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MOLECULAR DETECTION OF GENES ENCODING CLUMPING FACTOR *(CTFA*), THERMONUCLEASE *(NUC)* AND IMMUNOGLOBULIN G BINDING REGION OF PROTEIN A (*SPA*)FROM METHICILLIN RESISTANCE *STAPHYLOCOCCUS AUREUS* ISOLATED FROM BOVINE MASTITIS.

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ABSTRACT

The *Staphylococcus aureus* responsible for intramammary infection in bovine and is the main etiological agent of clinical and subclinical mastitis in dairy herds. In this study a total of 64 *Staphylococcus aureus* strain obtained from 112 samples from mastitis cow (57.14%). *S. aureus* strain were identified phenotypically and further characterized genotypically by polymerase chain reaction PCR. Amplification of genes encoding specific species for *S. aureus(Sau),* clumping factor *(ctfA*), thermonuclease *(Nuc)* and the gene segment encoding the immunoglobulin G binding region of protein A gene *spa*. The amplification of *Sau* gene produce amplicon in a molecular size proximally 530bp in all strain, the produce amplicon in a molecular size proximally size 980 bp in *ctfA* gene (73.43%) andImmunoglobulin G binding region of *spa* gene produce amplicon in a size proximally 950 bp was observed in 43 and 3 strain amplicon in a size proximally 390 bp (71.87%). The thermonuclease gene the amplicon in a size proximally 279 bp with (90.62%). After that methicillin resistance (MRSA) were detected in a percentage (21.87%), all of these strain of MRSA contain all virulence genes.

INTRODUCTION

Mastitis is one of the major causes of economic losses in dairy industry worldwide, microorganisms involved the major organism is *S. aureus*(1).*S. aureus*is the main etiological agent of clinical and subclinical mastitis in dairy herds (2).

S. aureus is a ubiquitous Gram-positive microorganism commonly isolated from raw milk of dairy cattle suffering from mastitis. So its presence in raw milk is a major concern for the safety and the quality of traditionally dairy products (3).

Bovine mastitis, a multi-factorial disease, is characterized by physical, chemical and microbiological changes in the milk and pathological changes in the glandular tissue of udder (4). In the control of mastitis, the improper use of antimicrobial agents on dairy farm animals is a major concern as it lead to the emergence of resistant zoonotic bacterial pathogens (5). The intra mammary administration of antibiotics used on farms has increased, as it was proved to be effective for treating subclinical mastitis in dry small ruminants (6).

The main reservoir of *S. aureus* seems to be the infected quarter and transmission between cows usually occurs during milking (7).

S. aureus causing wide spectrum of diseases in both human and animal. *S. aureus* expresses many potential virulence associated factors like surface proteins that promote colonization of host tissue surface factors that inhibit phagocytic engulfment (capsule protein A) biochemical properties that enhance their survival in phagocyte (catalase production) immunological disguises (protein A, clottin factor) inherent and acquired resistance to antimicrobial agent and membrane damaging toxins like hemolysis that lyse eukaryotic cell membranes (8). One of the major surface proteins is staphylococcoal protein A *(spa)* which bacterial cell wall product that bind immunoglobulin G and impair opsonisation by serum complement and phagocytosis by polymorphnuclear leukocytes (9) Protein A is a surface protein of *S. aureus* which binds IgG molecules by their Fc region in serum the bacteria will bind IgG molecules in the wrong orientation on their surface which disrupts opsonization and phagocytosis (10). The decrease of protein A on the cell on surface of *S. aureus* resulted in greater number of free receptor sites for complement C3b and in increase in phagocytosis (9).

Clumping factor A *(ctfA)*is the surface antigen which acts as adhesions factor, and considered to be one of the most important adhesion factors of *S. aureus* to host cells, (*ctfA)* is known to participate in the infection process by binding via soluble or immobilized fibrinogen (11). This factor has been shown to inhibit phagocytosis in the absence of fibrinogen and the inhibition was enhance in the presence of fibrinogen (12).

Methicillin-resistant *S. aureus* (MRSA) includes *S.aureus* that have acquired a gene, called *mec*A, giving them resistance to methicillin and essentially to all other beta-lactam antibiotics. MRSA was first reported as a nosocomial pathogen in 1961, soon after methicillin was introduced into human medicine to treat penicillin-resistant staphylococci (13). MRSA clones have particularly been detected in animal populations (14, 15). Although MRSA is associated with the acquired*mec*A gene, the role of inappropriate antibiotics use should also not be under estimated in formation of bacterial resistance and multidrug resistant strains (16). MRSA infection from mastitis cases is partly related with failure in dosage therapy and choice of antimicrobial substance (14, 17).

The aim of this study detection *S. aureus* strain phenotypically and genotypically from clinical and sub clinical mastitis and study virulence profile with determination Methicillin resistant *S. aureus* MRAS.

MATERIALS AND METHODS

Samples collection

One hundred twelve (112) samples were obtained from diseases pathogenic and clinically apparent healthy non-pathogenic cases of bovine. The isolation of staphylococcus was attempted depend on clinical sign and decrease milk production that tested by California mastitis test (CMT) for subclinical mastitis and were graded as negative, trace, weak, distinct, or strong positive (4). The specimens were transported to the laboratory directly and inoculated ontoMannitol salt agar (MSA)platesHimedia - India; and incubated at 37°C for 24 h. All colonies from primary cultures were purified by sub culturing onto MSA medium and incubated at 37°C for 24 to 48 h (18).

Biochemical testes

Different tests were performed for identification of *S. aureus*. The tests were catalase test; oxidase test; coagulase; clumping factor test; free coagulase test; Vogas-Proskauer test; ONPG ; Latex agglutination (MASTSTAPH); heamolysin production; DNAase production test; Urease test; O\F test; gelatin test; methyl red test and sugar fermentation test. The tests were done using the methods of (19, 20). API Staph test was done for the conformity of the identification of isolates. Homogenous bacterial suspension was prepared with a turbidity equivalent to 0.5 McFarland stander (21).

DNA isolation

KAPA Express Extract KK7100 (50 rxns) Transfer DNA containing supernatant to a fresh tube,and diluted in TE buffer for long term storage at -20 C^o**(KAPA BIOSYSTEMS USA).**Genotypic characterization Kit**, KAPA Taq Ready Mix,**DNA polymerase contain *Taq* DNA polymers (0.0 5U /1.25 U per 25 ul) Reaction buffer with Mg $^{+2}$ and o.4 mM each dNTP with or without dye. **(KAPA BIOSYSTEMSUSA).A ladder (KAPA BIOSYSTEMSUSA)**, size of amplicons KAPA Universal ladder contain (100 ng/µl) 1^{x}1 ml KAPA loading dye (60x) ^{x}1.5 ml contain eighteen DAN segment.

Sau* gene to specific species *S. aureus

PCR amplification was don as follow 5 µl of DNA was added to 20 µl of **master mix** with 0.6 µmol each primers for *Sau* gene to specific species *S. aureus* described by (22). The thermal cycling parameters were an initial denaturation step (94 C$^°$ 4min) 25 cycle amplification including denaturation (94 C^o 30 s) annealing (54 C^o, 30 s) DNA chain extension at 72 C^o for 7 min were done in thermal cycler 10 µl volumes of PCR products were electrophoreses in 1.5% (w/v) agarose gel 1h ethidium bromide (0.5 µg/ml TAE) stained DNA amplicons were visualized in UV transilluminator.

Clumping factor *(ctfA*) gene

This genesencoding clumping factor *(ctfA)* initial denaturation at 94 C^o for 4 min, followed by 38 cycle of denaturation, annealing and extension (94 C^o for 60s, 58 C^o for 60s 72C^o for 60s) and final extension at 72 C for 5 min (22).

IgG binding –region of protein A *(spa)* gene

The PCR amplification was don under Initial denaturation at 94 C^o for 2 min , followed by 30 cycle of denaturation ,annealing and extension (94 C^o for 3 min 58 C^o for 30s ,72 C^o for 45s) and final extension at 72 C^o for 10 min ,The sequences of the oligonucleotide primers summarized in Table 1 .

Thermonuclease *(nuc)*gene

Reaction mixtures (25 µl) included 2 µl template DNA, 20µl of master max**,** 10 pmol of each of the 2 primers 279 bp. Amplification and primer described by (22) A total of 37 PCR cycles were run under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 0.5 min, and extension at 72°C for 1.5 min. After the final cycle, the reaction was terminated final extension keeping it at 72°C for 3.5 min. Amplified products were separated by agarose gel electrophoresis (1.7% agarose) at 5 V/cm for 3 h and photographed under UV illumination.

Table 1 primers for amplification of *S. aureus* gene (sequence (5-3) size of amplified bp.

Gene	Sequence	Size bp	Reference
Sau	F:ATAAGAGATGGCGGTACTAAA R:TAAGGCGGATTACACGTTACT	530	(Ruzickova *et al.*, 2005)
CtfA	F:GGCTTCAGTGCTTGTAGG R:TTTTCAGGGTCAATATAAGC	980	(Stepan *et al.,* 2001)
Nuc	F:CGATTGATGGTGATACGGTT R:ACGCAAGCCTTGACGAACTAAAGC	279	(Stepan *et al.,* 2001)
Spa	F:CACCTGCTGCAAATGCTGCG R:GGCTTGTTGTTGTCTTCCTC	950	(Stepan *et al.,* 2001)
mec	F:5' AAA ATC GAT GGT AAA GGT TGG C 3' R: 5' AGT TCT GCA GTA CCG GAT TTG C 3'	533	(Murakami *et al.*, 1991)

Cefoxitin sensitivity testing

This test was done according to method of Kirby and Bauer(23) disc-diffusion method, *S. aureus* isolates were tested for their sensitivity to cefoxitin (cx 30 mg). A zone of inhibition with a diameter of ≤ 21 mm was considered as an indication for resistance to methicillin.

Genotypic screening (PCR amplification for detection of *mecA* gene)

All *S. aureus* isolates were screened for *mecA* gene by PCR assy. The *mecA* gene was described by (24). Reaction mixtures include 20µl of master max**,** 20 pmol concentrations of forward and reverse primers and 5 µl of DNA template. The cycling parameters were as follows: an initial denaturation at 94°C for 5 min; followed by 40 cycles of 94°C for 30 s, 55°C for 30 s, and 70°C for 1min; the final extension was at 72°C for 5 min . PCR products were visualized on 2% agarose gel with ethidium bromide under UV transilluminator. Amplicons of 533 bp were consistent with *mecA* gene amplification.

Statistical methods:

The significance of differences in proportions was analyzed by the chi-square test. Fisher’s exact test was used when there was a cell with a number less than 5. Data were performed with SPSS version 15 and P values equal or less than 0.05 were considered statistically significant.

RESULTS

Out of 112 milk samples which collected from dairy cows *S. aureus* isolates *were* 64 (57.14%) samples were found positive depend on biochemical test that used. *S. aureus* isolates were similar in some biochemical tests like catalase, oxidase, coagulase, O/F, ONPG, MR, sugar fermentation, gelatin liquefaction, latex agglutination and API Staph. All tests were positive at 100%. The different percentage in biochemical test, hemolysis on blood agar (90.62%) VP test (93.75%) urease test (92.18%), DNase 96.87%, *S. aureus* isolates of bovine origin produced beta- haemolysis.

Table 2:- Number and percentage of sample test with biochemical test detection S aureus.

Sample	Growth on MSA		Coagulase tube		Coagulase slide		Oxidase negative		Suspected *S aureus*	
112	64	100%	64	100%	47	73%	112	100%	64	57.14%
X^2 =42.749	P= 0.0004									
Suspected *S aureus*	Heamolysis		Urease		VP test		DNase		API staph	
64	58	90.62%	59	92.18%	60	93.75%	62	96.87%	64	100%
X^2 =0.479	P= 0.976									

However, all the isolates were subjected to PCR assay used test with *sua* gene to diagnostic specific species of *S. aureus* amplification yielded of 530 bp in all isolates this gene not product polymorphism (Fig.1).

Amplification of the clumping factor *(ctfA)* gene has yeild amplicon with a size of approximately 985 bp 47 strain from 64 *S. aureus* isolates were positive to *(ctfA)* gene (Fig. 2).

Out of 64, 43 produce single band a proximally of 950 bp was developed with PCR amplification of the gene segment encoding IgG binding region of protein A*Spa* (Fig. 3) and 3 isolated in 390 bp (Fig. 4), while the amplification of extracellular thermonuclease *Nuc* gene produced an amplicon of proximally279 bp in 58 out of 64 isolates (fig.5).All the gene with number of positive showing in (Table 2). While the *mec A* was appeared in 533 bp (Fig.6) the percentage of isolated of MRAS was appeared in (21.87%), all of these strain of MRSA contain all virulence genes.

Table 2:- Number and percentage of genes detection in the *S aureus*.

No.	***Sau* gene**	***ctfA* gene**	***spa* gene**		***Nuc gene***	***Mec A***
Strains	**530 bp**	**985 bp**	**950 bp**	**390 bp**	**278 bp**	**533bp**
64	64/ 64	47 / 64	43 / 64	3/ 64	58/ 64	14/64
Percentage	100%	73.43%	67.18%	4.68%	90.62%	21.87%
	X2 = 93.783 P= 0.0002					

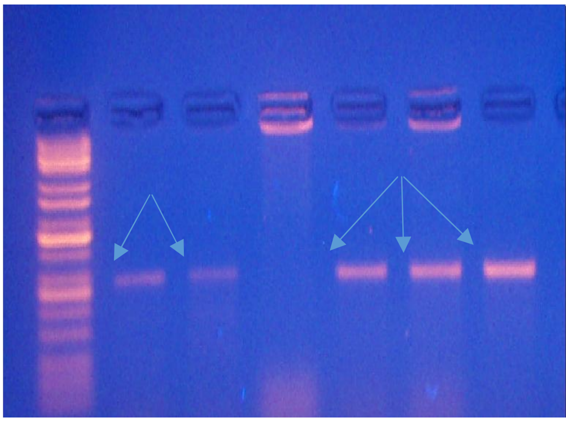

Fig.2. Gel electrophoresis (1% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for *(sua)* gene was appeared in 530 bp, L1:- 100bp DNA

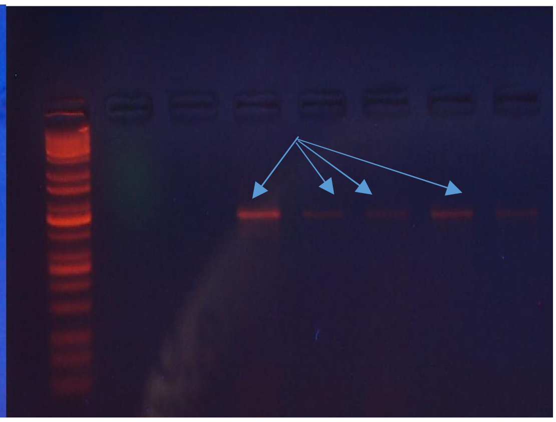

Fig.2 Gel electrophoresis (1% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for *(ctfA)* gene was appeared in 985 bp, L1:- 100bp DNA ladder.

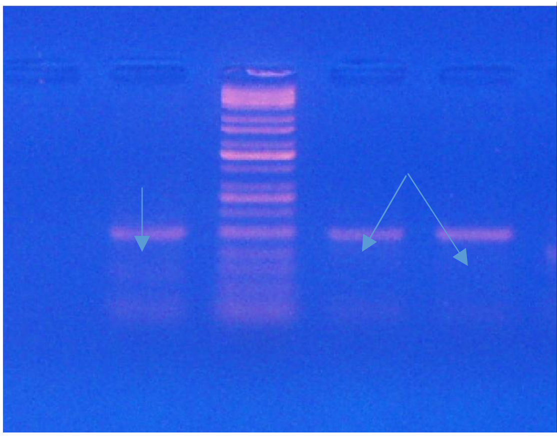

Fig.3 Gel electrophoresis (1% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for*Spa*genewas appeared in360 bp, L1:- 100bp

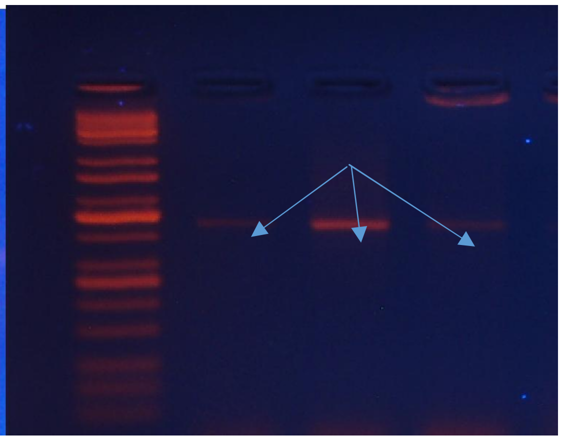

Fig.4 Gel electrophoresis (1% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for *Spa gene* was appeared in950 bp, L1:- 100bp DNA ladder.

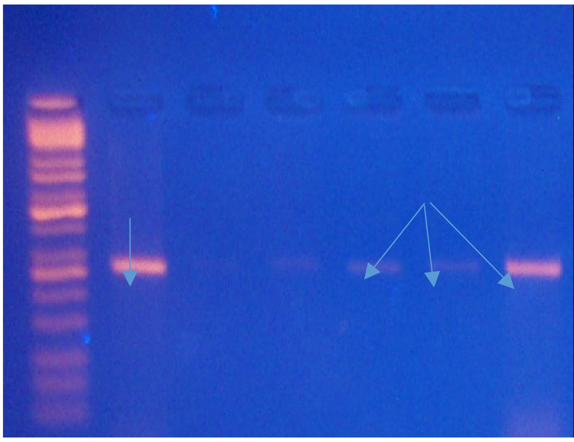

Fig.5. Gel electrophoresis (1% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for *mecA*gene was appeared in533 bp, L3:- 100bp DNA ladder

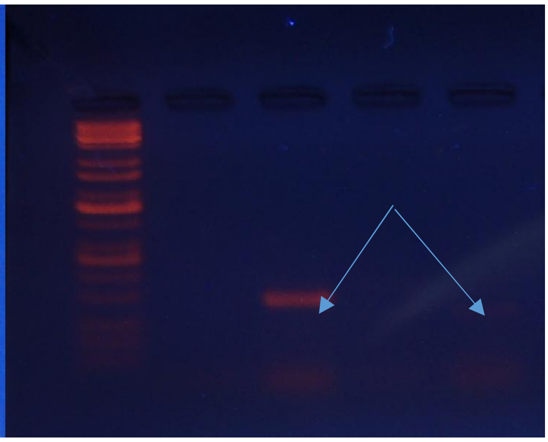

Fig.6 Gel electrophoresis (1% agarose, 7 v/cm^2, 1. hrs) of PCR positive products for *Nuc*gene was appeared in 279bp, L1:- 100bp DNA ladder.

DISCUSSION

In the present study *S. aureus* strains isolated from subclinical bovine mastitis cases were identified (phenotypically) by biochemical test and characterized by PCR amplification of several virulence genes encoding*Sau*for species of *S aureus* clumping factor *ctfA*, *Spa* gene segment encoding the immunoglobulin G-binding region and *Nuc* gene encoded stable of thermonuclease activity and *mec A* gene encoded for MRSA.

In this study combined with other study in Iraq Al –Kafaje (2008) (25) found that percentage of isolation (46.24%) in clinical and sub clinical mastitis. (26) Al – Marsomy (2008) recorded that the percentage (43.5%) while the study (27) Mustsafa (2007)found the percentage of isolation from clinical and subclinical mastitis (46.24%).

The isolation rates of *S. aureus* observed in the present study are consistent with findings in other studies(28) identified a total of 245 strains of *S. aureus,* among which (61.2%) were derived from milk. In another study (29) observed *S. aureus* in 67% of isolates from raw milk. Worldwide, several studies suggest that *S. aureus* isolation rates in milk can vary from (13.5%) to (64.7%) (30, 31).

The *Sua* gene product 533bp control amplicon derived from species-species genomic sequence was present in all *S. aureus*investigated in this study, this result combined with the study of (32) that used similar primer to code to specific *S. aureus*.

The *spa* gene 69 samples encoded to IgG binding region gene but in this study appear 43 strain product molecular weight 390 bp these genes are the most frequent in*S. aureus*. The PCR amplification of the gene encoding the IgG binding region of protein A *spa* revealed a size of 950 bp for most of the isolates investigated (9)

Spa gene produced different products ranging in size from 190bp to 320bp (33). PCR revealed that from all the coagulase positive *S. aureus* isolates (98.5%) had *spa (*spa-X) gene(34), while another study determine the *spa*. gene in(46.8%) (35).

The amplification of the clumping factor *(ctfA)* gene resulted in a single amplicon with a size of approximately 1,000 bp and eight isolated contain 950 bp (22), but in this study indicating no size polymorphism of this gene in all isolated had a size of 985 bp. Polymorphism with on information is available about the sequence variation of these strains. However (36, 37) reported amplicons at 900-950 bp in molecular weight in fewer isolated as well as amplicons at 985 bp in most of the isolated they examined even though sequence variances were reported in previous studies there is still insufficient knowledge related to the polymorphism in this gene.

Presence of the *clfA* gene and the gene encoding the X-region of the protein A are considered as the Staphylococcus spp. virulence genes in development and severity of mastitis (36, 38).

PCR amplification of the gene encoding the IgG binding region of protein A revealed 3 strain contain 390 bp amplicon this result combined with other study of Akineden(36). These three strains the PCR products were 390 bp smaller because the fragment size that is required to encode one IgG binding domain a alack of two domains is assumed for these strains Comparable *spa* gene polymorphisms were observed by (22, 39).

PCR analysis of *Nuc*and *ctfA* genes in the investigated strains suggested an important role of these elements in the pathogenicity of bovine mastitis. (9)

The *Nuc* gene PCR analysis of the other virulence genes revealed investigated important role of these elements in the pathogenicity role of mastitis (8)

Some studiesisolated *S aureus*in 69of 360 (19.16%) milk samples*,*63(91.30%) specimens contained the *clfA* gene, 69 specimens contained the *spa* gene 22(31.88%) specimens contained *spa* gene (IgG Binding region) (40).

The prevalence of *S. aureus* has been reported to vary with the size and geographic region in the world. The improper hygiene and poor management practices contributed to the presence of *S. aureus* in the milk (41, 42).

Molecular detection of methicillin resistance in Staphylococci from cases of bovine mastitis and persons handling the infected cattle the role of animals as reservoir for MRSA infection to humans (43).

A total of 151(31.45%) *S.aureus* isolates were identified by API-Staph® detection, (41.05%) isolates were determined as resistant to cephoxitin (30 ìg) demonstrates the distribution of *mecA* carrying *S. aureus* isolates and their locations in Turkey (44).

A total of 235 clinical mastitis milk samples from dairy cows were cultured for incidence of *S. aureus.* Methicillin resistant *S. aureus* was isolated from a total of 12 (44.25%) of the 116 *S. aureus* samples. Basedon the antimicrobial sensitivity and MIC results (45).

It has now become an increasingly urgent problem in veterinary medicine with MRSA infections reported in small animals and cattle (46).

The detection of *mecA* gene by PCR is accepted as “gold standard”. Detection of methicillin resistance is influenced by several factors as mec regulatory genes, β-lactamase regulatory genes (18, 47).

التحديد الجزيئي للجينات المشفرة (*spa* A) و*(Nuc)*,(*ctfA*)
في العنقودية الذهبية المقاومة للميثاسلين العزولة من التهاب الضرع في الابقار

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الخلاصة

تعتبر المكورات العنقودية الذهبية المسؤولة عن الاصابة داخل الثدي في الأبقار وهي المسبب المرضي الرئيسي لالتهاب الضرع السريري وتحت السريرية في قطعان الأبقار الحلوب. في هذه الدراسة تم الحصول على 64 عترة من المكورة العنقودية الذهبية من 112 عينة من ابقار مصابة بالتهاب الضرع وبنسبة (57.14٪). وقد تم

تحديد العنقودية ظاهريا بالاضافة الى ذلك تم تشخيصها جينيا بتفاعل سلسلة البلمريز. تضخيم الجين المشفر لتحديد النوع*Sua* و*ctfA* و *Nu*c و *Spa*. تضاعف الجين المشفر لتحديد النوع انتج حزم الحمض النووي بوزن جزيئي 530 في كل العتر وبوزن جزيئي تقريبا*CtfA* 980 وبنسبة عزل 73.43% . *Spa*فظهر التضاعف بوزن جزيئي 950 في 43 عترة و3 عتر تحتوي وزن جزيئي 390 وبنسبة 71.87% .*Nuc*تضاعف بوزن جزيئي 279 وبنسبة 90.62%. بعد ذالك تم تحديد المقاومة لميثاسلين فظهرت بنسبة 21.87% وظهرت جميع العتر المقومة للميثاسلين تحتوي على جينات الضراوة.

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