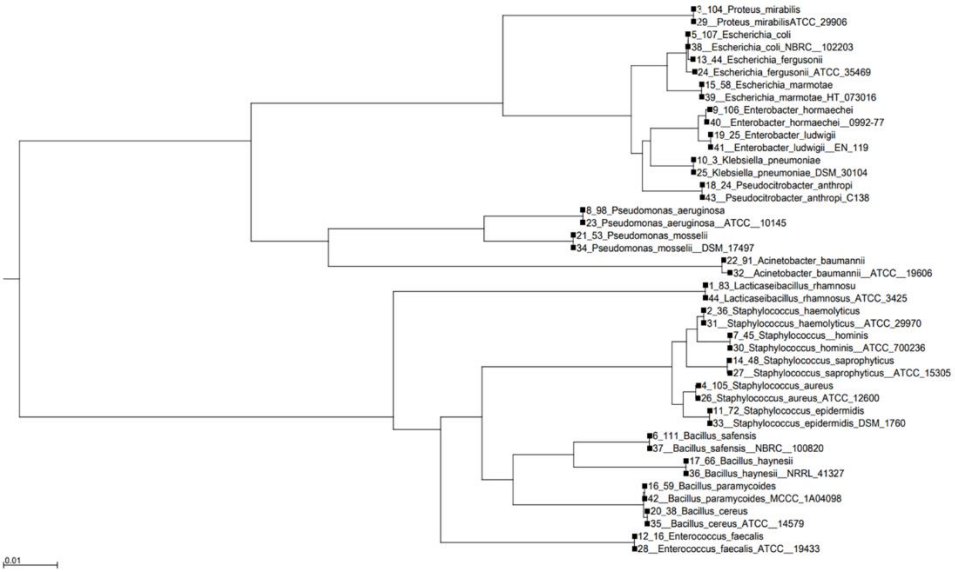

FIGURE 3: Rooted Neighbor Joining phylogenetic tree concatenated sequences of 1166bp for each strain (derived from an alignment of 16S rRNA sequences) produced from MAFFT program. This tree showing the distribution and phylogenetic relationship for a model of 22 species isolated from different clinical sources with their reference strain (ATCC, DSM, NRBC, MCCC, HT and EN) all horizontal length were drawn to scale.

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Twenty five bacterial isolates were recorded as new global strains in the National Center for Biotechnology Information (NCBI) as IRQBAS112, IRQBAS113, IRQBAS115, IRQBAS116, IRQBAS117, IRQBAS118, IRQBAS119, IRQBAS120, IRQBAS121, IRQBAS122, IRQBAS124, IRQBAS125, IRQBAS126, IRQBAS127, IRQBAS128, IRQBAS129, IRQBAS 130, IRQBAS131, IRQBAS132, IRQBAS133, IRQBAS134, IRQBAS135, IRQBAS136, IRQBAS137 and IRQBAS138.

Bacterial hemolysis on Blood Agar Base

Most bacterial isolates showed the ability to lyse the blood including different types of hemolysis (Figure 4). The synergistic hemolysis method showed that delta hemolysis was the most frequent type in 50 of 111 (45.04%) of isolates. In spite of that, the ordinary method showed gamma hemolysis in 47 (42.34 %), beta in 39 (35.14 %) and alpha in 37 (33.33 %) with no significant differences among the four types of hemolysis at $P \leq 0.05$.

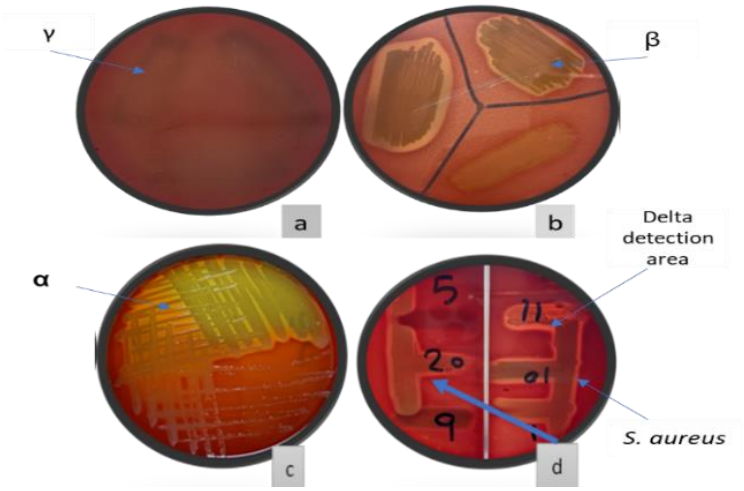

FIGURE 4: Bacterial hemolysis activity on Blood agar base. a: gamma hemolysis: no change on blood agar, b : beta hemolysis: clear zone around bacterial growth, c: alpha hemolysis : greenish zone around bacterial growth d : delta hemolysis : the increasing in hemolysis at the area closely related to the horizontal growth of S. aureus.

Bacterial hemolysins by molecular technique

All the five of the hemolysins genes were detected in the present study. The hla gene was detected in 46 of 111 (41.4%) bacterial isolates (Figure 5).

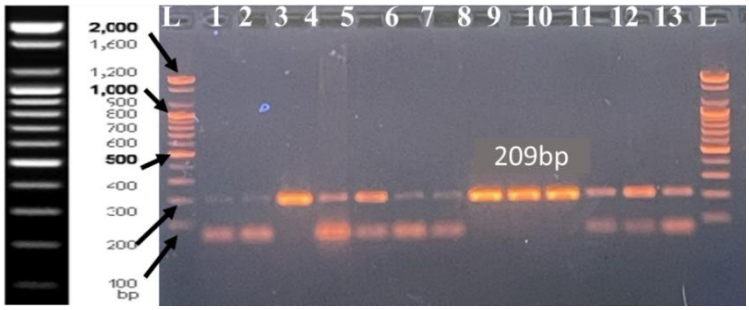

FIGURE 5 : Agarose gel electrophoresis (1.5%) showed a model of amplified hla gene (209 bp). Lane L: 100 bp Marker . Lane 1-13: hla gene bands for bacterial isolates .

The hlb was detected in 75 (67.57%) of bacterial isolates (Figure 6).

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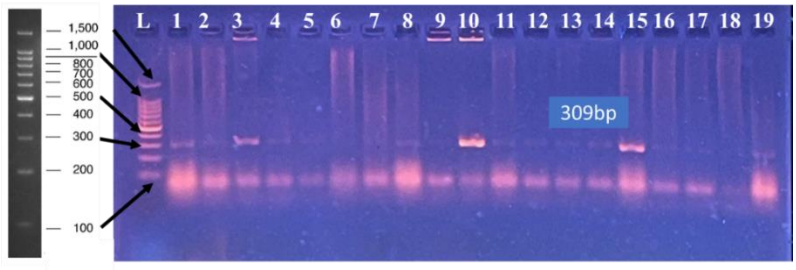

FIGURE 6: Agarose gel electrophoresis (1.5%) showed a model of amplified hlb gene (309 bp). Lane L: 100 bp Marker. Lane 1-19: hlb gene bands for bacterial isolates.

The hlgC was detected in 98 (88.28%) of bacterial isolates (Figure 7).

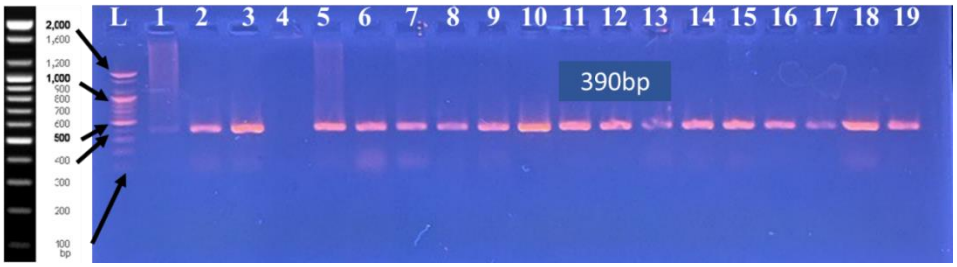

FIGURE 7 : Agarose gel electrophoresis (1.5%) showed a model of amplified hlgC gene (390 bp). Lane L: 100 bp Marker . Lane 1-19: hlgC gene bands for bacterial isolates .

The hld was detected in 97 (87.39%) of bacterial isolates. In detail, the hld of S. epidermidis was detected in 95 (85.59%) of isolates while the hld of S. aureus was detected in 13 (11.71%) of isolates and the gel electrophoresis showed the band size at 444 and 111bp respectively (Figure 8,9) .

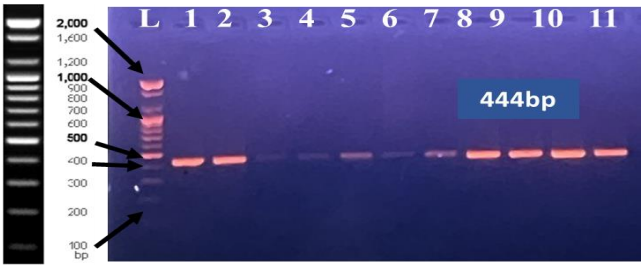

FIGURE 8: Agarose gel electrophoresis (1.5%) showed a model of amplified hld gene of S. epidermidis (444 bp). Lane L: 100 bp Marker . Lane 1-11: hld gene bands for bacterial isolates.

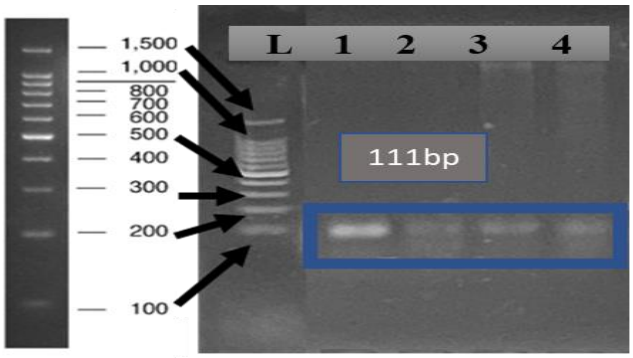

FIGURE 9: Agarose gel electrophoresis (1.5%) showed a model of amplified hld gene of S. aureus (111 bp). Lane L: 100 bp Marker . Lane 1-4: hld gene bands for bacterial isolates.

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The cylA was detected in 68 (61.26%) of bacterial isolates and the gel (Figure 10) .

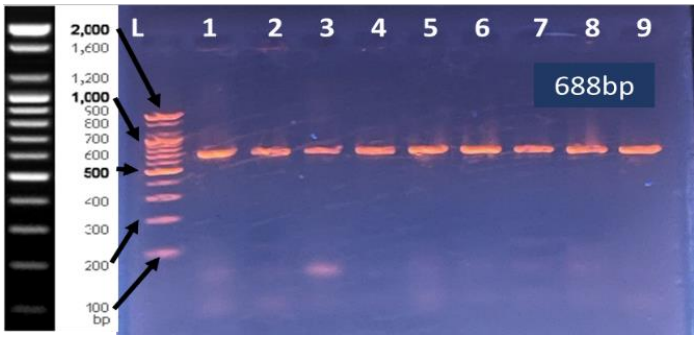

FIGURE 10: Agarose gel electrophoresis (1.5%) showed a model of amplified cylA gene (688 bp). Lane L: 100 bp Marker. Lane 1-9: cylA gene bands for bacterial isolates.

Gamma hemolysis on rabbit blood agar & Rabbit blood agarose

The bacterial isolate having the hlgC gene (98- P. aeruginosa) showed a hemolysis zone on rabbit blood agarose that is larger than the hemolysis zone on rabbit blood agar. At the same time, bacterial isolates having hlb and or hla and hlgC (20-S. aureus, 61-E. faecalis and 94- P. aeruginosa) produce two hemolysis zones, including a large zone (40 mm) and a small zone (15 mm), as shown in Figure (11).

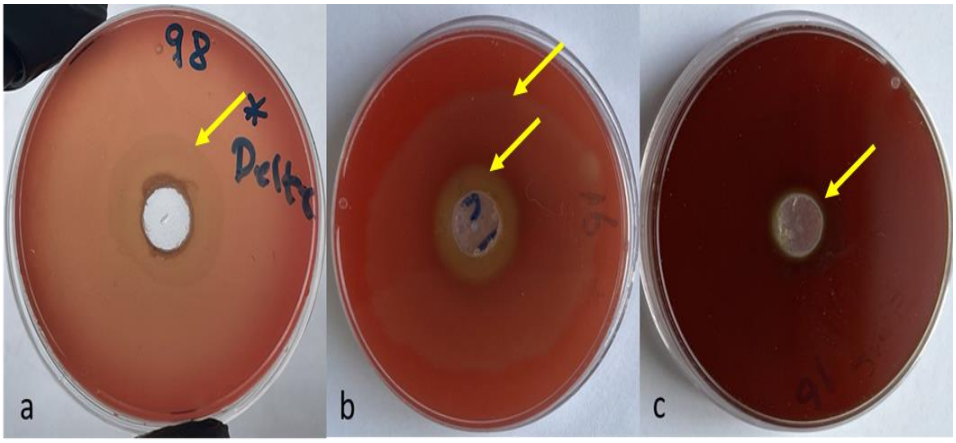

FIGURE 11: The lysis zone on blood agar and agarose (a) Gamma hemolysis zone of 98- P. aeruginosa on rabbit blood agarose. (b) Double lysis zone of 16 - E. faecalis on rabbit blood agarose.(c) Small lysis zone of 16- E. faecalis on rabbit blood agar.

Comparison between genetically and biochemically hemolysis

Except for alpha hemolysin, the phenotypic and genotypic detection of hemolysins revealed significant differences at $p \leq 0.05$ (Figure 12). Delta was showed 50 (45.05 %) of isolates positive on blood agar by cross culture, while 97 (87.39%) had the hld gene, followed by gamma hemolysis with 47(42.43%) positive on BA and 98 (88.28%) having the hlg. BA showed 39 (35.14%) of isolates as beta hemolysis and 75 (67.57%) had the hlb gene followed by alpha hemolysis 37(33.33%) positive on BA and 46 (41.4%) had the hla gene. Finally, 68 (61.26%) of the isolates had the cytolysin A gene (cylA).

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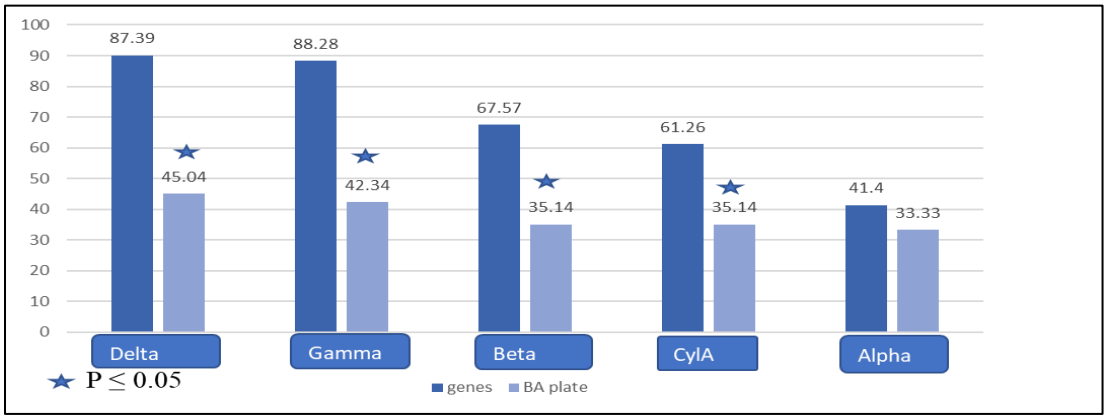

FIGURE 12: Comparison between phenotypic and genotypic hemolysis.

The 111 bacterial isolates were divided into nineteen groups according to the frequency of hemolysin genes in each isolate (Table 3). hlgC, hld and cylA were appear each gene alone in a single bacterium. hlb + hlgC, hlb + hld, hlb + cylA and hlgC+ hld showed each two genes together in a single bacterium. hla+ hld+ hlgC, hlb+ hlgC+ hld, hlb + hld + CylA, hla +hld + CylA, hlb+ hlgC+ CylA and hlgC +hld +cylA were all three genes together in a single bacterium. hla +hlb+ hld + CylA, hla +hlb + hlgC+ CylA, hla + hlgC +hld + CylA, hlb + hlgC + hld + CylA and hla + hlb + hlgC + hld were all four genes together in a single bacterium. All of the five genes hla + hlb+ hlgC + hld + cylA were found together in a single bacterium. At the same time, hla+ hlb +hlgC + hld + cylA, hlb +hlgC+ hld +CylA and hla+ hlb +hlgC + hld were significantly different than other group at p≤0.05.

TABLE 3: Bacterial species distribution according to the presence of hemolysin gene

No.	Hemolysin genes	Genes n (%)	Bacterial species	no. of bacteria
1	hlgC	1 (0.90)	P. aeruginosa	1
2	hld	3 (2.70)	S. hominis S. epidermidis E. hormaechei	1 1 1
3	CylA	1 (0.90)	E. coli	1
4	hlb & hlgC	1 (0.90)	E. faecalis	1
5	hlb & hld	1 (0.90)	E. marmotae	1
6	hlb & CylA	1 (0.90)	S. epidermidis	1
7	hlgC & hld	8 (7.21)	S. haemolyticus B. cereus L. rhamnosus P. aeruginosa B. safensis P. mirabilis E. coli	1 1 1 2 1 1 1
8	hla , hld & hlgC	4 (3.60)	P. aeruginosa S. epidermidis B. safensis S. aureus	1 1 1 1
9	hlb , hlgC & hld	11 (9.91)	P. aeruginosa K. pneumonia B. paramycoides S. epidermidis A. baumannii	3 2 1 3 2
10	hlb , hld & CylA	4 (3.60)	E. coli P. aeruginosa S. epidermidis	1 1 2
11	hla , hld & CylA	1 (0.90)	S. epidermidis	1

12	hlb , hlgC & CylA	8 (7.21)	E. faecalis E. bugandensis E. hormaechei E. coli P. mirabilis S. hominis P. aeruginosa	1 1 2 1 1 1 1
13	hlgC , hld & CylA	12 (10.81)	E. faecalis P. anthropi E. coli S. epidermidis E. fergusonii S. hominis P. mosselii B. haynesii	1 1 3 2 1 1 1 1
14	hla , hlb , hld & CylA	2 (1.80)	K. pneumonia S. aureus	1 1
15	hla , hlb , hlgC & CylA	2 (1.80)	E. coli E. faecalis	1 1
16	hla , hlgC , hld & CylA	6 (5.41)	P. aeruginosa S. epidermidis S. haemolyticus E. faecalis	1 3 2 1
17	hlb , hlgC , hld & CylA	15 (13.51)*	P. mirabilis K. pneumonia E. hormaechei E. coli S. epidermidis E. mori E faecalis A. baumannii	1 1 3 3 4 1 1 1
18	hla , hlb , hlgC & hld	14 (12.61)*	S. haemolyticus S. epidermidis E. hormaechei S. haemolyticus P. aeruginosa S. aureus B. safensis	1 1 2 1 4 4 1
19	hla , hlb , hlgC , hld & cylA	16 (14.41)*	S. aureus E. coli E. faecalis S. epidermidis E. ludwigii S. saprophyticus S. hominis K. pneumoniae A. baumannii	3 3 1 4 1 1 1 1 1

$P \leq 0.05$

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Hemolysin gene alleles

The hla gene was found to has two alleles (Figure 13) distributed among 33 bacterial isolates: the first one in 1-S. aureus and the second in 32 isolates. Also, the sequence of hlb was in 45 bacterial species contained two alleles groups (Figure 14), the first one in the isolate no.101- S. aureus while the second was found in 44 bacterial species. The hlgC for 25 bacterial isolates was contained two alleles (Figure 15), the first one in 22 isolates while the second one found in the three. The sequence of the hld gene for 46 isolates was divided into three groups according to the length of the different fragments. The first group including four alleles (Figure 16), the second group contained one allele in four species. The third group contained one allele too. Finally, the cylA gene for 21 bacterial species is owned by only one allele. The frequency of all these alleles were listed in Table (4). However, the number of alleles for each gene didn't show significant differences at $P \leq 0.05$.

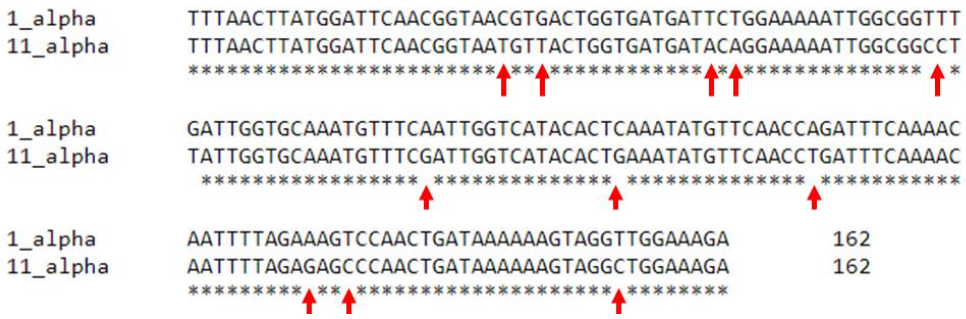

FIGURE 13: Multiple sequence alignments of 2 alleles groups of hla gene. The arrows refer to the nucleotide mutations.

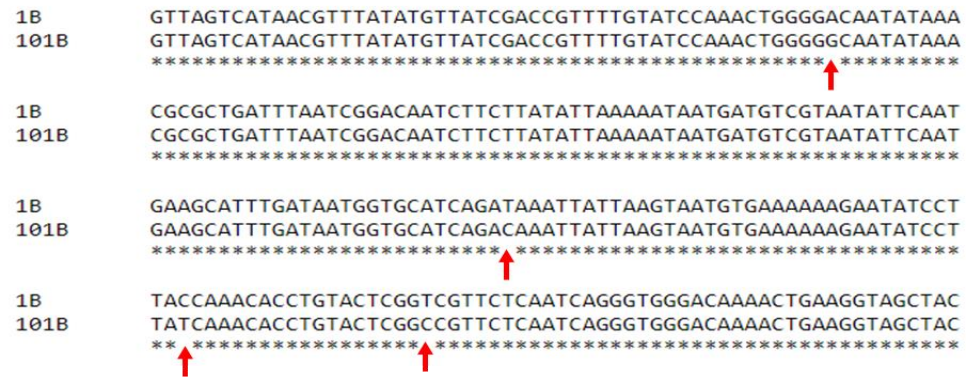

FIGURE 14: Multiple sequence alignments of 2 alleles groups of hlb gene. The arrows refer to the nucleotide mutations.

FIGURE 15: Multiple sequence alignments of 2 alleles groups of hlgC gene. The arrows refer to the nucleotide mutations.

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FIGURE 16: Multiple sequence alignments of 4 alleles of hld gene. The arrows refer to the nucleotide mutations.

TABLE 4: Distribution of hla, hlb, hlgC, hld and cylA alleles among different bacterial species from different sources.

Hemolysin genes (no. of sequences)	no. of alleles	Bacterial species	no. of isolates	Bacterial sources
hla (33)	1	S. aureus	1	Aspirate
	2	K. pneumonia	1	Wound swab
		E. faecalis	2	
		S. aureus	1	Body fluid
		E. coli	2	Urine
		B. safensis	1	
		P. aeruginosa	1	Ear swab
		S. aureus	1	
		S. haemolyticus	2	Sputum
		S. epidermidis	5	Nasal swab
		S. aureus	1	
		S. saprophyticus	1	
		E. ludwigii	1	
		E. hormaechei	1	Stool
		S. hominis	1	Burn swab
		S. haemolyticus	1	
		P. aeruginosa	2	
		S. aureus	2	
		E. hormaechei	1	
		P. aeruginosa	2	Blood
		S. epidermidis	1	
		S. aureus	1	
		B. safensis	1	
hlb (45)	1	S. aureus	1	Body fluid
	2	E. coli	5	Urine
		P. mirabilis	1	
		B. paramycoides	1	
		B. safensis	1	
		E. faecalis	2	Wound swab
		P. aeruginosa	1	
		S. aureus	1	Ear swab
		S. epidermidis	5	Nasal swab
		E. ludwigii	1	

		E. faecalis	1	Throat
		K. pneumonia	2	Stool
		E. hormaechei	4	
		P. aeruginosa	1	Body fluid
		S. epidermidis	3	Blood
		S. hominis	1	
		S. hominis	1	Burn swab
		K. pneumoniae	2	
		A. baumannii	4	
		P. aeruginosa	2	
		S. epidermidis	2	
		S. aureus	2	
		B. safensis	1	
hlgC (25)	1	S. aureus	1	Nasal swab
		P. aeruginosa	1	Burn swab
		P. aeruginosa	1	Blood
	2	E. coli	2	Urine
		B. safensis	1	
		P. aeruginosa	1	Ear swab
		E. hormaechei	1	Stool
		S. haemolyticus	1	Nasal swab
		S. hominis	1	
		S. aureus	4	Burn swab
		K .pneumonia	1	
		E. faecalis	1	
		L. rhamnosus	1	
		B. safensis	1	
		P. mirabilis	1	
		E. coli	1	
		E. hormaechei	1	
		p. aeruginosa	3	Blood
		S. aureus	1	
hld (46)	1	S. epidermidis	1	Blood
	2	S. epidermidis	1	
	3	S. epidermidis	1	
	4	E. coli	1	CSF
		E. faecalis	1	Urine
		E. coli	3	
		B. safensis	1	
		K. pneumoniae	1	Stool
		B. paramycoides	1	Nasal swab
		P. aeruginosa	1	Body fluid
		P. aeruginosa	1	Wound swab
		S. hominis	1	Blood
		S. epidermidis	2	
		P. aeruginosa	1	
		S. hominis	1	Burn swab
		K. pneumoniae	2	
		S. haemolyticus	1	
		A. baumannii	2	
		P. aeruginosa	5	
		L. rhamnosus	1	
		E. faecalis	1	
		S. epidermidis	1	
		P. mirabilis	1	

		E. hormaechei	2	
		E. coli	1	
	5	S. aureus	1	Aspirate
		S. aureus	1	Nasal swab
		P. aeruginosa	1	Burn swab
		S. aureus	1	
	6	S. aureus	1	Ear swab
		P. aeruginosa	2	Blood
		B. safensis	1	
		S. epidermidis	1	
		S. aureus	3	Burn swab
cylA (21)	1	P. anthropi	1	Stool
		E. hormaechei	2	
		E. bugandensis	1	
		E. coli	1	
		P. mosselii	1	
		S. epidermidis	1	
		S. epidermidis	10	Nasal swab
		S. saprophyticus	1	
		S. hominis	1	
		E. coli	1	Urine
		B. haynesii	1	Blood
		A. baumannii	1	Burn swab

Phylogenetic tree of hemolysin genes

The rooted phylogenetic tree for hemolysins sequences (n=170 from bacteria under the study and 5 reference sequence) was constructed (Figure 17). The tree showed the distribution and phylogenetic relationships of five clusters of hemolysin genes (hla=33, hlb= 45, hlg= 25, hld =46 and cylA=21) of different bacterial species (isolated from different clinical sources) with their reference strains. In spite of the nucleotide mutation found in hemolysin genes, each gene still exists as a group with its reference strain and there was no interfere among the different genes.

FIGURE 17 : Rooted Neighbor Joining phylogenetic tree showing phylogenetic relationship of 175 hemolysins gene (hla=33, hlb= 45, hlg= 25, hld =46 and cylA=21) in comparison with their type strains ATCC, JKD and NLR that is constructed from UPGMA MAFFT.

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Hemolysins alleles and amino acids alteration

The first allele of the hla represented by isolate No.1-S. aureus (identical to ATCC_25923) was differs from isolate No.11-S. aureus by one amino acid (serine instead of threonine respectively) as Figure (18).

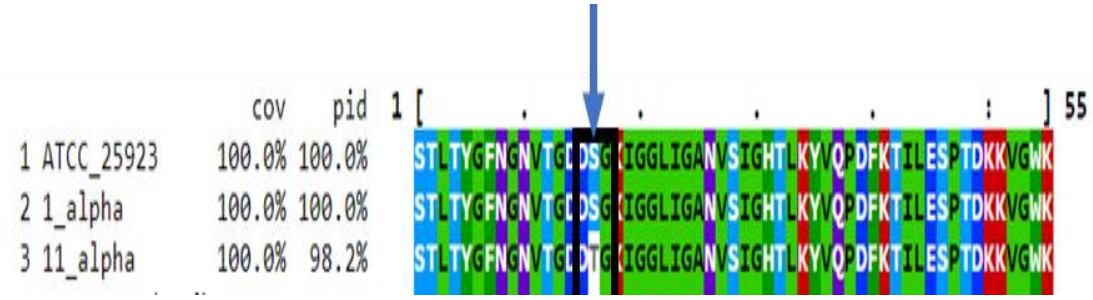

FIGURE 18: The amino acid sequence of 2 hla alleles including isolate No. 1- S. aureus with their type strain ATCC_25923 of serine and No. 11- S. aureus of threonine.

The hlb gene consists of two alleles (No. 1and 101-S. aureus) according to the sequence of nucleotides, while that difference doesn't lead to a change in the sequence of amino acids also that identical to ATCC _25923 (Figure19)

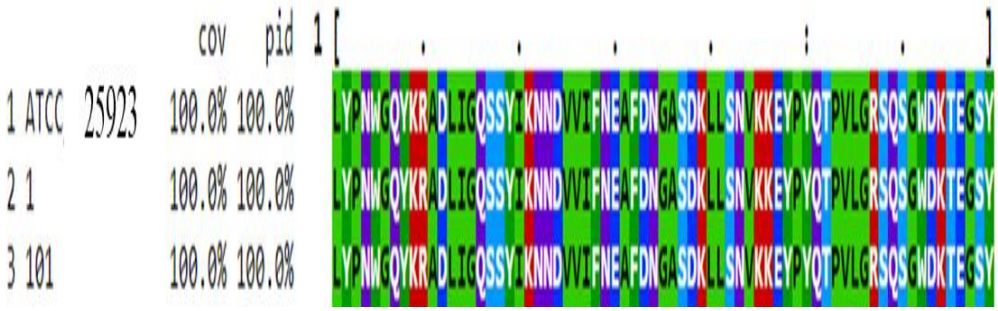

FIGURE 19: The amino acid sequence of hlb alleles for No.11- S. aureus and No.101- S. aureus with ATCC_25923 .

The difference in nucleotide sequence leading to differences in six amino acids of hlgC, the allele one in isolate No.81- P. aeruginosa (identical to ATCC 25923) and allele two in isolate No.6- P. aeruginosa (asparagine instead lysine, asparagine instead glycine, phenylalanine instead tyrosine, valine instead glutamate and lysine instead arginine respectively) as shown in Figure (20).

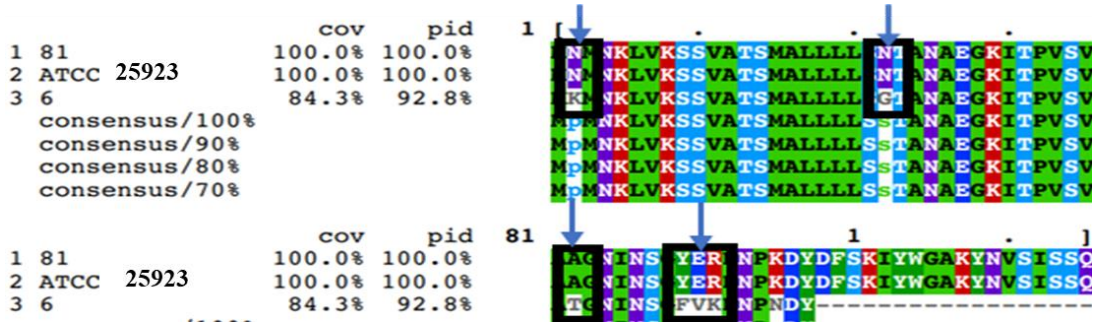

FIGURE 20 : The amino acid sequence of two hlgC alleles, including isolates No. 6 - P. aeruginosa and No. 81- P. aeruginosa showed differences in amino acid asparagine instead lysine , asparagine instead glycine, phenylalanine instead tyrosine, valine instead glutamate and lysine instead arginine, respectively) with type strain_ATCC25923 (identical to No. 81- P. aeruginosa)

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In spite of there being four alleles according to the difference of nucleotides in S. epidermidis, but there was only one different amino acid in isolate No. 68 (Isoleucine) in comparison to 69, 70,93 and ATCC (Valine) Figure (21).

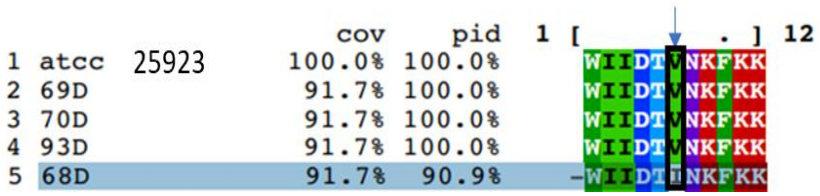

FIGURE 21: The amino acid sequence of four hld alleles in S. epidermidis, including isolates No. 69, 70, 93 and ATCC_25923 contain valine instead Isoleucine in 68.

Spontaneous mutation

Only two of S. aureus (22.2%) were susceptible to amikacin and showed spontaneous mutation by resistant to amikacin when growing in an antibiotic-rich environment (Figure22). The hla gene was detected in No.1 S. aureus and No. 102 S. aureus by PCR after the growth on a higher concentration of amikacin, then gene sequenced to compare it with hla sequences before the mutation. As a result of spontaneous mutation , the hla of mutated 1-S. aureus showed mutation (Figure23), while the isolate 102-S. aureus showed no difference before and after the mutation. The novel allele was recorded at the National Center for Biotechnology Information (NCBI) as 1–S. aureus strain IRQBAS-168-G (accession number =LC726267).

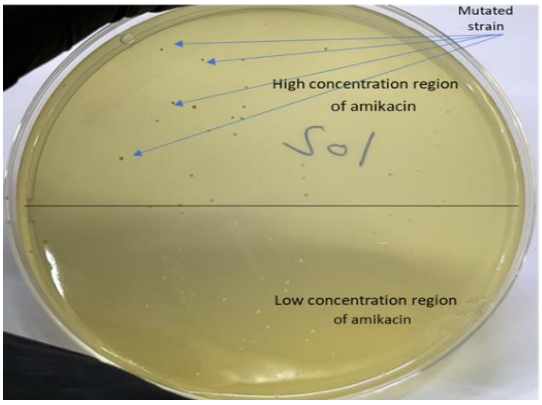

FIGURE 22: Spontaneous mutation for amikacin resistance showed the resistant colonies at higher concentration region of gradient BHIA plates.

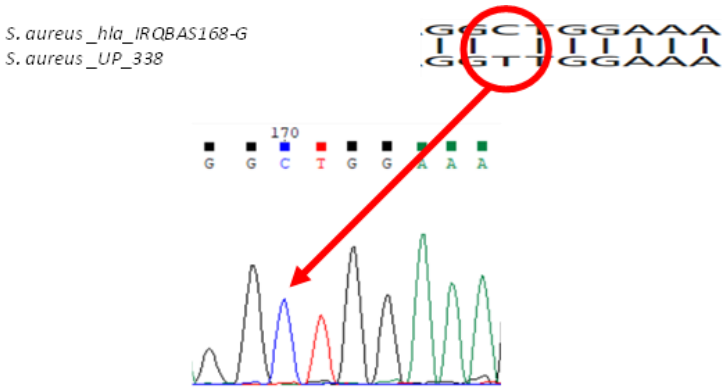

FIGURE 23: Comparison of hla nucleotide sequence for isolate No. 1-S.aureus before and after the mutation, a transition mutation (C instead T) at the position 170 bp.

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DISCUSSION

Most infectious diseases were caused by Staphylococcus including coagulase-positive and negative isolates having several virulence factors, especially hemolysins. The most important one that have the ability to invade the immune cells of the host such as macrophages (da Silva et al., 2005). From 97 of 111(87.39%) bacterial isolates, all S. aureus (n=9) were contained the hld gene, since 97% of S. aureus have the gene according to the study of Schmitz et al. (1997), but the present study showed the detection of hld in 24 different bacterial species in addition to S. aureus. Furthermore, hlg is the gene encoding gamma hemolysin in 99.5% of S. aureus isolates (Prevost, 1995), whereas the current study found hlg in 98 (88.28%) of all 111 isolates, which included in twenty-four bacterial species, followed by hlb in 75 (67.57%) and hla in 46 (41.4%) because the platelets and monocytes are more sensitive to alpha hemolysis (Todar, 2005). Moreover, the expression of beta hemolysis on BA is influenced by the concentration of magnesium and the chelating agent in the prepared medium (Boyce, 1985). As a result to the inhibitory effect of agar on gamma hemolysis activity, the rabbit blood agarose was used to detect their activity (Divyakolu et al., 2019).

The detection between phenotypic and genotypic for delta, gamma and beta hemolysin showed significant differences (Figure 11). In detail, the expression of hemolysins on blood agar plates showed less frequency in comparison to the genes coded for them, which may be affected by several factors. For example, if the gene was inactive, the expression was occurring in the exponential phase when the bacterial density was in high. Nevertheless, the surrounding conditions are improper for gene activity. In general, just because the bacteria do not produce hemolysis on blood agar does not mean they are not expressed indefinitely (Marconi et al., 2005; Moraveji et al., 2014). The detection of hemolysin genes without detection of the expression on blood agar in some isolates may refers to the presence of a combination of coding genes, so the expression on blood agar plates depends on which gene expressed was the predominant, therefore there is an indication of the false positive results if the detection was depend on biochemical testing only. However, there were no significant differences in the expression of alpha hemolysin on blood agar and the presence of the hla gene, which is a major virulence gene in S. aureus. Since, the toxin contributed in the pathogenesis and invasion of host tissue infection (Xiao et al., 2016). Most bacterial species showed combining of hemolysin genes in the some isolate rather than a single one increasing the virulence because the increase of virulence factors lead to increase of bacterial pathogenicity according to Jarraud et al. (2002). The four hemolysins specific for S. aureus detected in other bacterial species were recorded in NCBI. As a result of genetic transfer, whether via mobile genetic transfer or recombination, perhaps that will provide a way for bacterial species in general to adapt to their surrounding environment.

Hemolysins alleles

Two alleles were recorded in hla, hlb, and hlgC leading to amino acid alteration in hla and hlgC, four alleles were recorded in the hld while one allele recorded in the cylA, in spite of more than one nucleotide changed in some alleles, there is only one amino acid changed as a result of the fact that more than one codon encodes for a single amino acid (Turanov et al., 2009). The effect of nucleotide or even amino acid substitution depends on the site of the change, for instance, histidine substitution at site 35 causes loss of or decreases. The presence of the same alleles in more than one strain of the same bacterial species or even in other bacterial species means that all of them refer to the same common ancestor, which is S. aureus.

As a result of genetic transfer, whether via mobile genetic transfer or recombination, perhaps that will provide a way for bacterial species in general to adapt to their surrounding environment. This results in non-specific hlb for S. aureus, which interferes with using the phage-derived hlb gene as a marker and has a significant impact on S. aureus identification (Miruka et al., 2022). Importantly, the comparison among alleles in the present study were refer to the differences more than identities, because the present study didn't produce a complete sequence for all comparing gene. From that, the difference is true, but the identities between alleles need more studies.

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Spontaneous mutation

A spontaneous mutation was used to show the effect of mutation on alpha hemolysin because the bacteria may be exposed to misuse and overuse of antibiotics inside the body leading to invention of a new hla allele by the occurrence of point mutations in the nucleotide sequence which may be more dangerous due to the increase of the bacterial pathogenicity.

CONCLUSION

The five types of hemolysins were recognized together in S. aureus, E. coli, E. faecalis, S. epidermidis, E. ludwigii, S. saprophyticus, S. hominis, K. pneumoniae and A. baumannii by the molecular detection of hemolysins referring to the horizontal gene transfer (HGT). More different alleles were distinguished in the hld gene when compared to other hemolysin genes leading to the variation of amino acids sequences. Furthermore, Antibiotic over and misuse consider as predisposing factor in the mutations of the hemolysin genes that establish new alleles.

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