



Original Research Article

Molecular Study of Virulence Genes of *Staphylococcus aureus* Isolates from Various Clinical Origins by PCR

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Abstract

Staphylococci pathogen was blamable for a huge scale of infections in human because of their invention of secreted and other cell-surface related virulence factors that regulate by various genes. This study was carrying out to realize the frequency of Staphylococcus species in Hillah city using specific genes for that purpose.

PCR technique was applied to amplify the staphylococcal virulence genes like *sea*, *see*, *eta*, *tst*, *coa*, *nuc*, *seb*, *femA*, *mecA*, *etb*, *cna*, *fib* and *fnbA*, and they determined either multiplex or uniplex PCRs depending on the sizes of the PCR products amplicons.

Out of 229 collected samples from different clinical sources, only 100 *Staphylococcus aureus* (43.7%) were revealed. According to phenotypic and biochemical tests, the isolates can be distributed as follow; Urine 22 (9.6%), Pus 19 (8.3 %), Sputum 12 (5.2%), CSF 4 (1.7%), Blood 32 (14%), Pericardial fluid 9 (3.9%) and Peritoneal fluid 2 (0.9%). From the 13 examined genes, most abundant gene was *fib* (27 cases) followed by *femA* (25 cases). While *tst* gene reported as the least frequently detected gene. *S. aureus* isolated from Blood infection formed the highest ratio among genes reveals in this study (194; 38.6%) followed by pus infections isolates (120; 23.9%), urine infections isolates (102; 20.3%), sputum isolates (32; 6.4%), Pericardial fluid (28; 5.6%), CSF (19; 3.8%) and finally Peritoneal fluid (8; 1.6%).

Key words: *Staphylococcus aureus*; PCR; virulence genes; *femA*; phenotypic, MGEs.

الخلاصة

تعتبر بكتريا المكورات العنقودية المسبب الرئيسي لكثير من الامراض التي تصيب الانسان بسبب عوامل الضراوة المفردة والعوامل الاخرى المرتبطة بسطح الخلية والتي تنظم بمختلف الجينات. اجريت هذه الدراسة للتعرف على جنس المكورات العنقودية في مدينة الحلة باستخدام جينات خاصة لهذا الغرض.

استخدمت تقنية PCR للتعرف على جينات الضراوة الموجودة في بكتريا المكورات العنقودية مثل *sea*, *see*, *eta*, *tst*, *coa*, *nuc*, *seb*, *femA*, *mecA*, *etb*, *cna*, *fib* and *fnbA*, وتحديد استخدام تقنية multiplex PCRs او uniplex اعتمادا على حجم الناتج من PCR. تم جمع 229 عينة من مصادر سريرية مختلفة, وقد وجد ان 100 عينة كانت تعود الى جنس المكورات العنقودية (43,7%). وطبقا للاختبارات المظهرية والبايو كيميائية كان توزيع العزلات كالآتي: Urine 22 (9.6%), Pus 19 (8.3 %), Sputum 12 (5.2%), CSF 4 (1.7%), Blood 32 (14%), Pericardial . fluid 9 (3.9%) and Peritoneal fluid 2 (0.9%). *fib* (27 حالة) هي الأكثر تواجد ثم الجين *femA* (25 حالة). بينما الجين *tst* سجل الاقل تكرارا.

وقد شكلت بكتريا *S. aureus* المعزولة من اصابات الدم الاعلى نسبة ١٩٤ (٣٨.٦%) ثم عزلات اصابات القيح ١٢٠ (٢٣.٩%) عزلات الاصابات البولية ١٠٢ (٢٠.٣%) عزلات sputum ٣٢ (٦.٤%) Pericardial fluid ٢٨ (٥.٦%) CSF ١٩ (٣.٨%) واخيرا Peritoneal fluid ٨ (١.٦%).

Introduction

Staphylococcus aureus was documented as essential for causing disease among hospitals and the community for human in addition it consider for a comprehensive variety of diseases, including wound, bacteremia, abscesses infections [1]. Adherence of *S. aureus* to extracellular environment thought to be vital for colonization. Many of these toxins are absorbed systemically producing indications in persons who are deficient in defensive antitoxin antibody [2]. There is a wide ecological deviation in the circulating types of enterotoxigenic strains; the common serotypes are SEA, SEB, SEC, and SED [3, 4]. It is well acknowledged that bacterial components and products, including the capsule, surface-associated adhesins, secreted proteins and exotoxins, play a role in the process such as coagulase, hemolysins (encoded by *hl* genes), exfoliative toxin (ET, *et* genes) [5], toxin of toxic shock syndrome 1 (TSST-1, *tst* gene), bicomponent leukotoxins (LukS–LukF, encoded by *luk* genes), enterotoxin-like toxins (SEIs, *sel* genes) and enterotoxins (SEs, *se* genes) [6, 7].

Other virulence elements complicated the pathogenicity of *S. aureus* was revealed by previous researches [10, 11] like Hyaluronidase and Lipases enzyme [12, 13], which intricate in incursion of cells and tissue. Many of these virulence-encoding genes are irregularly represented in *S. aureus* isolates and most are carried by mobile genetic elements (MGEs), which including prophage, pathogenicity islands and plasmids [14, 15]. *S. aureus* was recurrently found and environmentally hard to eradicate. Also it considered of main causative agent of food poisoning by their enterotoxins [16].

Specific strain of *S. aureus* was unusual for produce more than one category of a toxin. In other words, the gene of some toxin might be located in the chromosomal DNA, while others may located in mobile and transferable extra chromosomal DNA which is able to translocate between bacteria by horizontal gene transfer (HGT) (Cunha et al., 2008). Genes for SEA (*sea*) are harbored by a bacteriophage vector, SED (*sed*) are carried by a plasmid (pIB485) and genes for SEB (*seb*) and SEC (*sec*) are located on the chromosomes [17].

Present study was aimed to characterize of *S. aureus* isolates gathered from variant clinical cases. The ability of the bacterial isolates to revealed their virulence factors belong to staphylococcal. Furthermore, to estimate the association between these clinical samples of isolates with their capability to produce virulence factors.

Materials and Methods

Bacterial Isolates

In total, stock cultures of 100 different clinical *S. aureus* were collected from the Medical Microbiology Laboratory at the Babylon maternity and children hospital and Al-Hillah educational Hospital, Iraq. All the isolates were confirmed cultural methods and using vitek 2 compact for microbial detection (BioMérieux, France). The source of the isolates was from different systemic infection sites of clinically ill patients of mentioned Hospital. All these strains were analyzed for virulence gene content and for the correlation of certain genes or gene combinations with known mobile genetic elements (MGEs) as well as chromosomal genes.

All isolates involved in bloodstream infections and isolates collected from patients Urine, Pus, Sputum, CSF, Blood,

Pericardial fluid, peritoneal fluid were included in the study.

Collection and identification of clinical samples: Samples from the hospitals were gained with sterile media swabs that were subsequently placed into tubes containing Trypticase Soy Broth (TSB) with 6.5% NaCl, incubated for 24 hours in an incubator then cultivated on Petri dishes containing mannitol salt agar (MSA) (Himedia, India) [18].

After incubation at 37°C for 24-48 hours, Gram staining [19] was performed on suspected *S. aureus* colonies, and those identified as clustered Gram-positive cocci were subjected to the coagulase test [20]. After identification, samples were stored in TSB medium containing 20% glycerol at -20° C.

Slime Assay

Slime-producing clones of *S. aureus* were recognized using Congo red agar subsequent the technique as defined previously [21]. The isolates were incubated at 37°C for 24-48 hr. with aerobic conditions. The colonies shaped over Congo red agar medium were characterized as strong black, black, and fragile black. Normal slime-producing strains can be recognized with black colonies whereas other colonies (red colonies) were categorized as non-slime-producing strains [22].

Phenotypic characterization of *Staphylococcus* spp,

S. aureus were identified by conventional cultural methods. All of the 100 strains cultivated on mannitol salt agar were Gram positive cocci, arranged in clusters like grape, no spore forming catalase positive and non-motile. A total number of 100 *S.*

aureus strains obtained from urine (22), pus (19), sputum (12) and CSF (4) Blood (32), pericardial fluid (9) and peritoneal fluid (2) were randomly chosen from the available 229 isolates from various locations. They were tested using uniplex and multiplex PCR for amplification of *S. aureus* species-specific PCRs for detection of *S. aureus* genes like *sea*, *see*, *eta*, *tst*, *coa*, *nuc*, *seb*, *femA*, *mecA*, *etb*, *cna*, *fib* and *fnbA* genes to investigate a possible relationship between these groups and the existence of toxin genes in *S. aureus* isolates.

Polymerase Chain Reaction (PCR)

Using simplex and multiplex PCR was aimed to conclude the distribution of *S. aureus* and biofilm genes in 100 genotypically different clones of *S. aureus* isolates. Chromosomal nucleic acid was purified from *S. aureus* with a PureLink® Genomic DNA Kits for purification of genomic DNA kit (Invitrogen) according to the manufacturer instructions. No reference strains were available for the *S. aureus* genes (*sea*, *see*, *eta*, *tst*, *coa*, *nuc*, *seb*, *femA*, *mecA*, *etb*, *cna*, *fib* and *fnbA*), all primers used in this study was summarized in table1. A multiplex PCR was performed in a final volume of 20 µl containing 100 ng of DNA template, 1X of buffer, 0.3 µm of each primers, 200 µm of dNTP mix, 1.5 mM of MgCl₂ and 1U of Taq DNA polymerase (Pioneer, Korea). The PCR products were resolved on a 1.5 % (w/v) agarose gel stained with ethidium bromide; DNA bands were visualized and photographed under an ultraviolet transilluminator.

Detection of toxin genes

Program for PCR involved in this research was as followed:

Step	Temp. and time	No. of cycles
Initial denaturation temperature	94°C for 4 min	1 cycle
Denaturation	94°C for 3 min	40 cycles
Annealing	60°C for 1.30 min	
Extension	72°C for 2 min	
Final extension	72°C for 10 min	1 cycle
Cooling	4°C	∞

All PCR products were stored in the cycler at 4°C until they were collected. DNA polymerase using Viriti (APPLIED BIOSYSTEM). Aliquots of all amplified PCR products (5 µl), along with a 100-bp molecular weight DNA ladder (Promega, USA and Pioneer, S. Korea) were

subsequently separated by electrophoresis on 1.5% molecular biology grade agarose gel (Promega, USA) stained with 0.8 µg/ml ethidium bromide (Sigma, USA) on a mini slab horizontal electrophoresis unit (Bio-Rad, USA) at 100 V for 30 min.

Table 1: Oligonucleotide primers used in this study

Primer	Oligonucleotide sequence, 5' to 3'	(bp)
tst	GTSSTR-1 ACCCCTGTTCCCTTATCATC GTSSTR-2 TTTTCAGTATTTGTAACGCC	326
femA	Fem-A1 AGACAAATAGGAGTAATGAT Fem-A2 AAATCTAACACTGAGTGATA	509
fimB	5-CATAAATTGGGAGCAGCATCA-3 5- ATCAGCAGCTGAATTCCCATT-3	128
sea	GSEAR-1 GGTTATCAATGTGCGGGTGG GSEAR-2 CGGCACTTTTTTCTCTTCGG	102
fib	5-CTACAACTACAATTGCGTCAACAG-3 5-GCTCTTGTAAGACCATTTTCTTCAC-3	405
see	SEE1 5'CAG TAC CTA TAG ATA AAG TTA AAA CAA GC SEE2 5' TAA CTT ACC GTG GAC CCT TCA G 3'	178
coa	coa 1: CCAGACCAAGATTCAATAAQ coa2: AAAGAAAACCACTCACATCGT	700
eta	GETAR-1 GCAGGTGTTGATTTAGCATT GETAR-2 AGATGTCCCTATTTTGTCTG	93
cna	5-AAAGCGTTGCCTAGTGGAGA-3 5-AGTGCCTTCCCAAACCTTTT-3	192
nuc	NUC1 5' CTG GCA TAT GTA TGG CAA TTG 3' NUC2 5' AAT GCA CTT GCT TCA GGA CC 3'	397
etb	GETBR-1 ACAAGCAAAAGAATACAGCG GETBR-2 GTTTTTGGCTGCTTCTCTTG	582
mecA	Mec-A1 AAAATCGATGGTAAAGGTTGGC Mec-A2 AGTTCTGCAGTACCGGATTTC	533
seb	SEB-1 tcgcatcaaactgacaaacg SEB-2 gcaggtactctataagtgc	478

Results and Discussion

Out of 229 collected samples from different clinical sources, only 100 *Staphylococcus aureus* (43.7%) were revealed. Prevalence of *S. aureus* in samples collected from various human medical sources were revealed in figure 1. According to phenotypic and biochemical tests, the isolates can be distributed as follows; Urine 22 (9.6%), Pus 19 (8.3 %), Sputum 12 (5.2%), CSF 4 (1.7%),

Blood 32 (14%), Pericardial fluid 9 (3.9%) and Peritoneal fluid 2 (0.9%).

Many genes like *sea*, *see*, *eta*, *tst*, *coa*, *nuc*, *seb*, *femA*, *mecA*, *etb*, *cna*, *fib* and *fnbA* genes was determined by either multiplex or uniplex PCRs depending on the sizes of the PCR products amplicon. All the results of PCR are shown in figures 3, 4, 5, 6, 7, 8 and 9.

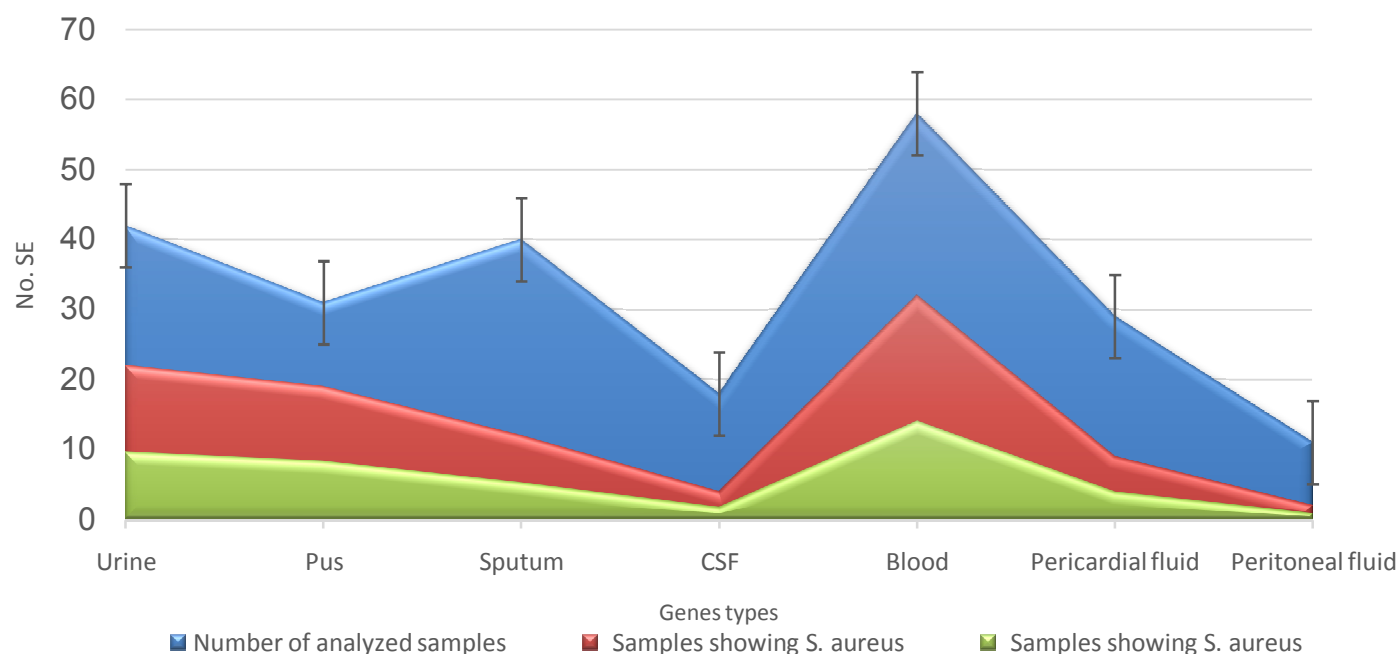


Figure (1) Distribution of *S. aureus* in different samples collected from various human medical sources in Hillah province

From the 13 examined genes, most abundant gene was *fib* (27 cases) followed by *femA* (25 cases) as revealed in figure 2. While *tst* gene reported as the least frequently detected gene. *S. aureus* isolated from Blood infection formed the highest ratio among genes reveals in this study (194; 38.6%) followed by pus infections isolates (120; 23.9%), urine infections isolates (102; 20.3%), sputum isolates (32; 6.4%), Pericardial fluid (28; 5.6%), CSF (19; 3.8%) and finally Peritoneal fluid (8; 1.6%).

Most of *S. aureus* strains have the ability to yield one or more toxins including *sea*, *see*, *eta* genes and these toxins characterize as highest source of food poisoning staphylococcal. 95% of these outbreaks were due to classical toxins such as *sea*, *see* and *eta* [23].

Furthermore, *S. aureus* isolates from men medical sources is delightful with other researchers reported in worldwide [24]. Maximum isolates of *S. aureus* were gained

from blood (194; 38.6%), followed by pus (120; 23.9%), urine (102; 20.3%), sputum isolates (32; 6.4%), Pericardial fluid (28; 5.6%), CSF (19; 3.8%) and finally Peritoneal fluid (8; 1.6%). These results as revealed in both figure 1 and 2 were partially conformity with those reported in Nigeria [25]. As previously mentioned, CSF, peritoneal and pericardial fluid samples compared to other clinical samples considered as lowest incidence and that's agreed with [26, 27].

Our results revealed that many genes were identified more frequently in MRSA isolates and these outcomes numbers were agreed with the earlier researches which indicated that *sea*, *see*, *eta*, *tst*, *coa*, *nuc*, *seb*, *femA*, *mecA*, *etb*, *cna*, *fib* and *fnbA* genes were detected more frequently in MRSA isolates [28, 29 and 30]. *mecA* Genetic factor can be repression of by *mecI* and *mecR1*, which considered as its co-repressors. *mecA* gene stimulation follows a indicating passageway instigated by the collaboration of β -lactams and *mecR1* [30].

The set of *nuc* genes standard most of *S. aureus* recognized with predictable techniques and these results confirm those acquired by other procedural methods [31]. Products of these genes with PCR explored in our research revealed classical polymorphism of the gene and it will allowed characterization genetic of these isolates [32].

The deviation in frequency of these genes among many other researches could be explained according to diverse factors like geographical variance, which may be further affected by ecological origins of *S.*

aureus strains, type of disease and clinical samples (urine, pus, sputum CSF, Blood, pericardial fluid and peritoneal fluid), quantity and quality of collected samples and procedure used to detect these genes and to isolation and purification the bacteria.

In conclusion, the incidence of coding genes which cause infection which recorded in the present study specify that the control of these genes appears to be a characteristic feature of *S. aureus*. Furthermore, control of numerous virulence genes seems to be more communal than it's believed.

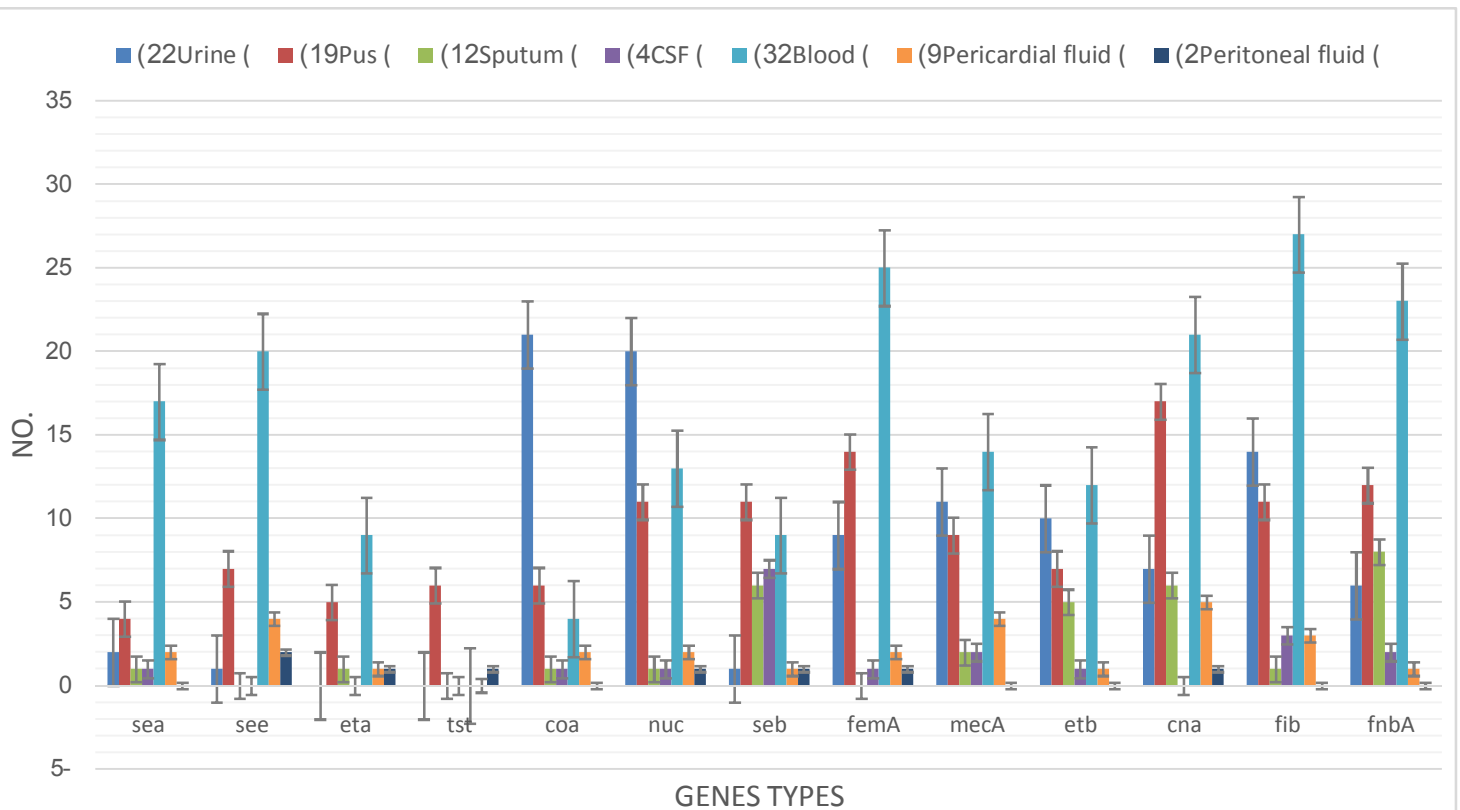


Figure (2) Virulence genes distribution among *S. aureus* isolates gathered from different medical samples.

Figures from A – I are Ethidium bromide-stained Agarose Gel Electrophoresis of PCR-amplified products from extracted (1.5%) patterns showing typical PCR amplification products in uniplex PCR for *finb*, *mecA*, *sea*, *nuc*, *cna* and *see* genes and in multiplex PCRs for A, B and g including *coa*, *eta*, *fema*, *seb*, *etb* and *tst* genes. Lane 1, was DNA ladder (bioneer)

Figure a: line s.1-5 was positive to *coa* gene (700 bp.), while for *eta* gene (93 bp) the samples from s.2-6 was positive.

Figure b: line s.1-6 was positive to *fimb* gene (128 bp.).

Figure c: line s.1-5 was positive to *cna* gene (192 bp.).

Figure d: line s.1-7 was positive to *fima* gene (509 bp.), while only 3 samples s.5-7 was positive for *seb* gene (478 bp).

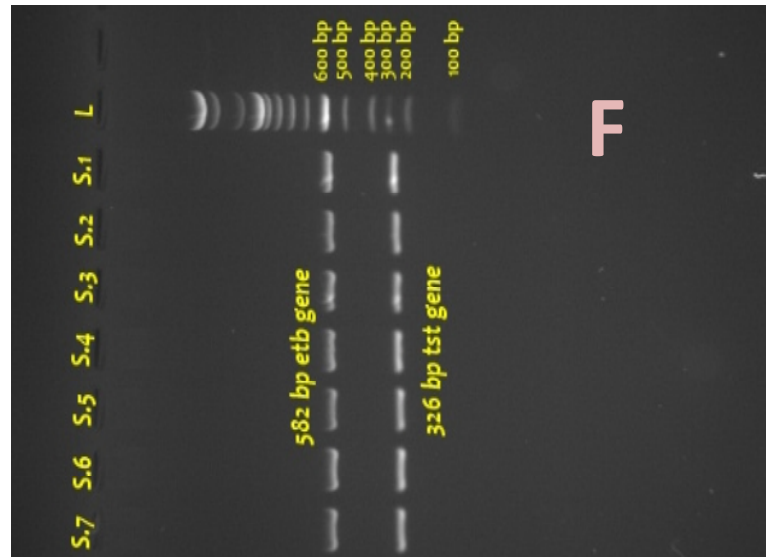
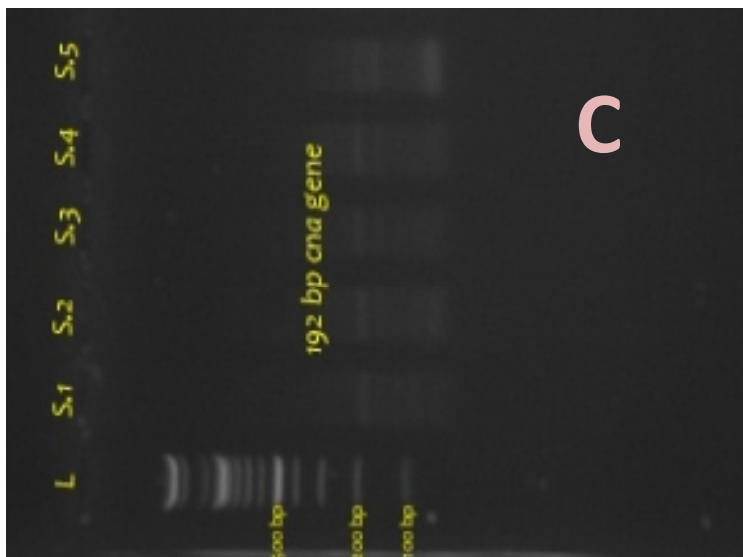
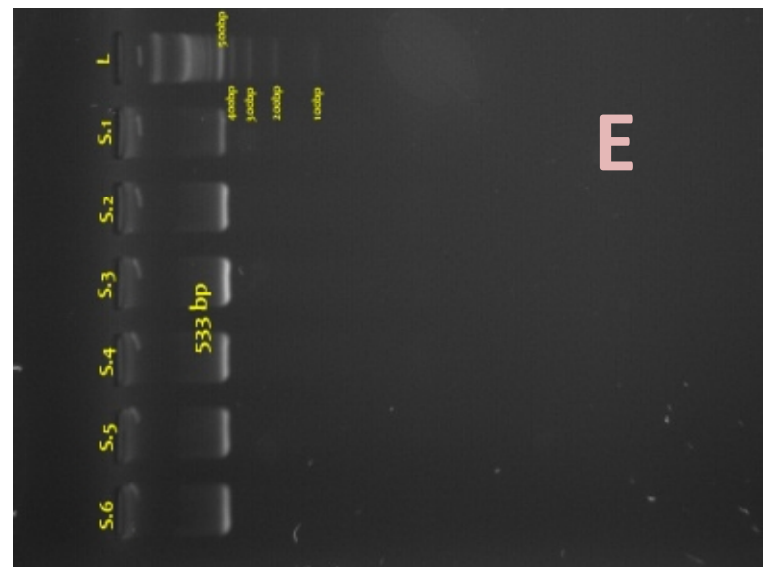
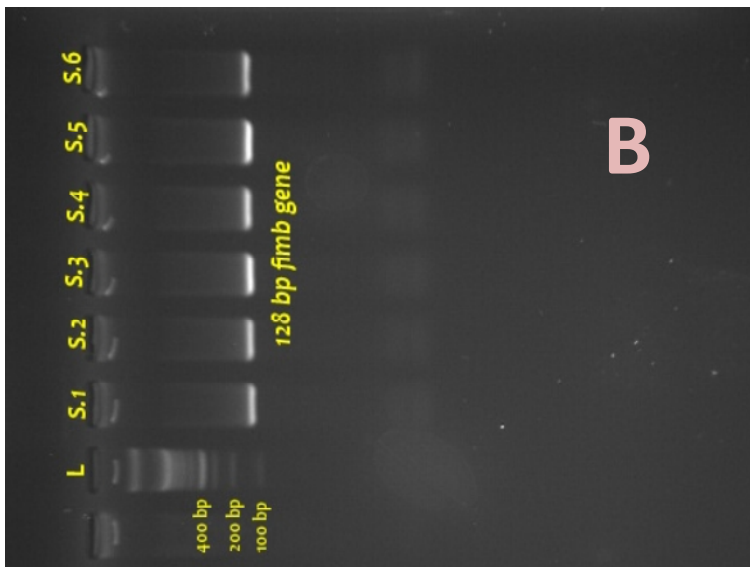
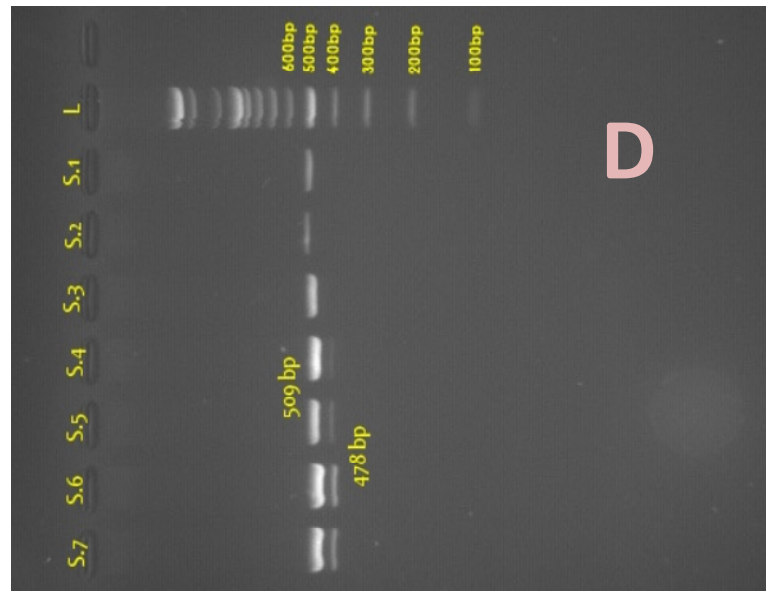
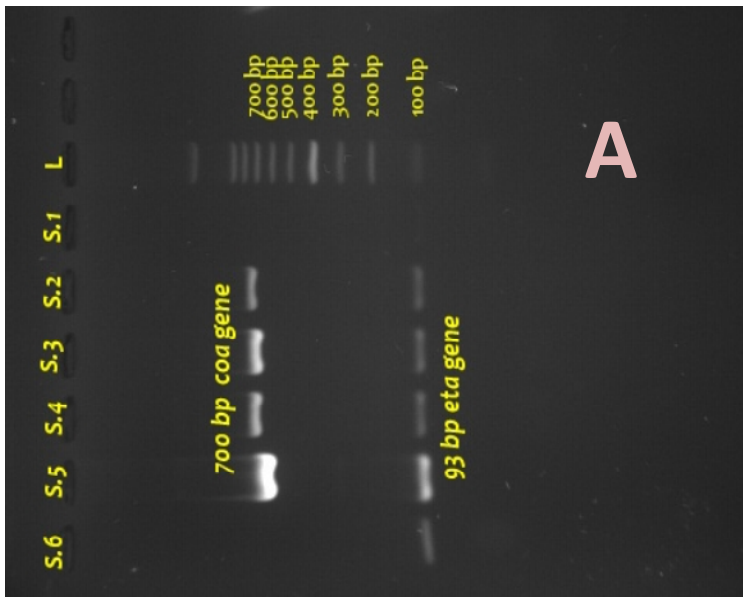
Figure e: line s.1-6 was positive to *meca* gene (533 bp.).

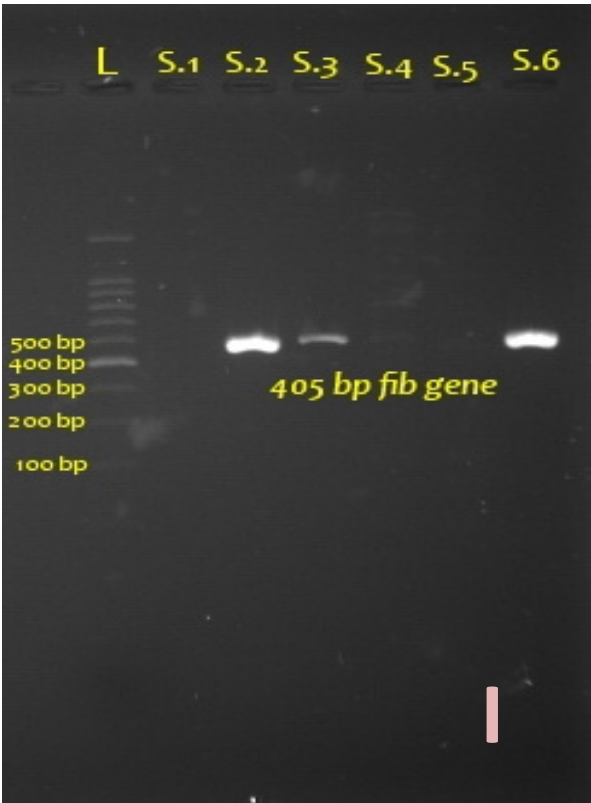
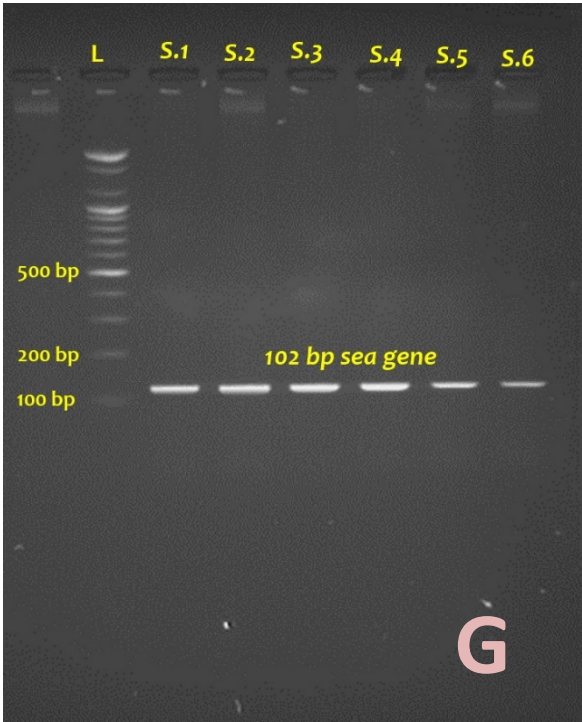
Figure f: line s.1-6 was positive to both *etb* gene (582 bp.) And *tst* gene (326 bp)

Figure g: line s.1-6 was positive to *sea* gene (102 bp.).

Figure h: line s.3-4 was positive to *nuc* gene (397 bp.), while s.5-8 was positive for *see* gene (178 bp)

Figure i: line s.2, 3 and 6 was positive to *fib* gene (405 bp.).





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