In Silico and *in Vitro* Evaluation of Real Time PCR Assay for Detection of *Staphylococcus aureus*

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Abstract

Many research groups developed real time PCR assays for identification of Methicillin-resistant *Staphylococcus aureus* (MRSA). They designed different primers and probes in their assays. Gene *mec*A is the target identification of MRSA by PCR assay. The aim of this study is using *in silico* approach to identify the best primers and probe for real time PCR identification *mec*A gene. Published primers and probes were analyzed *in silico* to select the best for real time PCR identification of *mec*A gene. The selected primers and probe successfully used for real time amplification of twenty MRSA tested. This study reveals the importance of *in silico* approach for designing diagnostic assays shorten the cost and the time. [DOI: 10.22401/JNUS.20.1.18]

Keywords: Methicillin-resistant *Staphylococcus aureus* (MRSA), real time PCR, *in silico.*

Introduction

Methicillin-resistance *staphylococcus aureus* (MRSA) is a serious threat capable of causing different diseases worldwide [1]. Traditional detection of *staphylococcus aureus* isolates performed by using bacteriological and biochemical methods. While identification of methicillin resistance done by disk diffusion assay [2]. Modern methods used polymerase chain reaction (PCR) techniques for rapid identification of MRSA. Many genes used for specific detection *staphylococcus aureus* such as *fem*A [3] and *nuc* [4] genes. The *mec*A gene used for detection of methicillin resistance [3]. Many researchers designed primers and probes for detection MRSA by real time PCR [5,6,7]. *In silico* approach was used in recent study to select the best primers and probe [8]. In this study *in silico* approach used to select the best primers and probe for detection *mec*A gene in local MRSA isolates using real time PCR assay.

Materials and Methods

Bacterial isolates

Total of 20 clinical isolates primary were identified as Staphylococcus species obtained from hospitalized patients at Child Central Hospital in Baghdad city. The isolates were primary cultivated on Mannitol agar medium then incubated at 37°C for 18-24h in aerobic condition.

DNA Extraction

Boiling method was used for bacterial DNA extraction as described by [9] with some modifications. Extracted DNA subjected to 0.8% agarose gel electrophoresis then stained with Ethidium bromide. Purity and concentration determined by Nanodrop Spectrophotometer [7].

Identification of MRSA by conventional Duplex PCR

Two genes were selected to be amplified together in a duplex PCR technique (*mec*A and *fem*A). PCR was performed to amplify *fem*A gene as a specific genomic marker for *S. aureus.* Forward primer was (5'-CATGATGGCGAGATTACAGGT-3') and Reverse primer was (5'-GTCATCACGACCAGCGAAAGC-3') [8]. The length of PCR product is 314bp. PCR amplification was performed for 35 cycles with initial denaturation (95ºC/5min), denaturation (94ºC/ 1min), annealing (60ºC/ 30sec), extension (72 ºC/ 45sec) and final extension (72ºC/ 5min) using Accupower master mix kit (Bioneer/ Korea).

The gene *mec*A was amplified because it encodes methicillin resistance. Forward primer was (5'-AAAATCGATGGTAAAGGTTGGC-3') and Reverse primer was (5'-AGTTCTGCAGTACCGGATTTGC-3') (8). The length of PCR product is 533bp. PCR

amplification was performed for 35 cycles with initial denaturation (95ºC/5min), denaturation (94ºC/ 1min), annealing (60ºC/ 30sec), extension (72ºC/ 45sec) and final extension (72ºC/ 5min) using Accupower master mix kit (Bioneer/ Korea).

In-silico real time PCR identification of MRSA

Twenty five primers and probes published by different researches online collected and analyzed by the in silico PCR online software (http://insilico.ehu.es/PCR/) to select the primers that *in silico* amplify most *Staphylococcus aureus* strains. The selected primers and collected probes analyzed by oligoanalyzer 3.1. https://eu.idtdna.com/analyzer/Applications/OligoAnalyzer/ to select the best forward and reverse prime and probe for Taqman real time PCR assay detection of *mec*A gene. The best *in silico* primes and probe analyzed finally by Blast (http://blast.ncbi.nlm.nih.gov) to identify the specificity for *Staphylococcus aureus.*

Real time PCR for *mec*A gene

The selected forward primer was (5'- GAATGCAGAAAGACCAAAGCA-3') and reverse primer was (5'- TTTGGAACGATGCCTATCTCA-3') and probe was (FAM-5'-ACCGAAACAATGTGGAATTGGCCA-3'-BHQ). The PCR product was 124bp. Real time PCR conditions were initial denaturation (95ºC/3min), denaturation (94ºC/15sec), annealing (60ºC/30sec), extension (72ºC/ 45sec) and final extension (72ºC/ 5min) for cycles using Accupower master mix kit (Bioneer/ Korea).

Results and Discussion

The identity of Staphylococcus isolates were verified as *S. aureus* by cultivation on mannitol salt agar which considered selective and differential medium for the genus *Staphylococcus* [10]. The isolates had the ability to ferment mannitol sugar and form large, round, smooth, raised, mucoid and glistening. More over only *S. aureus* forms large golden colonies surrounded by wide yellow zones due to fermenting the mannitol and producing acid which turned the color of the medium from pink to Fig.(1). Antibiotic disk assay was used to confirm the methicillin sensitivity of isolates Fig.(2).

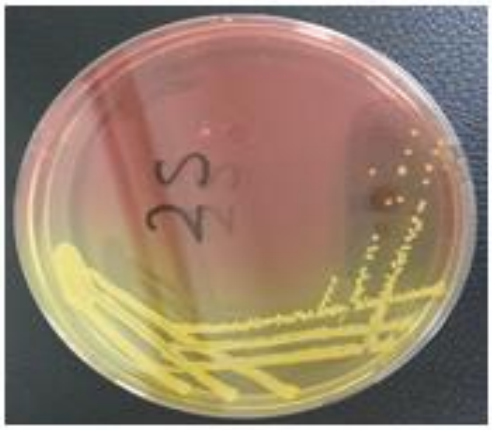


Fig.(1): Staphylococcus aureus colonies cultures on Mannitol.

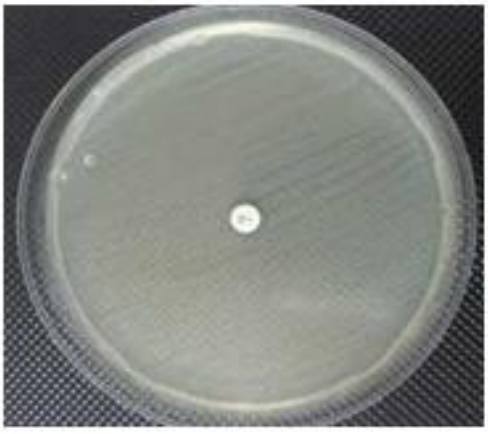


Fig.(2) Methicillin (5mg) resistance of S. aureus on Muller-Hinton agar.

To confirm the identity of MRSA isolates, DNA extracted from bacterial isolates Fig (3), and then Duplex PCR was used for amplification *mecA and femA* genes [3]. The gene *mec*A did not amplified in methicillin sensitive *S. aureus* isolates Fig (4).

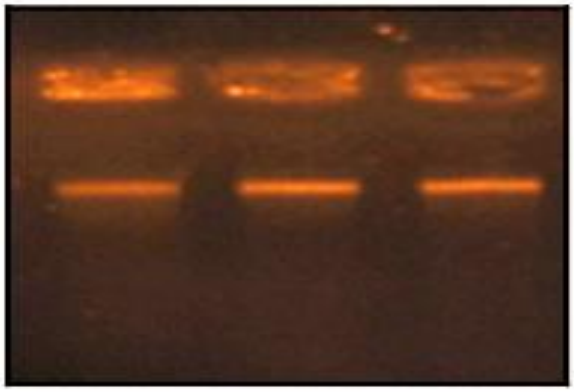


Fig.(3): Agarose gel electrophoresis 0.8% of DNA extracted from Staphylococcal isolates stained with ethidium bromide.

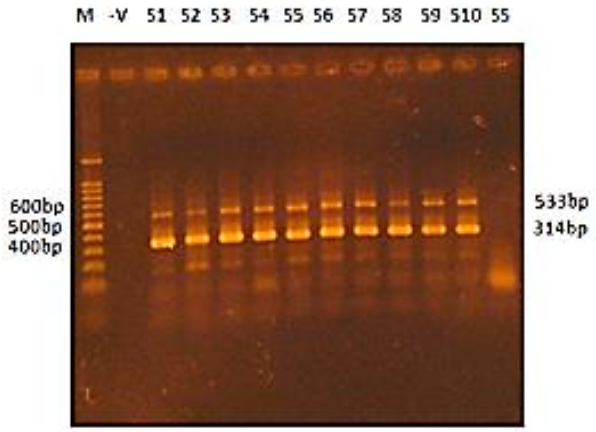


Fig.(4) Ethedium bromide stained agarose gel electrophoresis 1.5% of amplified PCR products of FemA (314bp) and mecA (533bp) genes of S. aureus isolates. M: 100bp marker, -v: negative control, S3-S12:samples, SS: sensitive isolate.

Many research groups published their primers and probes for detection of *mec*A efficiently in real time PCR assay. Twenty five published primers sequences were collected and analyzed by *in silico* PCR online software Table (1).

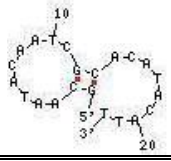
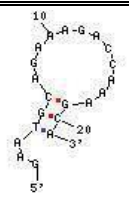
Table (1)
In silico PCR amplification results for primers verification.

	Staphylococci species and strains (Accession number)	Amplification results
		mecA
1	*Staphylococcus aureus* RF122 (AJ938182.1)	-
2	*Staphylococcus aureus* strain Mu50 (BA000017.4)	+
3	*Staphylococcus aureus* subsp. *aureus* 11819-97 (CP003194.1)	+
4	*Staphylococcus aureus* subsp. *aureus* 71193 (CP003045.1)	-
5	*Staphylococcus aureus* subsp. *aureus COL* (CP000046.1)	+
6	*Staphylococcus aureus* subsp. *aureus* ECT-R 2 (FR714927.1)	-
7	*Staphylococcus aureus* subsp. *aureus* ED133 (CP001996.1)	-
8	*Staphylococcus aureus* subsp. *aureus* ED98 (CP001781.1)	-
9	*Staphylococcus aureus* subsp. *aureus* JH1 (CP000736.1)	+
10	*Staphylococcus aureus* subsp. *aureus* JH9 (CP000703.1)	+
11	*Staphylococcus aureus* subsp. *aureus* JKD6159 (CP002114.2)	+
12	*Staphylococcus aureus* subsp. *aureus* M013 (CP003166.1)	+
13	*Staphylococcus aureus* subsp. *aureus* MRSA252 (NC002952.2)	+
14	*Staphylococcus aureus* subsp. *aureus* MSHR1132 (NC016941.1)	+
15	*Staphylococcus aureus* subsp. *aureus* MSSA476 (BX571857.1)	-
16	*Staphylococcus aureus* subsp. *aureus* MW2 (NC003923.1)	+
17	*Staphylococcus aureus* subsp. *aureus* Mu3 (NC009782.1)	+
18	*Staphylococcus aureus* subsp. *aureus* N315 (NC002745.2)	+
19	*Staphylococcus aureus* subsp. *aureus* NCTC 8325 (JN571546.1)	-
20	*Staphylococcus aureus* subsp. *aureus* T0131 (CP002643.1)	+
21	*Staphylococcus aureus* subsp. *aureus* TCH60 (NC017342.1)	+
22	*Staphylococcus aureus* subsp. *aureus* USA300_FPR3757 (CP000255.1)	+
23	*Staphylococcus aureus* subsp. *aureus* USA300_TCH1516 (CP000730.1)	+
24	*Staphylococcus aureus* subsp. *aureus* VC40 (CP003033.1)	-
25	*Staphylococcus aureus* subsp. *aureus* str. JKD6008 (NC_017341.1)	+
26	*Staphylococcus aureus* subsp. *aureus* str. Newman (NC_009641.1)	-
27	*Staphylococcus carnosus* subsp. *carnosus* TM300 (AM295250.1)	-
28	*Staphylococcus epidermidis* ATCC 12228 (NC004461.1)	-
29	*Staphylococcus epidermidis* RP62A (NC004461.1)	-
30	*Staphylococcus haemolyticus* JCSC1435 (NC007168.1)	-
31	*Staphylococcus lugdunensis* HKU09-01 (NC007168.1)	-
32	*Staphylococcus lugdunensis* N920143 (NC017353.1)	-
33	*Staphylococcus pseudintermedius* ED99 (NC017568.1)	-
34	*Staphylococcus pseudintermedius* HKU10-03 (NC014925.1)	-
35	*Staphylococcus saprophyticus* subsp. *Saprophyticus* (AP008934.1)	-

Selection primers that *in silico* amplify most *S. aureus* isolates increase the chance that these primers will amplify genes in our local isolates.

The six primer pairs amplified in most MRSA strains were analyzed by oligoanalyzer 3.1 software to select the best primer pair Table (2).

Table (2)
The best six primers pairs analyzed by oligoanalyzer 3.1 software.

primers	Length	Melting Temp.	GC content	hairpin	Maximum G	Hybridization Temp.	Mismatch Tm
Forward	22	51.8 ºC	36.4 %		-38.91 kcal/mole	51.8 ºC	51.8ºC
Reverse	20	55.4 ºC	55.0 %		-38.23 kcal/mole	55.4 ºC	55.4ºC
Forward	22	54.5 ºC	40.9 %	7 structures	-38.54 kcal/mole G -46.2 kcal/mole	54.5 ºC	54.5ºC
Reverse	26	53.9 ºC	30.8 %	5 structures	-46.2 kcal/mole	53.9ºC	53.9ºC
Forward	21	53.9 ºC	42.9 %		-39.22 kcal/mole -39.33 kcal/mole	53.8 ºC	53.9ºC
Reverse	21	53.8 ºC	42.9 %	3 structure	-39.33 kcal/mole	53.8ºC	53.8 ºC
Forward	20	52.3 ºC	45.0 %	One structure	-37.27 kcal/mole -37.75 kcal/mole	52.3 ºC	52.3 ºC
Reverse	20	51.7 ºC	40.0 %	3 structures	-37.75 kcal/mole	51.7ºC	51.7 ºC
Forward	22	52.6 ºC	36.4 %	One structure	-41.68 kcal/mole	52.6 ºC	52.6ºC
Reverse	21	56.3 ºC	47.6 %	3 structures	-39.91 kcal/mole	56.3ºC	56.3ºC
Forward	20	56.1 ºC	50.0 %	6 structures	-39.68 kcal/mole -42.35 kcal/mole	56.1ºC	56.1ºC
Reverse	24	54.8 ºC	37.5 %	5 structure	-42.35 kcal/mole	54.8ºC	54.8 ºC

Probes are the most expensive item in real time PCR assay, so *in silico* prediction of the best probe will reduce the cost for development and evaluation of these assays. Table 3 shows the six probes amplified in most MRSA strains were analyzed by oligoanalyzer 3.1 software to select the best probe. The optimum values selected for parameters testes is according to the guidelines of probe design [10].

Table (3)
The best six probes analyzed by oligoanalyzer 3.1 software.

probe	length	MeltTemp	GC content	hairpin	Maximum ΔG	Hybridization Temp	Probe sequence
1	35	62.7ºC	45.7%	One structure	G -68.25 kcal/mole -68.25 kcal/mole	62.7ºC	GATGGCAAAGATATTC AACTAACTA
2	28	58.7ºC	42.9%	One structure	-48.96 kcal/mole	58.7ºC	CC AGA TTA CAA CTT CAC CAG GTT CAA CT
3	24	59.8ºC	45.8%	One structure	-48.64 kcal/mole	59.8ºC	ACCGAAACAATGTGGA ATTGGCCA
4	21	57.1ºC	47.6%	One structure	-40.98 kcal/mole	57.1ºC	TTGGCCAATACAGGAA CAGCA
5	29	59.2ºC	41.4%	One structure	-52.78 kcal/mole	59.2ºC	TGGAAGTTAGATTGGG ATCATAGCGTCAT
6	31	61.1ºC	45.2%	One structure	-58.26 kcal/mole	61.1ºC	CCTTGTTTCATTTTGAG TTCTGCAGTACCGG

Real time PCR amplification of *in silico* selected primers and probe were analyzed by fast 7500 real time PCR Fig.(5). The gene *mec*A amplified in all MRSA isolates detected by disk diffusion method which indicate the usefulness of *in silico* analysis before real time PCR assay performance.

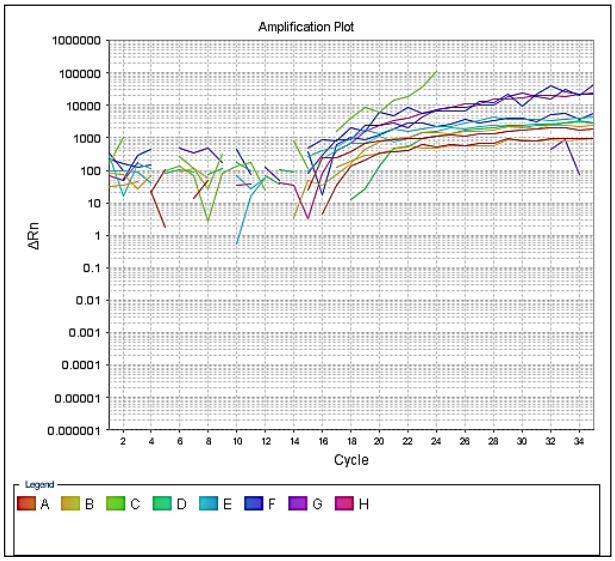


Fig.(5): Real-time amplification curves of mecA gene.

To confirm real time amplification of *mecA* gene result, PCR products were analyzed by agarose gel electrophoresis stained with ethidium bromide Fig.(6).

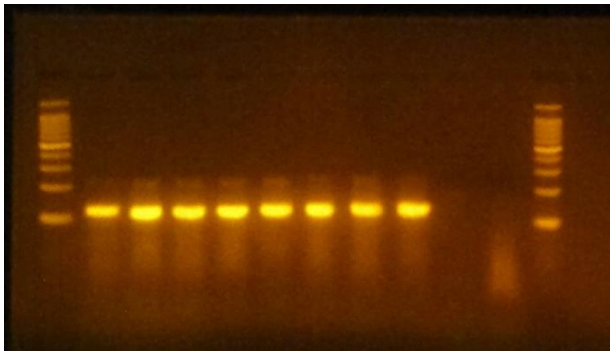


Fig.(6): Agarose gel electrophoresis (1.5%) of mecA (124bp) gene products (amplified in real time PCR).

In silico PCR approach in this work act as prescreen for custom-designed primers. This will reduce the cost for purchasing unwanted primer pairs as well as decrease the time spent on trial and error of the primer selection. The *in silico* primers and probe showed a good correlation with actual real-time PCR detection *mec*A genes towards identification of local methicillin-resistant *S. aureus* (MRSA).

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الخلاصة

قامت عدد من المجاميع البحثية بتطوير عدد تفاعل الكوثرة اللحضي للكشف عن بكتريا المكورات الذهبية المقاومة للمضاد المثسيلين. قاموا بتصميم بادئات ومجسات في عددهم. جين *mec*A يعتبر هدف الكشف لعدة تفاعل الكوثرة. تهدف الدراسة الى استخدام *in silico* لاختيار افضل بادئين ومجس لعدة تفاعل الكوثرة اللحضي للكشف عن جين *mec*A. تم تحليل البائات والمجسات المنشورة في البحوث لاختيار افضل بادئين ومجس لعدة تفاعل الكوثرة اللحضي للكشف عن جين *mec*A. البادئات والمجس التي تم اختيارها استخدمت بنجاح في عدة تضخيم البوليمر المتسلسل اللحضي التي طبقت على عزلات محلية. هذه الدراسة اشارت الى اهمية الـ *in silico* في تصميم عدد التشخيص لتقليل المال والوقت.

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