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# Isolation, characterization and clonal identification of auxotrophic mutants of *Staphylococcus aureus* application to vaccine development

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## Abstract

Numerous attempts have been made to develop a vaccine to prevent *Staphylococcus aureus* mastitis in cattle, but so far the results have not been entirely effective. This paper investigated the isolation, identifying, and cloning the auxotrophic mutant *S.aureus* in order to make the vaccines. The investigation's goal was to help increase the herd's stability, immunity, and effectiveness of treatments. *S.aureus* was first identified in bovine milk in which bovine mastitis was present. Approximately 85% of Bovine Clinical Isolates, the genomic macro-strict and pulsed field electrophoresis (MECPs) fragments have been identified as type A or their subclasses for these isolates (group 1). Group-1 isolates shared 80% of the genetic similarities, with the most prevalent being MECP-type A. Conversely, automated ribotyping discriminated against bovine clinical isolates into 13 ribotypes. Two generic species were discovered between the examined Bovine isolates (SCC-MEC and MSSA & MRSA), whose prevalence has varied over time and has extensively diffused across the nation. 86.3% of the *S. aureus* bovine clinical isolates have the specific medications targeted against the capsular serotypes S (PCS) and 8 (nR) (PC8). PCS and PC8 prevalence in both cases was 10% or below. PC serotypes 5 and 8 aren't viable candidates for a bovine mastitis preventative vaccine in Iraq. In contrast, MECP type B was discovered in the time period with the expression PC8. Rather, MECP type A was not linked to capsular NR phenotype or PCS or PC8 expression. The Aromatic amino acid auxotrophic mutant *S. aureus* was found in the second half of the experiment. *Sa*306 was produced by inserting the *Tn917* transposon mutagenesis into the genome of the *S.aureus* virulent strain *RN*6390.

**Key words:***S. aureus,* Bovine Samples, auxotrophic mutants, vaccine development

## Introduction

Mastitis is the inflammation of the mammary gland and is still the most important disease of dairy herds due to the losses it causes. Although several bacterial species can cause mastitis, both in our country and in most countries of the world, about 80% of breast infections occur due to *S. aureus.* Calzolari et al., (1997)developed a vaccine against staphylococcal and streptococcal mastitis composed of an extract of exopolysaccharides from *S. aureus* together with inactivated capsulated strains (serotype 2 and serotype A) of *S. aureus* and by dead strains of *Streptococcus ubersy S. agalactiae*.(Giraudo, et al., 1997).

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

In general, capsulated bacteria are resistant to phagocytosis by leukocytes. Because the antibodies directed towards the capsule neutralize the antiphagocytic properties of the capsular polysaccharide, this antigen has become the target of study for the formulation of numerous vaccines. Most of the *Staphylococcus aureus* produce capsular polysaccharide, in general they present a microcapsule or pseudo capsule that is smaller than the capsule expressed by highly capsulated isolates. Of the 11 capsular serotypes described, only serotypes 1 and 2 are eminently capsulated and the rest are microcapsulated. Most clinical isolates from humans produce serotype-S (PCS) or serotype-8 (PC8) of the capsular polysaccharide, while a variable prevalence has been reported in the capsular serotype of isolates of animal origin from different areas. Geographical areas of the world, in the United States, as in France, 70% of clinical isolates of *S. aureus*have PCS or PC8. It is because of this that the use of some of these serotypes (PCS or PC8) began to be relevant in the preparation of vaccines that prevent bovine mastitis. The ideal vaccine for the prophylaxis of breast infections by *S. aureus* should induce the humeral response by means of antibodies that prevent adherence, that promote bacterial death by opsonic-mediated phagocytosis and that neutralize the toxic exo-proteins produced by the bacteria. Similarly, it should induce the antibacterial, cytotoxic and suppressive cellular immune response; and fundamentally generate immunological memory.

Because staphylococcal infections involve multiple virulence determinants, new strategies are needed to combat infections caused by *Staphylococcus aureus*. In this regard, our work team proposes using live attenuated strains as vaccines, which when administered by the same route as that of natural infection could induce not only humoral but also cellular antibacterial immunity. Therefore, this research proposes to isolate stable and attenuated auxotrophic mutants (with *Aro* phenotype) through the insertion of a transposable element in the *S. aureus* genome. Such mutants could be immunogenic after local application in a murine model of intramammary infection. *Aro* deficiency would confer an attenuation of virulence, since *Aro* mutants would replicate in a limited way due to the low availability of p-aminobenzoic acid in mammalian tissues. Likewise, the local antigenic stimulation of the mucosa produced by the limited replication of the *Aro* mutants would induce a protective immune response towards all types of antigens, even those that are synthesized only in vivo. Lamente, in what to the development of a vaccine is concerned, the final design of an attenuated strain of *Staphylococcus aureus* to be used as a live vaccine will depend on the genoupic and phenobpic characteristics of higher incidence in the environment where the vaccine will be applied. The proposed work will contribute with basic information that can be applied to the development of a vaccine to prevent staphylococcal mastitis in cattle in our country. Since the epidemiological situation of the strains of *Staphylococcus aureus*isolated from milk of mastitis cows, it was necessary to investigate the genotypes present in the country and causing clinical mastitis in bovines. In this regard, the prevalent genotype causing the disease in Iraq cattle should be considered to design a prototype vaccine. For this reason, this work is aimed to evaluate the genotype of *S. aureus* isolates from the milk of cows with clinical mastitis in Iraq using two molecular typing methods and identify the laboratory strains of *S. aureus* that were used as the parental genome, Obtain and isolate *Aro* mutants of *S. aureus* by mutagenesis by transposition and to characterize the auxotrophic phenotype of *S. aureusAro* mutants.

## Materials and Methods

**Bacterial strains and culture:** 195 isolates of *S. aureus* obtained, between 1989 and 1996, from milk of bovines with mastitis from different dairy farms located in Duhak and Erbil, Iraq. In all clinical isolates, the capsular serotype was evaluated. A group of 127 isolates were

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

randomly selected to be analyzed by ribotyping. The bovine clinical isolates are representative of bovine mastitis in Iraq since they were obtained from the most important dairy regions. The species *S. aureus* was identified by standard microbiological procedures (Kojima, 1990). All the strains were preserved until their use in brain-heart broth (CC) with 20% glycerol and at -20°C. Bacteria were grown on CC agar. In some cases, the culture medium was supplemented with 10 pg/ml of erythromycin (Em) or with 10 pg / ml of chloramphenicol (Cm) (Sigma, St Louis, USA). When appropriate, the bacteria were cultured in the minimal defined medium (MDM) for *S. aureus* (153).

**Animals:** In all experiments, 68-week-old Swiss strain exorcized mice were used. The mice were kept during the experiments under standard conditions with a maintenance diet and tap acidified water.

Genomic DNA extraction and enzymatic digestionas described by Murphy et al (2002), Genomic macrorestriction and pulsed field electrophoresis (MECP) done by following the procedure developed by Rato et al (2009).

**Plasmid DNA Extraction and Enzyme Digestion:** Plasmid DNA was isolated by alkaline lysis after treatment of the bacteria with isozyme (10 mg / ml) and lysostafin (5 mg / ml) (Sigma, St Louis, USA). Plasmid DNA was digested with the appropriate endonuclease following the manufacturer's recommendations. Fragments were separated by 0.7% ag*Aro*se gel electrophoresis in 0.5x TBE buffer. The molecular size of each band was estimated by comparison with the molecular weight marker (1 Kb DNAladder, Promega). For the analysis of the restriction fragments of plasmid DNA (REAP) of the bovine clinical isolates of *S. aureus*, the endonuclease *EcaRI* was used. Isolates with differences of one band in the restriction profile were considered as different types and were designated with a letter P followed by an Arabic number. When necessary, the plasmid *pTV1ts* (12.4 Kb) was digested with the endonucleases *EcaRI* (a single cleavage site) or *Bg/II* (two cleavage sites that give rise to the 10.6 and 1.8 fragments Kb) (Promega, Madison, USA). This 1.8 Kb fragment was conveniently labelled and used as a specific probe for the *Tn917* transposon. The labeling of the specific probes for the capsular polysaccharide genes (*cap5ABCD*, *cap5IJK*, *cap5LMNOP*, *capóHIJK*) and for *Tn917* (1.8 Kb *BgIII* fragment) was performed with digoxigenin using the DIG DNA IabeIing kit (Boehringer Mannheim ). Briefly, the denatured DNA was mixed with hexanucleotides, dNTPs (with labeled dCl'Ps), water and the Klenow enzyme.

**Analysis of similarity, discriminatory power and concordance**: The similarity between the ribotypes was established by the coefficient of Dice. The similarity between the MECP types was evaluated by the coefficient of Nei and Li (1997) using the TREECOM program (version 2.1) for Windows.

Capsular polysaccharide serotyping done by the procedure used by Khudhair,(2016), Transfer, genomic hybridization and detection by flowing the method developed by Alsaad, et al (2012), Transduction transformation, Capsular polysaccharide serotyping and Growth rate of cultures in liquid medium identified by using the method developed by Shamoon, (2006) in Iraq bovine samples.

Reverse-mode polymerase chain reaction (PCR): Initially, the mapping of the insertion site of *Tn917* in the chromosome of mutant *Sa306* by hybridization with the specific probe (1.8 Kb fragment of *Tn917*) of the genomic fragments obtained with *HindIII* (Promega, Madison, USA). This allowed to have chromosomal DNA fragments flanked by a portion of the transposon (Figure 1). The right flanking region was amplified by reverse PCR (209). Briefly,

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

the genomic DNA of the *HindIII*-digested mutant *Sa306* was self-ligated, creating the plasmid pHIND. Then, for each case, the PCR reaction was carried out using the enzyme *Pfu* polymerase (Promega, Madison, USA) and 50 pmol of the respective primers. The primers were designed according to the sequence of the *Tn917* transposon available in Gene Bank using the OLIGO program (version 3.3). The 5'-3 'nucleotide sequences of the primers were the following: primer P3 (identical to the 5227-5244 nt region of *Tn917t* GACTGITATCTATTCCT), primer P4 (complementary to the 4393-4410 nt region of *Tn9175* AACATCGGATHTAGAGC) (primer *P3bis* identical to the 5485-5503 nt region of *Tn917i* GAAATAATGGAACAAGGA3 '). Amplification was carried out for 30 cycles of 94 ° C for 1 min, 55 ° C for 1 min and 72 ° C for 1 min. The fragment thus amplified was purified with the PCR Preps DNA kit (Promega, Madison, USA) to be sequenced.

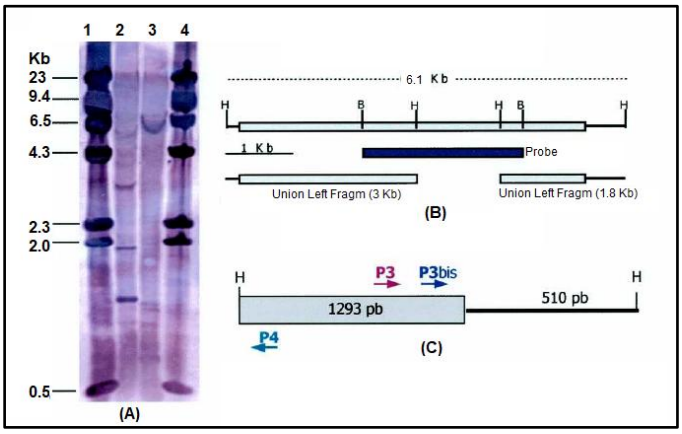


Figure 1.Mapping of the insertion site of *Tn917* in *Sa306*. (A) Genomic DNA fragments of *Sa306* (lane 2: HinaIII and lane 3: EcoRI) hybridized with specific labeled probe (Bg/II fragment of *Tn917*). Molecular weight marker: Iambda phage digested with HinaIII (lanes 1 and 4). (B) Schematic representation of the insertion of *Tn917* (gray rectangle) in the chromosomal fragment of *Sa306* (thick black lines) obtained by digestion with *H / naIII.* Relevant restriction sites in *Tn917* are shown. The blue rectangle represents the probe (*BglII* fragment of *Tn917*). (C) Schematic representation of the right binding fragment and primers *P3*, *P3bis* and P4. H: *HindIII*; B: *BglII*

Study of Nucleotide sequencing (Hawa Al-Khuzai,, 2018), Lethal Dose 50 ($DL_{50}$) (Al-Mossawei, 2016), Models of systemic infection, Bacterial permanence in tissue, Histopathological studies, Production of myeloperoxidase (MPO), Systemic protection and Systemic protection done by the standard procedures (Chen, 2019).

**Statistical considerations:** Statistical comparisons of samples without normal distribution were carried out by means of Mann-Whitney rank sum contrast using the GraphPad program (PRISM, version 2.0). For the statistical comparison of proportions, Fisher's exact test of the EPISTAT statistical package was used. To contrast the independence of the results with respect to the conditions under which they were observed, the Chi-square independence test was used using the GraphPad program.

## Results

Genotypic evaluation of *S. aureus* isolated from milk of cows with clinical mastitis from Iraq using two molecular typing methods.

In order to formulate strategies to reduce the spread of the infection of the mammary gland, the distribution of the prevalent genotype of *S. aureus* was established in the herds of cows

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

with clinical mastitis. In a first stage of the work, the clonal relationship between clinical isolates of *S. aureus* from milk of mastitic cows from different dairy basins in Argentina was evaluated. For this purpose, the following molecular typing methods were chosen: macrorestriction of the genome with *SmaI* and subsequent separation of the fragments by pulsed field electrophoresis (MECP) and automated ribotyping of the bacterial chromosome digested with *EcaRI*.

The molecular epidemiological study was carried out on 127 isolates of *S. aureus* from the milk of cows with clinical mastitis located in two governorates of the country. By analyzing the genomic DNA band profiles by MECP, a total of 24 different patterns were found (including types and subtypes) with bands with a molecular weight between 48.5 and 485 Kb. Based on the typing of the results of MECP, a dendrogram was constructed, taking into consideration the levels of similarity according to Nei and Li (1979) (Figure 2). The band profiles of the type A and subtype A11 isolates differed by 4 bands (Figure 3).

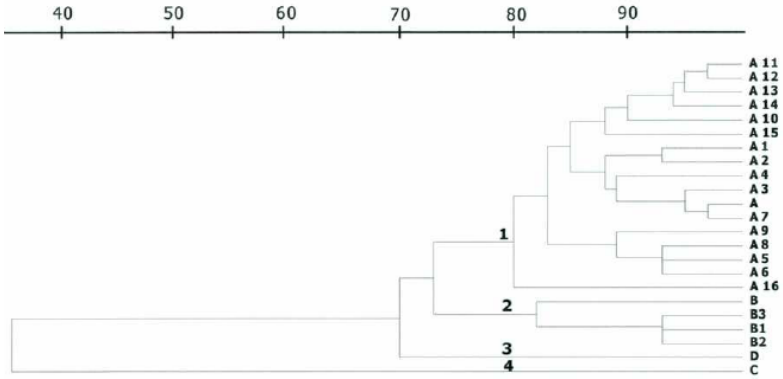


Figure 2: Dendrogram of similarity between the MECP patterns of the clinical isolates of *S. aureus.*

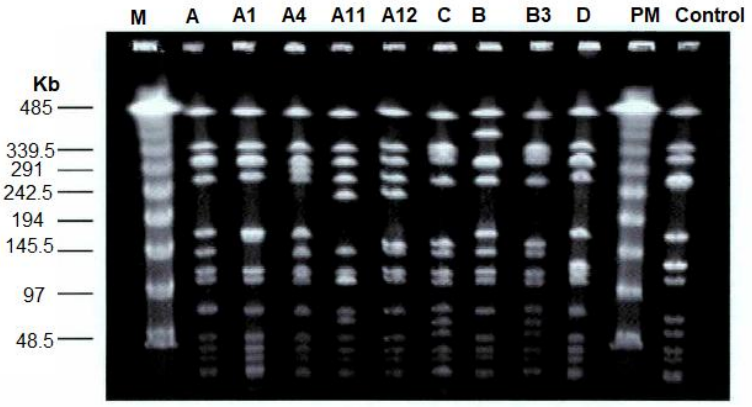


Figure 2: Separation of *SmaI* genomic fragments or pulsed field electrophoresis (MECP). Lines 1 and 11: (PM) Lambda phage DNA prepared for MECP. Lanes 2 to 10 patterns of the major types and subtypes of MECP corresponding to the clinical isolates of *S. aureus*. Lines 12 Ctrl (strain 8325).

Only 2 isolates were identified in group 3 (type D) and another 2 isolates in group 4 (type C). In conclusion, more than 85% of the bovine clinical isolates were identified by MECP in group 1 and the so-called *Afue* type was the most prevalent.

**Ribotyping of *S. aureus* isolates:** The 127 *S. aureus* isolates are typed according to Materials and Methods, using the automated molecular characterisation technique

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

Ribotyping. The band pattern of each isolate was compared to that of a database by means of a computerised system. The results were acquired using traditional biochemical procedures and an automated ribotyping approach by similar species identification. Eight groupings were formed while evaluating 80 percent similarity (group 3 belongs to laboratory strains of *S. aureus*). The system in 13 ribotypes discriminated against the isolate *S. aureus* included in the research (Figure 4).

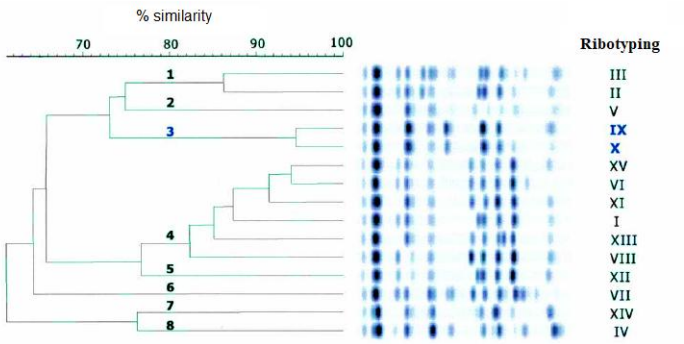


Figure 4: Similarity dendrogram based on automated ribotyping and digitized profiles of ribotypes.

**Identification of *S. aureus* isolates by combined application of both molecular typing methods**

The findings were examined using both techniques to better distinguish between genotypes acquired by MECP and ribotyping. On the one hand, MECP 44 isolates of type A were classified into three groups of ribotypes I, II, III and XII, comprised of 31, 7, 3 and 3 isolates. However, this distinction was not obtained if MECP subtype SCC-MEC (19 isolates) was examined using ribotyping technique. Of the 19 A11, 18 was ribotype VI, and only the rest was recognised as ribotype VII. In reality, 18 was isolates of SCC-MEC in all. In the two big groups consisting of Ribotypes I and VI isolates, however, the typing of MECP was beneficial to provide further differentiation. In fact, the MECP type A isolates are 31 out of 53 ribotype I isolates. The rest of the 22 isolates have been differentiated against in seven MECP subtypes of 1-5 isolates (A1-A5, B1 and B2). Similarly, 18 out of 37 ribotype VI isolates belonged to MECP type SCC-MECand 11 smaller MECP groups discriminated against in the remaining 19 isolates. Finally, two major genotypes, A/L and MSSA & MRSA, were identified among the bovine clinical isolates of *S. aureus* by an examination of the data through a combination of both techniques.

**3.1.5 Distribution of *S. aureus* strains SCC-MEC and MSSA & MRSA**

The findings were examined for the time and location of the *S. aureus* clinic isolates in order to ascertain if the majority of genotypes identified were localised in a single region. It has been discovered in most cases to be extensively disseminated throughout the country with genotype SCC-MEC (Defined by MECP and ribotyping). In fact, in 15 of the 22 sites examined, 31 SCC-MEC-genotype strains were identified. The second most frequent MSSA & MRSA genotype was likewise found to be widespread in remote areas around the nation. There has been considerable genotypical diversity in isolates of each district from various cows. In the La Vacherie region, eleven distinct genotyping strains were recovered from a total of 22 isolates, while a total of twenty Duhak isolates were discovered to consist of 14 different genotypes. Similarly, six distinct genotypes characterised by MECP and ribotypes were detected among the 6 isolates obtained from the Duhak in a 2-month period. Also, among seven La Vacherie isolates collected during a 4 months' time span, six strains with

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

distinct genotypes were discovered. In short, both of the primary genotypes have been widespread across the country and have not been confined in one or very near location. When all locations have been examined jointly, the prevalence of SCC-MEC and MSSA & MRSA strains differed significantly in the time period examined. Similar findings were seen among the isolates from 2 locations (60 from Duhak and 72 from Erbil). Genetic testing indicated that in Erbil from 2019 (high prevalence) to 2020 (low prevalence) and in Duhak (high prevalence) between 2019 (high prevalence) to 2020 (low prevalence), the SCC-MEC strain was identified. From 2019 to 2020, 11 separate locations isolated the second most frequent strain (MSSA & MRSA) (high prevalence). Therefore, the predominance of the two main genotypes was fluctuated over the investigated time.

**Typing of *S. aureus* isolates by plasmid DNA restriction**

Two dominant clones, the designated SCC-MEC and MSSE & MRSA, were identified by the combination of MECP and ribotyping. The enzyme restriction patterns of DNA plasmid (REAP) were examined in order to better identify the strains matching to these genotypes. The isolates acquired for a maximum duration of 5 months have been examined using REAP in order to prevent differences due to plasmid instability. In view of this consideration, two strain groups were selected, namely SCC-MEC and A11NI. The presence of plasmid DNA was demonstrated in 11 MSSA & MRSA strains from chosen locations. For each of the MSSA and MRSA strains, the respective REAP patterns were distinct. Each profile consisted of 3 to 11 molecular bands from 9500 to 650 bp (Figure 5). It is important to note that 3 distinct REAP patterns were identified in 3 MSSA & MRSA strains derived from separate herds of the same area. Over a four-month time frame the 14 SCC-MEC strains from nine locales were evaluated and 6 REAP profiles were identified, which did not correspond with those of the investigated MSSA & MRSA strains. These profiles showed between 2 and 11 bands of molecular weight between 900 and 18000 bp (Figure5) with a plasmid not present in the SCC-MEC strain. On the other hand, REAP-profiles distinguished two groups of three and two SCC-MEC strains, each with a size of 2 (Erbil, Duhak). On the other hand, there have been no discrimination between 2 Duhak SCC-MEC strains (Reaptype P15) and 2 Erbil strains (Reaptype P14) respectively. Taking into account the results gained by analysing the REAP patterns, MECP isolates with the same genotyper ribotype were better identified.

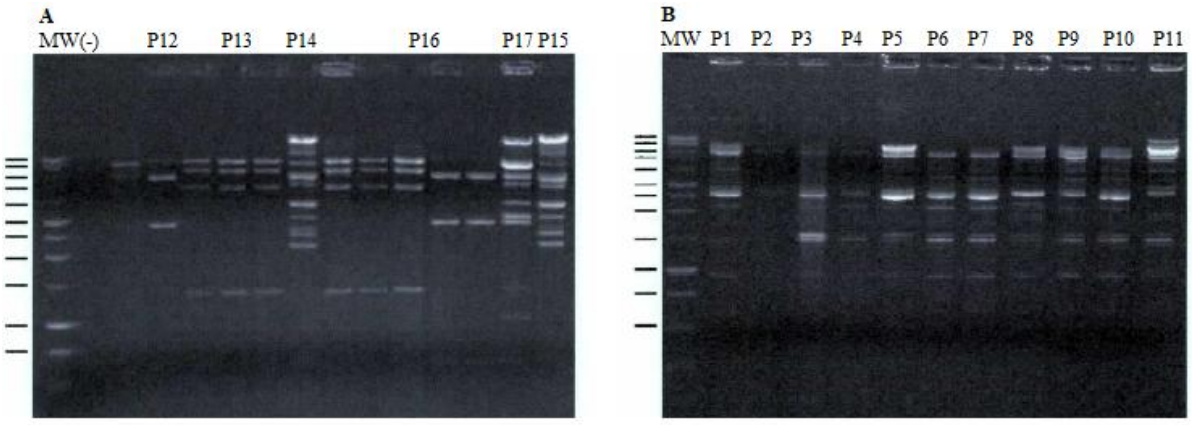


Figure 5: Electrophoresis of the plasmid DNA digested with *EcoRI*.

According to the data reported, the MECP Analysis revealed the predominant group (group 1) of bovine isolates in the *S. aureus*. 85.5% of the isolates of *S. aureus* examined were in this group. In Argentina there have been two prominent clones (SCC-MEC and MSSA & MRSA) which represent 39% of isolates. In identifying the two generally common genotypes, MECP

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

and ribotyping agreed. The SCC-MEC strain was established over a period of eight years, with significant decreases in its frequency and a concomitant increase in the prevalence of MSSA & MRSA strain. The SCC-MEC genotype was discovered over the whole period in which the investigation was done despite this shift in prevalence. By analysing REAP pattern, a collection of SCC-MEC and MSSA&MRSA strains collected over a period of many months may be discriminated against. Based on the data, two predominant genotypes were found over the examined time, which were also spread across the country. The majority genotype of *S.aureus* population producing such a disease should thus be taken into account for a time while designing a vaccination that prevents mastitis in bovines.

**Obtaining and isolating auxotrophic mutants of *S. aureus* by transposition mutagenesis**

Live attenuated strains provide better protection due to the limited replication of the bacteria in vivo that induces immunity against the pathogen(Novick, et al., 2001). Different pathogenic bacterial species have been attenuated by the introduction of a stable mutation in the biosynthetic pathway of amino acids, purines and pyrimidines or riboflavin. These attenuated mutants have also been able to stimulate protective immunity in different animal models. Therefore, in this stage of the work, the dependence for the growth of Aromatic amino acids was evaluated as a form of attenuation of S.*aureus* and its potential contribution to the design of a vaccine that prevents mastitis in bovines. Initially, and in order to have a live attenuated strain of S.*aureus*, a series of experiments were carried out to mutagenize, isolate and characterize the auxotrophic mutants.

***Mutagenesis by transposition with Tn917:*** The main risk of using mutants as live vaccines is their reversion to the parental pathogenic phenotype. In order to obtain the basic knowledge for the possible future construction of an auxotrophic mutant by deletion, that is, without the possibility of reversion, the methodology of mutagenesis by transposition was used.

***Characterization assays:*** The study began using the *S. aureus* 8325-4 strain as the parental genome. The relative ease of applying mutagenesis techniques on this strain favored the development of the methods to be used. For this purpose, the strain of *S. aureus* 8325-4 was transformed with the plasmid pTVIts, carrying the transposon Tn917, by transduction with phage phi-11. For this, a culture of the strain of *S. aureus* K511024 (carrier of pTV1ts) was infected with phage phi-11. The subsequently obtained phi-11 isolate carried the plasmid. Then, the *S. aureus* RN4220 strain was transduced with the previously obtained isolate. The RN4220 strain is used as an intermediary in the genetic transfer between S.*aureus* strains. Finally, the transduction of the 8325-4 strain was carried out with the phi11 isolate from the RN4220 strain. This lysis carries the pTVIts modified by methylation, therefore it can now be accepted by any strain of *S. aureus*.In this way, 134 CmR (plasmid marker) transductants were obtained, which were EmR (transposon marker), which were named 8325 -T. Likewise, the presence of the plasmid in strain 8325-4 was confirmed by extraction and electrophoretic running of plasmid DNA digested with the *EcaRI* endonuclease. A single molecular weight band of N12 Kb was observed corresponding to the linearized pTV1Ls.

*Growth of the S. aureus 8325-4 strain in liquid medium:* In order to establish the time it takes for the culture in minimal medium to double its OD, defined as the mean generation time or TMG, the growth kinetics of the strain was investigated. 8325-4 in minimal defined medium (MDM) at 37 ° C and 200 rpm. Bacterial growth was recorded spectrophotometrically. Under the above-mentioned culture conditions, a TMG of 120 min was observed. However, when the 8325-4 strain was cultivated in a nutrient medium, such as brain-heart broth, the TMG was 30 min. On the other hand, the number of duplications carried out by the 8325-4 strain in MDM was established. The doubling index for 8325-4 in MDM was 4.81 and in brain-heart

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

broth was 4.08. Therefore, the strain 8325-4 in a minimal defined medium had a moderate growth delay with respect to that observed in a nutrient medium.

***Transformation of the S. aureus RN6390 strain***: In preliminary tests it was found that inoculation with high doses of the *S. aureus* 8325-4 strain did not cause the death of most of the infected animals. Therefore, the impossibility of having a murine model of lethal infection due to the systemic inoculation of the 8325-4 strain was the reason why this strain was not considered appropriate to be used as the parental genome of possible attenuated mutants derived from it. . Then, and in order to isolate attenuated auxotrophic mutants, it was decided to use the *S. aureus* strain RN6390 as the parental genome. Also, the presence of the pTV1ts extraction and analyzed by electrophoretic run of the plasmid DNA digested with the restriction enzyme *EcaRI*. In this way, the RN6390 strain was transformed with the plasmid pTV1ts for its subsequent mutagenesis.

With the S.*aureus* 8325-4 strain, the optimal conditions for demutagenesis by transposition of Tn917 were set. However, the 8325-4 strain could not be used as a parental genome to obtain auxotrophic mutants because a murine model of lethal infection could not be established. Therefore, it was decided to use the S.*aureus* RN6390 strain as the parental genome since it is considered highly virulent(Feng, et al 2008). After obtaining a library of inserts of the Tn917 transposon in the RN6390 strain, the growth of 3493 EmR colonies was analyzed in minimal defined medium (MDM) plates lacking Trp, Phe and Tyr. 328 colonies with apparent *Aro* phenotype were isolated whose growth was evaluated in different MDM plates. Then, it was determined that only 10 colonies presented a strong dependent phenotype for their growth of the Aromatic amino acids /I. These isolated mutants deserved further characterization.

**Characterization of the auxotrophic phenotype of isolated *S. aureus* mutants**

Growth of auxotrophic mutants in minimal defined medium: In order to characterize the auxotrophic phenotype (*Aro*') of the 10 isolated mutants, these were seeded in several streaks in sets of plates II (Figure 6) and III (Figure 7) of MDM. After 20 h of incubation at 37 ° C, bacterial development was evaluated. Bacteria that showed deficient or no growth in some of the different MDM agar plates were registered as dependent mutants for their growth of the amino acid / s absent in the medium.

Results shows the growth results of the different mutants isolated in the sets of plates II and III. In total, 6 mutants dependent on the amino acid phenylalanine (Phe ') were isolated. However, only in the Sa68 mutant was a marked Phe 'phenotype observed. Likewise, in the mutants Sa68 and *Sa306* a marked phenotype Tyr 'Phe' and Tyr 'Phe' Trp ', respectively, was registered. The mutant *Sa306* deserved further study due to the strong dependence for its growth on the three Aromatic amino acids. Therefore, the phenotypic characterization of the enzyme of the possibly affected biosynthetic pathway was carried out.

For this, the studies carried out in this regard in *Bacillus subtilis* were taken into account. Considering the results mentioned above, mutant *Sa306* and parental strain RN6390 were seeded in several streaks in sets of plates IV and V. After incubation at 37°C for 20 h, bacterial development was evaluated. The total absence of growth of mutant *Sa306* in plate # 14 suggests that the enzyme affected and responsible for the observed phenotype would be one of those involved in the first steps of the metabolic pathway (in the formation of acid and / or chorismic acid); and it would not be any of the enzymes that, from chorismic acid, lead to the formation of each of the Aromatic amino acids (Phe, Tyr and Trp). It is worth mentioning that the growth of the parental strain RN6390 was observed in plate # 14. On the other hand, neither mutant *Sa306* nor strain RN6390 growth was observed in plate # 15, therefore

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

nicotinic acid would be an essential component for the growth of strain RN6390 and its derivatives. Some growth of the mutant *Sa306* was observed in the rest of the plates. Therefore, the phenotype dependent on Aromatic amino acids for growth that mutant *Sa306* presented would be due to a defect in some of the *Aro* genes and would have a deficient *Aro* phenotype (*Aro* '). In order to establish whether the deficient *Aro* phenotype of the *Sa306* mutant was due to the genomic insertion of the Tn917 transposon, the cotransduction of the Tn917 marker gene (EmR) and of the *Aro* 'auxotrophy was evaluated. For this, a phi-11 phage was prepared from a culture of the mutant *Sa306*. The isolate thus obtained was used to infect a culture of the parental strain RN6390. Aliquots were then taken to seed the appropriate dilutions. 100% of the transducing colonies were EmRy *Aro* '. Therefore, the *Aro* deficiency is due to a modification of the gene affected by the insertion of the transposon. After initial analysis for auxotrophic mutants, 10 RN6390 mutants with deficient *Aro* phenotype were isolated. Mutant *Sa306* showed a strong Tyr 'Phe' Trp 'phenotype and was therefore selected for further characterization. The evaluation of the growth of *Sa306* in plate # 14 of MDM, would allow to suggest that the enzyme affected and responsible for the *Aro* 'phenotype would be one of those involved in the steps that give rise to the formation of shikimic acid and / or chorismic acid. On the other hand, the resistance to Em and the *Aro* 'phenotype of the mutant *Sa306* cotransduced 100% to the parental strain RN6390. The comparison of the growth kinetics established that there were no differences in the in vitro growth speed between the *Sa306* mutant and the RN6390 parental strain. The reversion frequency of mutant *Sa306* was $5x10^{13}$. Furthermore, the *Aro* deficinet phenotype of mutant *Sa306* proved to be stable in vitro after 25 chimes and in vivo after inoculation and bacterial recovery. Considering the results found, it can be speculated that the *Aro*-phenotype of the mutant *Sa306* is due to a genetic change resulting from the insertion of the transposon and not to a physiological adaptation.

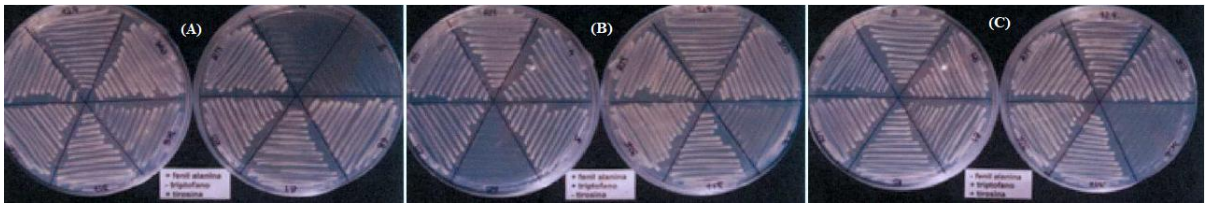


Figure 6: Growth of auxotrophic mutants in different lakes of a minimal defined medium (MDM). A: plate # 2 (without Trp); B: plate # 3 (without Tyr); C: plate # 4 (without Phe). The photographs were taken on the 5th day of the bacterial seeding. Colonies were seeded on replica plates. Trp: tryptophan; Tyr: tyrosine; Phe: phenialanine.

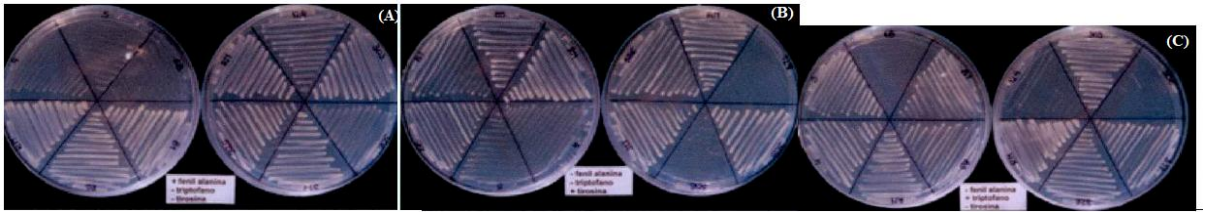


Figure 7: Growth of auxotrophic mutants on different minimal defined medium (MDM) agar plates. A: MDM plate # 5 (without Trp, without Tyr); B: MDM plate # 6 (no Trp, no Phe); C: MDM plate # 7 (no Phe, no Tyr). The photographs were taken on the 5th day of the bacterial seeding. Colonies were seeded on replica plates. Trp: tryptophan, Tyr: tyrosine, Phe: phenylalanine.

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

**Molecular characterization of isolated *S. aureus* auxotrophic mutants**

By sequencing the right flanking region of the transposon, it was established that the unknown sequence presented 94% nucleotide identity with the enzyme 5-enolpyruviIshiquimate-3-phosphate synthetase (EPSPS) of *S. aureus* and 49% homology with the gene of The EPSPS (called *Aro*E) from *Bacillus subtitlis* Also, the comparison of the amino acid sequences revealed 79% homology with the EPSPS enzyme from S*aureus*. The comparison of the unknown nucleotide sequence with the different databases of the unfinished genome of *S. aureus* determined significant homology percentages with certain "contig" sequences of each data bank. Furthermore, these were coincident with the "contig" sequences resulting from the comparison of the araA gene of *S. aureus* in the same databases. Therefore, despite the lack of published information, it was established that the unknown sequence would have a significant percentage of identity with the *Aro*A gene of S *aureus*. The alignment of the amino acid sequences of the araA genes of *S. aureus* and *B.subtilis* with the unknown sequence revealed a high homology between them. Therefore, it could be suggested that the insertion of the Tn917 transposon in the arm gene is responsible for the deficient *Aro* phenotype observed in the *Sa306* mutant. Indeed, a mutant of S.*aureusAro*A, designated *Sa306*, was isolated by insertional mutagenesis.

**Determination of the degree of attenuation of the auxotrophic mutant of *S. aureus***

The use of a lethal murine model of inoculation made it possible to establish the influence of auxotrophy, *Aro*A, on stafllococcal virulence. The previously described results showed that the *Sa306* mutant was attenuated ($LD_{50}$: $1x10^6$ CFU / mouse) with respect to the virulent parental strain RN6390 (LD50: $6.3x10^4$ CFU / mouse) since it was determined that a dose of the mutant *Sa306* is required 16 times higher than the $LD_{50}$ of RN6390 to cause the death of half of the animals infected by the ip route. On the other hand, 0% survival was observed in the mice inoculated with the parental strain RN6390 by the ip route. In contrast, 46% survival was determined in mice infected with the mutant by the ip route. Furthermore, it was established in the murine model of hematogenous infection that the *Sa306* mutant was attenuated with respect to the parental strain RN6390. This conclusion arises from the fact that 22 d after the inoculation occurred, a significantly higher survival percentage (43%) was observed in the mice inoculated with 55306 compared to that established for the animals infected with RN6390 (0%). Likewise, the histopathological evaluation revealed that the process that led to the death of the animals infected by iv route with the mutant was less severe than in the animals inoculated with the parental strain. In conclusion, the results suggest that the mutation in the *Aro*A gene of mutant *Sa306* could be the cause of the attenuation of virulence.

**Evaluation of the immunogenic capacity of the attenuated mutant *Sa306***

These experiments were carried out with the purpose of determining the immunogenicity of the mutant *Sa306* (*Aro*A) of *S. aureus*.

The mutant *Sa306* was able to grant systemic protection against challenge with the parental strain RN6390. The protective index (52%) was statistically significant ($p = 2x10^6$, Fisher's exact test). Likewise, the *Sa306* mutant conferred local protection that was indirectly related to the significant decrease in the colonization of the mammary gland by the parental strain RN6390 and by a heterologous strain of *S. aureus* (MB319). It is worth mentioning that the typing of the MB319 strain showed that it had the most prevalent genotype (SCC-MEC) of the studied population and that it was classified as non-reactive (NR) because it did not express PCS or PC8. Therefore, the isolated araA mutant would induce protection of the

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

murine mammary gland against the homologous strain (RN6390) and against a virulent strain of S.*aureus* with the most prevalent capsular genotype and phenotype in our region during the period under scrutiny.

**Identification of the auxotrophic mutant *Sa306* among clinical isolates of *S. aureus***

The genome of the mutant *Sa306* could be easily discriminated among the bovine clinical isolates of S.*aureus* using the MECP as a molecular typing method. It was established that the MECP genotype for *Sa306*, called Q1, differed in more than 7 bands with respect to the genotypes established for each isolate. Therefore, according to the most rigorous criteria for the analysis of MECP polymorphisms, the *Sa306* mutant was genetically different from the rest of the isolates studied.

**Discussion**

Mastitis caused by *Staphylococcus aureus* continues to be a serious problem worldwide due to the significant economic losses it causes in the dairy industry. One way to lessen the impact of mastitis on dairy farms is to induce immunity in cattle to resist infection by S.*aureus*. Interest in an effective immunoprophylactic procedure that prevents the disease is continuing. In this regard, during the last decade contradictory and not entirely satisfactory conclusions have been reached regarding the efficiency of vaccines(Wedlock, 2002). At present, the most common practice is based on appropriate hygiene measures, on reducing exposure to environmental pathogens and on antibiotic therapy in the dry period of the cow. The information available on the subject indicates that new strategies are required for the control and / or prevention of bovine mastitis.

Based on current knowledge about the pathogenesis of S.*aureus* infections, it has not yet been possible to establish with certainty which antigen or combination of them is capable of providing the best protection against this bacterium. In addition, the complex regulation of the virulence factors involved must be taken into account in the pathogenesis of different infections (Novick, 1993). For this reason, the preservation of the greatest quantity of bacterial antigens in the composition of a vaccine could be essential to promote a favourable host immune response against infections by *S. aureus* AI. Regarding, live attenuated strains could be good immunogens since they would present antigens in their native conformation. Furthermore, due to limited growth in the host, they could express antigens that are only synthesized *in vivo*.

An important point to take into account in vaccination studies with live *S. aureus* bacteria is the reason for choosing immunization test. Indeed, the vast majority of infections are caused by microorganisms that are acquired through the mucosa. Therefore, it is especially important to consider the immunity provided by the mucosa as a vaccination strategy, due to the immune response produced in the antigen deposition state, as well as in remote mucosal sites. It is surprising that despite the current level of knowledge about the common mucosal immune system, the *S. aureus* vaccines that other researchers have tried in recent years have been administered by the parenteral route. Even though it is known that systemic immunization is essentially ineffective for the induction of mucosal immune responses. In the present work, it was established that immunizations in the mammary gland with the mutant *Sa306* confined local protection through the significant decrease in the degree of colonization of the mammary tissue of the virulent parental strain. Similar results were observed when mice immunized with *Sa306* were challenged by ima route with a virulent strain of *S. aureus* isolated from the milk of a bovine with Mastitis. Probably, immunization in the bovine mammary gland with an arm mutant of *S. aureus* could confer protection against infection by

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

a strain of *S. aureus* whose genotype (SCC-MEC) is responsible for most of the cases of mastib's of the cattle of our country in the period of study.

Unambiguous identification of vaccine strains from clinical isolates is necessary before considering the use of prototype vaccines in field trials. Certainly, the genetic base of the bovine strain has to be easily recognized from those of the bovine clinical isolates to ensure if the appearance of new cases of infection are caused by possible derivatives of the bovine strain. Taking into account that the incorporation of antibiotic resistance genes in the genome of the attenuated *Aro*A strain should be avoided in the design of the future vaccine, it was necessary to consider a strategy for a reliable discrimination of the vaccine strain.

By using the MECP band profiles the mutant and parental stress of the Sa306 from the bovine clinical isolates of *S. aureus* could be identified with confidence. The lab strains could also be differentiated against from milk obtained from the bovines using automated ribotyping of the remainder of *S. aureus*. Therefore it might be beneficial to analysis a vaccination strain from the location under investigation using the molecular typing methods outlined in the present research. According to Fitzgerald et al (1997), there might be a sluggish gene flow rate in the underlying natural *S.aureus* population of just 2 predominant genotypes (SCC-MEC and MSSA & MRSA). The findings of these works might therefore be valid for several years and valuable. A similar studying using live-attenuated vaccinations produced on the same genetic foundation and within an identical geographic region may thus not be needed before every field investigation. The relevance of this study is that they give information on a feasible process prior to the introduction into the environment of a *S. aureus* vaccine strain. The potential additional incidences of illness, probably caused by its reversers, are a serious worry when a live attenuated vaccination is being administered. It should not be considered out that mastitis cows emerge in vaccinated flocks since 100% protection against infection is not acceptable. The effectiveness of *S. aureus* Live Attenuated Strain immunisation must certainly be established in field experiments in order to prevent bovine mastitis. The technique presented in this study may certainly be used to assure the stability of vaccination strains in bovines, just as if this event were occurring in nature, it can offer information on the dissemination of a vaccine strain derivatives.

## Conclusion

Mastitis is an infectious disease that affects dairy cattle, affecting the production and quality of milk. In Iraq country and in the world, the main etiological agent of bovine mastitis is the pathogenic bacterium *Staphylococcus aureus*. Infection of the mammary gland of milking cows continues to be a serious problem for producers due to the significant economic losses it causes in the dairy industry. In addition, it involves serious sanitary and epidemiological difficulties, due to the presence of toxigenic strains and the possible content of antibiotic residues in the milk. One strategy to lessen the impact of mastitis in cattle is to induce the animals' immunity to resist *S. aureus* infection.The research focused on isolating, characterising and cloning the auxotrophic mutant *S.aureus* in order to be used to produce the vaccines. The major aims of the investigation were stability, safety, immunogenicity and the discovery of the auxotrophic mutant in the bovine clinical isolate population. The clonal ratio and the predominance of the *S. aureus* capsular serotype of bovine milk with mastitis were initially assessed. In that respect it has been shown that the genomic macro-strict and pulsed field electrophoresis (MECPs) fragments have been recognised as type A or their subtypes for 85% of Bovine Clinical Isolates tested (group 1). Moreover, group-1 isolates shared 80% of the genetic similarities and the most prevalent was the so-called MECP-type A. Automated ribotyping, on the other hand, discriminated against bovine clinical isolates into 13 ribotypes. Two generic species were identified between the investigated Bovine isolates (SCC-MEC

ISSN:0975-3583,0976-2833 VOL12,ISSUE06,2021

and MSSA & MRSA), whose prevalence has changed over time and also extensively dispersed in the nation, by the joint analyse of the data obtained by both typing techniques. Surprisingly, 86.3% of *S. aureus* bovine clinical isolates showed the particular antibiotics aimed against capsular serotypes S(PCS) and 8 as capsular non-reactive phenotype (nR) (PC8). The PCS and PC8 prevalence in both cases was less than 10%. PC serotypes S and 8 are therefore not excellent candidates for taking part in a bovine mastitis preventive vaccination in Iraq. In contrast, MECP type B was identified in the time investigated in association with the expression PC8. Conversely, the prevailing MECP type A was not related to the capsular NR phenotype or to PCS or PC8 expression. An auxotrophic mutant *S.aureus*, which depends on the phenotype of Aromatic amino acids, was identified in the second part of the experiment. This *Aro*-deficient mutant was called *Sa306*and was achieved by insertion of the *Tn917* transposon mutagenesis into the genome of the *S. aureus* virulent strain RN6390.The phenotype observed in mutant *Sa306* was due to a single insertion of the transposon *Tn917* in the gene for the enzyme 5-enolpyruvilshiquimate-3-phosphate synthetase, therefore it would be a *3raA* mutant. Mutant *Sa306* was shown to be stable in vitro and *in vivo*. The reversion frequency of *Sa306* was $<5\times10^{13}$.Furthermore, it should not include capsular polysaccharide serotypes 5 or 8 since they exist in a very low percentage within the *S. aureus* population under scrutiny. Certainly, an attenuated and immunogenic arm mutant makes possible the potential use of this strategy in the design of live vaccines to prevent some of the infections caused by *S. aureus*.

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