

Molecular Confirmation of Staphylococci Strain's Identification Isolated in the Hospital and University Center of Brazzaville, Republic of Congo

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Abstract

Bacteria of the genus *Staphylococcus* are pathogenic Gram-positive bacteria responsible for various infections, including skin suppuration, which can be severe or chronic. The objective of this study was to confirm Staphylococci strain's identification isolated by bacteriological methods from biological products of CHU-B patients, by molecular methods based on the analysis of the gene coding for 16S rRNA. In total, 30 strains of Staphylococci were isolated including 8 (26.66%) community strains, 22 (73.33%) hospital strains. The products of the amplification of gene fragments encoding 16S rRNA from 10 strains of Staphylococci including 6 strains of *Staphylococcus aureus* (*S. aureus*) and 4 Coagulase Negative Staphylococci (CNS) were sequenced. The sequences obtained were subjected to bioinformatics analysis to confirm the results of conventional bacteriological methods. Six (6) *S. aureus* strains, 2 *Staphylococcus haemolyticus* strains, 1 uncultured bacterium clone nbw618g09c1, and one

Staphylococcus sp. have been identified. These results made it possible to confirm the effectiveness of the molecular method and to show the limits of traditional bacteriological methods in the complete identification of bacteria.

Introduction

Bacteria are micro-organisms or unicellular living beings without a nucleus. Human body harbors several microorganisms, which can be commensal or pathogenic. As soon as they become pathogenic, they can be responsible for many infectious diseases [1] According to the World Health Organization (WHO) bacterial infectious diseases are the 3rd cause of death in developing countries malaria and malnutrition [2]. Among the bacteria we cite Staphylococci, which are bacteria responsible for numerous infectious foci which can disseminate and cause septicemia, endocarditis (infection of the endocardium), osteomyelitis (infection of the bone tissue) [3], [4]. The species *Staphylococcus aureus* is the most isolated species and in recent decades has been one of the main causes of hospital or clinical and community infections [5].

Traditional bacteriological methods, namely the study of morphological, cultural and biochemical characters, remain the reference for the identification of the *Staphylococcus* genus as for all other bacteria. On the other hand, molecular biology techniques are more advantageous for the detection of different bacteria [6], [7]; and have been able to find their place in routine in a large number of medical analysis laboratories. There is a common molecular biology method for the identification of bacterial strains consisting of bioinformatics analyzes of the nucleotide sequences of the gene encoding 16S ribosomal RNA (16S rRNA), an essential component of the 30S ribosomal complex in prokaryotes, due to conservation of this gene between different species of bacteria. *Staphylococcus* strain's identification being an important factor in the management of hospitalized and non-hospitalized infected patients and also a means of overcoming therapeutic failures, this work is therefore

part of the aim of confirming by molecular methods the identification of bacteria of the genus *Staphylococcus* produced by conventional bacteriological methods.

Materials and Methods

Site and Collection of Strains

Staphylococci strains collected at the biomedical analysis laboratory of the Center Hospital and University Center of Brazzaville (CHU-B) using various biological fluids from hospitalized patients (hospital strains) and non-hospitalized patients (community strains) were used. Strains were isolated during the period of April 2020 to July 2020.

Isolation of Strains

The isolation of the strains was carried out on nutrient agar associated with a selective Chapman agar.

Identification of Strains

Classical Bacteriological Methods

The identification of the strains was made on the basis of the morphological characters and the production of a catalase and a staphylocoagulase by the bacterial strain [8].

Molecular Identification

16S rRNA Gene Analysis

Ten strains of *Staphylococci* identified by conventional bacteriological methods were the subject of a molecular analysis on the analysis of the gene coding for the 16S rRNA.

Extraction of Genomic DNA

DNA extracts were isolated from *Staphylococcus* strains using the ZR DNA Card extraction Kit while following the manufacturer's instructions.

Amplification of the 16S rRNA Gene

The DNA extracts were amplified by PCR (polymerase chain reaction) using specific primers for the 16S rRNA gene, the universal primers used are those designated by [9] and also used by [10], [11]. The sequences and directions of the primers are: Forward

F 5'-AGA GTT TGA TCC TGG CTC AG-3' and Reverse R 5'-ACG GCT ACC TTG TTA CGA CTT-3'.

The PCR reaction was used in a total volume of 50 microliters comprising 39.5 μ L of PCR water (Nuclease-free water), 2 μ L (20 μ M) of each primer, 1 μ L (10 μ M) of dNTPs, 5 μ L of buffer (10X), 2 μ L (20ng) of genomic DNA and 0.5 μ L (5Unit/ μ L) of Taq polymerase. The PCR reaction was carried out in a Biometra thermocycler under the following conditions: pre-denaturation 95°C at 5 min, followed by 30 cycles, each cycle comprising denaturation 95°C at 30 sec, hybridization 55°C, 30 sec and elongation 72°C, 1 min 30s and finally a final elongation 72°C, 5 min.

Agarose Gel Electrophoresis of PCR Product

The PCR products were demonstrated by electrophoresis on a 1% agarose gel at 100 volts for 45 minutes with TBE buffer. The staining was carried out with a 1 μ g/ml solution of ethidium bromide. The gel was visualized under a UV lamp, by fluorescence. The size of the sought gene was around 1500-1300 bp.

Sequencing of PCR Products and Assembly of Sequences

The PCR products resulting from the amplification of the gene encoding the 16S rRNA of the ten strains were purified using the NucleoFast 96 PCR plate (Macherey-Nagel EURL, France) and sequenced by the company Masrogen using the BigDye terminator chemistry on an ABI sequencer. 3730 (Applied Biosystems, Foster City, CA, USA). Baser DNA sequence assembler was used for sequence assembly.

In Silico Sequence Analysis

The analysis of the sequences A2, A3, A4, A5, A6, A10 respectively sequences of the strains S2, S3, S4, S5, S6, S10, *S. aureus*) and A1, A7, A8 A10 of the S1, S7, S8, S10, SCN) was performed using the Local Baseline Alignment Search Tool (BLAST) available at the National Database, Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The alignment of the nucleotide sequences was done on ClustalW. Molecular phylogenetic and evolutionary analyzes were performed using MEGA version 7 [12].

Results

Isolation and Identification of Strains

Isolation of Strains

Figure 1 represents the percentage of strains of community and clinical Staphylococci isolated and identified. 30 strains of Staphylococci were isolated, including 8 (26.66%) community strains, 22 (73.33%) strains from samples taken from the services in the Hospital and University Center of Brazzaville.

Identification of Strains

All strains isolated were Gram positive and catalase positive bacteria. The fresh state showed that all the stumps were cocci in immobile clusters.

Figure 2 shows the distribution of Staphylococci identified by different coagulase assays. Of the 30 strains of staphylococci isolated from hospitalized and outpatients, 21 (70%) were able to produce a free coagulase (*S. aureus*), while 9 (30%) were not able to produce it (Coagulase Negative Staphylococcus).

Molecular Identification

Ten strains of clinical Staphylococci, including 6 *S. aureus* and 4 with Negative Coagulase by classical bacteriological methods, were the subject of a molecular analysis for confirmation on the analysis of the gene coding for 16S rRNA.

DNA extracts from each strain were amplified by PCR using 16S rRNA gene-specific primers.

Electrophoresis of PCR Products

Figure 3 shows the DNA fragments (gene encoding RNA16S) obtained by 1% agarose gel electrophoresis of the 10 strains. The bands are about 1500 Pb in size.

Bioinformatic Analysis of 16S rDNA Sequences and Phylogenetic Inference Test

BLASTN Analysis and Sequences Submission in GenBank

PCR fragments were sequenced and assembled. Table 1 and Table 2 describes the analyzes relating to the similarity of the 16s rRNA sequences between our query sequences and the sequences present in the Gene Bank

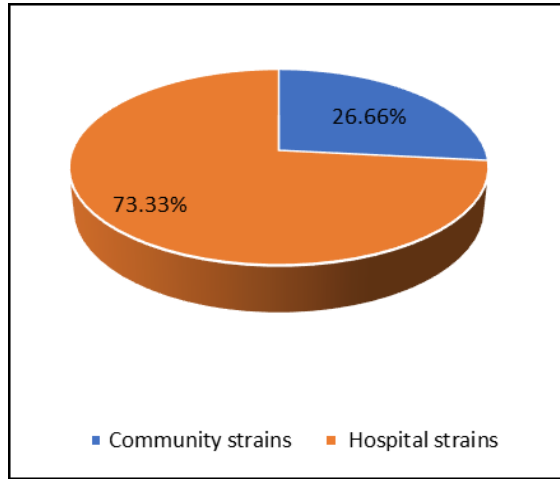


Figure 1. Distribution of isolated strains of Staphylococci.

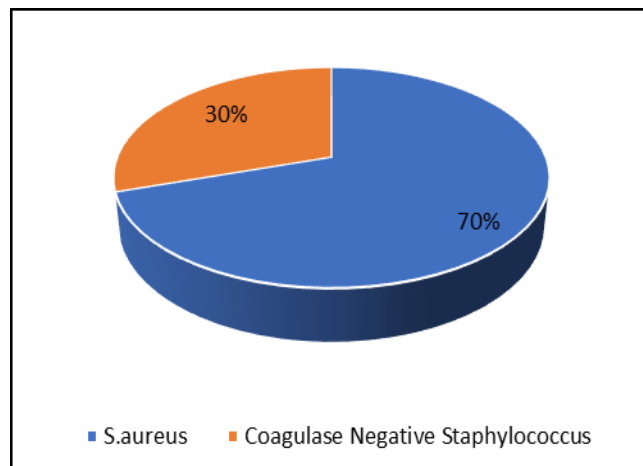


Figure 2. Distribution of strains of Coagulase Negative Staphylococcus and S. aureus identified.

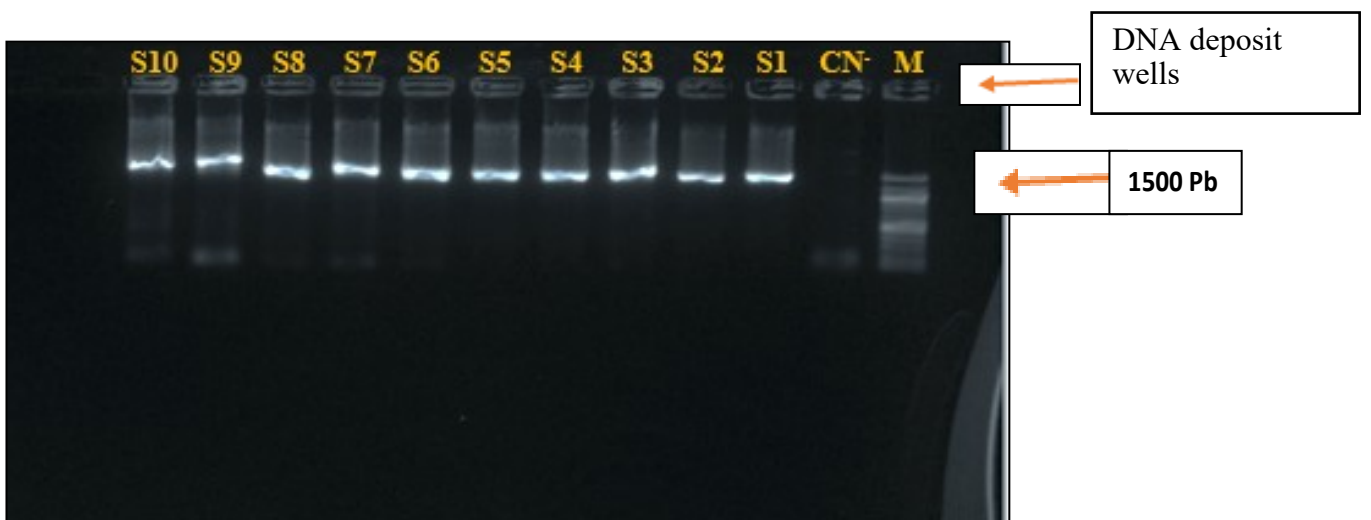


Figure 3. Electrophoresis on Agarose Gel at 1% of the PCR Product of the rRNA16S gene of strains. S1-S10 : Strains of Staphylococci ; M : ladder marker DNA (100), CN- : Negative control.

Table 1. Blastn results for strain identification by 16S rRNA gene similarity

Codes	Max Score	E. Value	% de similarité	Accession numbers	Equivalent strains
A1	1714	0.0	94,5%	OM281812.1.	Staphylococcus sp. strain PYCC 8255 16S ribosomal
A2	1725	0.0	94 %	MW308320.1.	Staphylococcus aureus strain s18 16S ribosomal RNA gene
A3	1720	0.0	94 %	MW308321.1.	Staphylococcus aureus strain s18 16S ribosomal RNA gene
A4	1727	0.0	91,2%	OP067888.1.	Staphylococcus aureus strain s20 16S ribosomal RNA gene
A5	1742	0.0	91,2%	MK809243.1.	Staphylococcus aureus strain RM_AST_SA012 Ribosomal RNA gene 16
A6	1705	0.0	92%	MK809241.1.	Staphylococcus aureus strain RM_AST_SA001 16S ribosomal RNA gene
A7	1881	0.0	93,4%	KY218856.1	Staphylococcus haemolyticus strain AP BFT16 16S ribosomal RNA gene
A8	1882	0.0	92,4%	MK934564.1.	Staphylococcus haemolyticus strain AP BFT16 16S ribosomal RNA gene
A9	1714	0.0	93,5%	GQ110719.1	Uncultured bacteria clone nbw618g09c1 16S ribosomal RNA gene
A10	1716	0.0	94,5 %	AF015929.1.	Staphylococcus aureus 16S ribosomal RNA gene

Table 2. Sequences of isolates identified by BLASTn 16S rRNA gene analysis.

Codes	Sequences of strains identified
A1	Staphylococcus sp. Strain BMGLG21A1
A2	Staphylococcus aureus Strain BMGLG21A2
A3	Staphylococcus aureus Strain BMGLG21A3
A4	Staphylococcus aureus Strain BMGLG21A4
A5	Staphylococcus aureus Strain BMGLG21A5
A6	Staphylococcus aureus Strain BMGLG21A6
A7	Staphylococcus haemolyticus Strain BMGLG21A7
A8	Staphylococcus haemolyticus Strain BMGLG21A8
A9	Uncultured bacteria Strain BMGLG21A9
A10	Staphylococcus aureus Strain BMGLG21A10

database, using the bioinformatics algorithm BLASTn. The analysis of this table shows, according to the percentage of similarity, a diversity of species of the identified strains.

Multiple Alignment of the Sequences Obtained and Their Homologs in The Databases

Figure 4 represents part of the multiple alignment of the sequences of the RNA16S gene corresponding to all the strains of staphylococci identified and the homologs in the database in particular by using the CLUSTAL W algorithm. These results highlight the highly conserved regions at the level of all the strains due to the T-T-T-T-T-T motif present at the level of all the identified strains, absent at the level of the strain (unidentified clone), as well as mutations (loss of nucleotides).

Phylogenetic Classification of the Strains Identified by the Sequencing of 16S rDNA

Figure 5 shows the phylogenetic tree of the identified strains, the species diversity is clearly visible. This tree shows distances of 0.2% (0.002) between strains and that the strains have a common ancestor.

The evolutionary history was deduced using the UPGMA method [13]. The optimal tree with sum of branch length = 0.05128113 is displayed. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were calculated using the p-distance method [14] and are expressed as the number of baseline differences per site. The analysis focused on 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with gaps and missing data have been eliminated. There were a total of 1156 positions in the final dataset. Evolutionary analyzes have been carried out in MEGA7 [12].

Discussion

Although classical bacteriological methods remain the reference for bacterial identification, we used the molecular method based on the analysis of the gene coding for 16S rRNA for the confirmation of the isolated staphylococcus strains and the different related species identified in this work. Analysis of the results by conventional bacteriological methods show a high

percentage of *staphylococcus* strains from hospital settings (clinical strains) with a prevalence of 67.50%, these results are close to those of [15] and [16]. All strains of Staphylococcus were identified as Gram positive and catalase positive bacteria. The results of the fresh state showed Cocci in immobile clusters at the level of all the strains.

1% agarose gel electrophoresis of the strains' 16S rRNA gene PCR products showed a length of approximately 1500 bp. This size (1500Pb) has been endorsed by several studies which have shown the importance of the 16S rRNA gene in the classification and identification of the molecular genus and species of prokaryotes identical to the results obtained by, [17] [18]. [19]. This size indicates that the strains belong to the large group of prokaryotes and certainly to the genus Staphylococcus. Analysis by BLASTn of the sequences of the PCR products, A2, A3, A4, A5, A6, A10 and A7, A8, A9, A10 originating respectively from the strains S2, S3, S4, S5, S6, S10 (*S.aureus*) s and S7,S8,S9,S6 (SCN) gave E values of 0.0 for all sequences, for each isolate the equivalent based on % similarity of 94.5% with A1 *Staphylococcus*.sp (OM281812.1), A2 94 % *S.aureus* (MW308320.1), A3 94 % *S.aureus* (MW308321.1), A4 91.2 % *S.aureus* (OP067888.1), A5 91.2 % *S .aureus* (MK809243.1), A6 92 % *S. aureus* (MK809241.1), A7 93.4 % *Staphylococcus haemolyticus*, A8 92.4% *Staphylococcus haemolyticus* (MK934564.1 ,A9 93.5% Bacterium clone uncultivated nbw618g09c1 (GQ110719.1), A10 94.5% *S.aureus* (AF815929.1) . The E value as well as the percentage of sequence similarity of the rRNA16S gene of the identified strains show that the results obtained are significant, and make it possible to infer the belonging of the strains, S2, S3, S4, S5, S6, S10 to the species *S. aureus*, confirming the identification par the coagulase test; from S7 and S8 to the *Staphylococcus haemolyticus* species and the S9 strain to a bacterial clone. The results are different from those presented by [20] during the 15th National Day of Infectiology on the limits and indications of universal PCR of 16S rDNA, indicating the identification of isolates of *S. aureus* and *S. epidermidis* at a similarity percentage > 98%. Blast analysis also allowed the identification of two

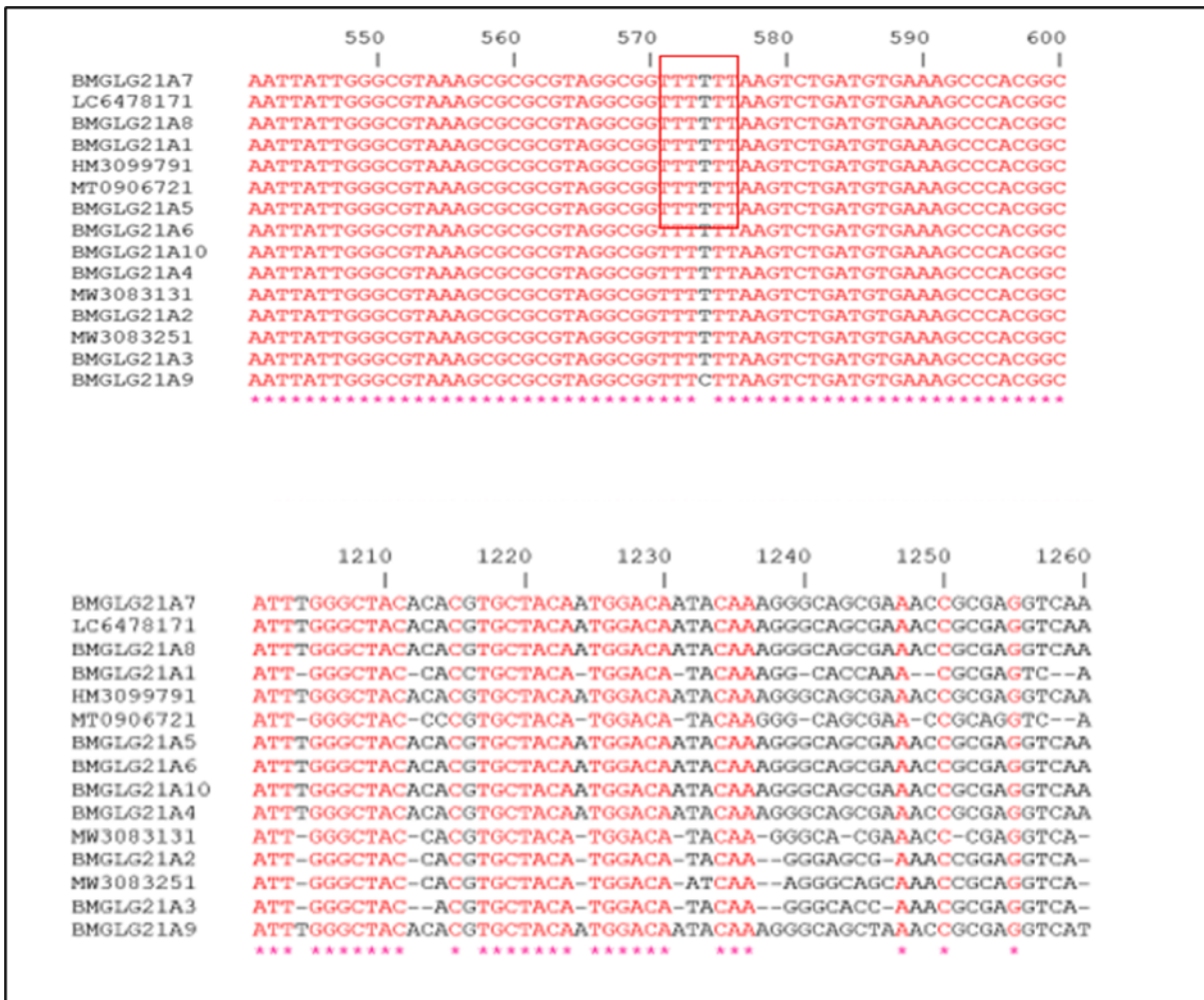


Figure 4. Part of the multiple alignment of the gene sequences encoding the 16S rRNA of the identified strains and the homologs sequences from the databases.

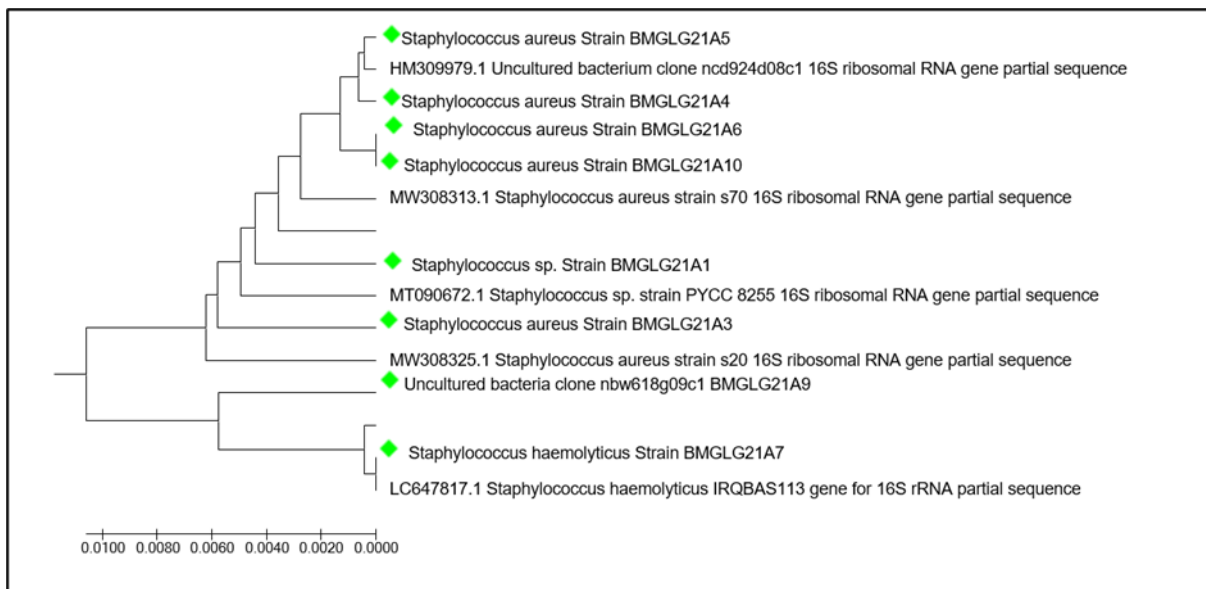


Figure 5. Phylogenetics of strains identified by the 16S rRNA gene and their homologs from databases.

Staphylococcus haemolyticus species and of a bacterial clone of the 4 SCN strains identified by the coagulase test; these results show the limits of traditional bacteriological methods in the complete identification of bacteria.

The analysis of the multiple alignment of the sequences of the RNA16S gene corresponding to all the strains of staphylococci identified and the homologs in the database using the CLUSTAL W algorithm allowed the highlighting of the mutations (losses of nucleotides). These results could explain the low rate of the percentage of similarity observed between the identified sequences and the homologs in Gen Bank. The multiple alignment of the sequences also made it possible to highlight highly conserved regions between the sequences, reflecting the belonging of the identified strains to the same family, to the same genus. These results also show that the strains have undergone evolutionary changes over time, evolutionary changes that are confirmed by the established Phylogenetic tree.

Conclusion

This study made it possible to confirm the identification of Staphylococcus strains made by microbiological methods based on morphological and biochemical characters (coagulase test) by molecular biology techniques, more precisely by the amplification of the 16S rRNA gene from DNA extracts from ten strains. The amplification of the 16S RNA gene is a universal, precise and objective method, whose inter-operator variability is limited compared to conventional techniques, it has enabled us to broaden our knowledge of the bacterial world, more precisely of the genus staphylococcus both at the level of the species and of the genus by highlighting the existing phylogenetic interferences.

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