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Identification of Quinolones/Fluoroquinolones Resistance Genes from Staphylococci Strains Isolated at the University Hospital of Brazzaville, Republic of the Congo

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Abstract

Staphylococci strains, like the majority of bacterial strains, have developed the resistance to several antibiotics, including Quinolones and Fluoroquinolones In the Republic of the Congo, cases of resistance leading to treatment failures have been observed during the treatment of staphylococcal infections with antibiotics in hospitals. The objective of this study was to identify the Quinolones/Fluoroquinolones resistance genes from staphylococci strains isolated in hospitals. A total of 51 strains of Staphylococci were isolated, including 16 (31.37%) community strains, and 35 (68.62%) clinical strains. 46 strains of *Staphylococcus aureus* (*S. aureus*) and 5 SCNs were identified. A total of 34 DNA fragments from different strains resistant to Quinolones/Fluoroquinolones, including 21 (61.67%) DNA fragments from clinical *S. aureus* and 13 (38.23%) from community SCN strains were analyzed by the molecular method (genotypic detection) by PCR. The genotypic results made it possible to identify the gyrA, grLA and norA genes and to show that these genes are involved in the resistance of the strains to the various antibiotics used. The grLA gene was the most identified gene with a frequency of 75%. The gyrA and grLA genes have been identified in *Staphylococcus aureus* and Coagulase Negative Staphylococci. The norA gene, on the other hand, has only been identified in

Staphylococcus aureus. Two mechanisms are essentially involved in the resistance of Staphylococci to quinolones/Fluoroquinolones, the mechanism of resistance by efflux, which takes place thanks to a transmembrane protein coded by the norA gene and by point mutations (substitution and deletion of acids or nucleotides) observed within the protein and nucleic sequences of the chromosomal gyrA and grLA genes.

Keywords

Genes, Resistance, Quinolones/Fluoroquinolones, Staphylococci

1. Introduction

The human body harbors several microorganisms, which can be commensal or pathogenic. Once they become pathogens, they are responsible for many infectious diseases [1]. This is the case of Staphylococci bacteria responsible for many infectious foci which can disseminate and cause sepsis, endocarditis (infection of the endocardium), osteomyelitis (infection of the bone tissue) [2] [3] of these infections is based on the use of several families of antibiotics (ATBs), which are molecules capable of killing the bacteria (bactericidal) or preventing its multiplication (bacteriostatic). These infections are not at all easy to treat, and treatment failures occur.

Indeed, bacteria have been able to develop many resistance mechanisms. Currently, we are seeing an increase in resistance reaching alarming levels against antibiotics [4]; these resistances could be due to the acquisition of a growing number of resistance determinants to multiple antibiotics, among which we cite genomic mutations, responsible for the production of enzymes that destroy antibiotics and, on the other hand, their invasive and toxigenic power of the secretion of enzymes and toxins allowing them to escape the control of the immune system.

Staphylococcal germs have been able to develop resistance to several antibiotic families, including the Quinolones/Fluoroquinolones family which are classified into 4 generations, 1st generation (nalidixic acid), 2nd generation (norfloxacin, ciprofloxacin, Ofloxacin), 3rd generation (levofloxacin and 4th generation (moxifloxacin) [5]. Although they are antibiotics whose use as a last resort both in human medicine and in veterinary medicine should have been privileged, they have not been spared by this phenomenon [6]. In addition, the frequent and wide use of these molecules is one of the causes of the emergence and spread of resistant strains of staphylococci; any previous intake of Fluoroquinolones (FQ) is also a higher individual risk of being infected with FQ-resistant bacteria [7]. These antibiotics are potent antibacterial agents whose targets are bacterial enzymes, bacterial DNA topoisomerases d e type II (DNA gyrAse and topoisomerase IV). The gyrAses are encoded by the gyrA and gyrB genes and the topoisomerases IV on the one hand by the parC, parE and grLA and grLB genes (in *Staphylococcus aureus*) at the chromosomal level [8].

Three mechanisms are essentially involved in the resistance of these strains to the ATBs used. Modification of the target which involves a mutation in the chromosomal genes grLA or grLB of topoisomerase IV and the alteration of the A or B subunits of gyrAse by introducing a mutation within the gyrA or gyrB genes [9] [10].

The mechanism of resistance by efflux occurs through a transmembrane protein encoded by the chromosomal norA gene [11]. Resistance genes can also be brought by plasmids or other mobile genetic elements such as transposons [12]. Several studies have been carried out on the resistance mechanisms of staphylococci to antibiotics [13] [14] [15] [16]. In the Republic of the Congo, cases of resistance leading to treatment failures have been observed during the treatment of Staphylococcal infections by antibiotics in hospitals and in the community [17]. Thus, several phenotypic studies of resistance by antibiogram method using strains of staphylococci of hospital and community origin have been carried out with Quinolones/Fluoroquinolones, making it possible to confirm the resistance of these strains to these molecules [18] [19] [20]. However, to explain the causes of resistance of strains of staphylococci to antibiotics observed in our country, to our knowledge only one study on the determinism of resistance of staphylococci to antibiotics is currently cited. This study is limited to the families of Macrolides and related Lincosamides and Streptogramins B [21]. No study on the genetic determinism of resistance of staphylococci to Quinolones/Fluoroquinolones has been carried out to date. Thus the objective of this study is to identify the genes responsible for the resistance of staphylococci to Quinolones/Fluoroquinolones of strains isolated at CHUB.

2. Materials and Methods

2.1. Isolation, Identification and Antibiotic Resistance of Strains

Fifty-one (51) strains of staphylococci including thirty-eight (35) clinical and thirteen (16) communities were isolated on a selective agar medium called Chapman* agar, and 46 strains of *S. aureus* and 5 SCN were identified by following the morphological characters by the Gram staining technique, and by biochemical characters obtained by the catalase and coagulase test [22]. The results of the antibiotic resistance of 40 strains of *S. aureus* (27 clinical and 13 community) of the 51 strains isolated are presented in [20]. The resistance profile of the 11 community strains including 5 SCN and 6 *S. aureus* was evaluated by standard antibiogram (by the phenotypic method) [23] [24] [25].

2.2. Molecular Detection of Resistance Genes

2.2.1. Choice of Resistance Genes to Be Studied

According to the diversity of genes encoding for the resistance of staphylococci to quinolones/Fluoroquinolones, a silico analysis was conducted in order to select a few genes. The genes selected for the study are: parC, grLA, gyrA, and the norA gene.

2.2.2. DNA Extraction

DNA from 34 strains of Staphylococci exhibiting resistance to Quinolones/Fluoroquinolones was extracted using the standard method of chromosomal DNA extraction [26]

2.2.3. PCR Amplification of Genes Encoding Quinolones/Fluoroquinolones Resistance

1) Primers design

Primers used in this study have been already selected and used by some authors [27] [28] [29]. These primers used are shown in **Table 1**.

2) Dilution of Primers, Mix Preparation and PCR Conditions

The primers being freeze-dried, a volume of sterile water was added according to the manufacturer's recommendations in order to obtain the primers concentrated at 100 µM. The PCR amplification reaction mixture (25 µL) contained 4 µL of DNA template and 21 µL of the mix. For all types of genes, the mix consisted of 4 µL of PCR water, 1 µL of F primer, 1 µL of R primer, and 15 µL of max mix. PCR amplification was performed according to the nature of the gene. An initial denaturation at 94˚C for 10 min, 25 cycles each of the cycle comprising: denaturation at 94˚C for 20 seconds, hybridization at 55˚C for 20 seconds, elongation at 72˚C, 20 seconds and a final elongation at 72˚C for 50 seconds for the parC and gyrA genes; initial denaturation at 95˚C for 2min, 30 cycles each of the cycle comprising: denaturation at 95˚C for 15 seconds, hybridization at 60˚C for 20 seconds, elongation at 72˚C, 20 seconds and a final elongation at 72˚C for 10 minutes for the norA gene and for the grLA gene 25 cycles each of the cycle comprising: denaturation at 94˚C for 30 seconds, hybridization at 52˚C for 30 seconds, elongation at 70˚C, 1 minute.

3) Agarose gel electrophoresis of PCR products

The PCR products were submitted to agarose gels electrophoresis, depending on the size of the gene fragment to be visualized (0.5% for the genomic DNA-fragments, 1% and 1.5% for the fragment ParC, 2% for the gyrA and grLA fragments, 2.5% for the norA fragment) at 100 volts for 40 minutes with TBE buffer.

Table 1. PCR primers used in this study.

Target genes	Primers	Sequences (5 → 3')	Size of amplified fragment	Position	Références
parC	parC F	ACTTGAAGATGTTTTAGGTGAT	559	2402	
	parC R	TTAGGAAATCTTGATGGCAA	559	559	
gyrA	gyrA F	AATGAACAAGGTATGACACC-	223	2311	[27]
	gyrA R	TACGCGCTTCAGTATAACGC	223	2533	
grLA	grLA F	TTCCGTAAAAGTGCGAAAACAG	176	178 - 199	
	grLA R	CGCATTGCCGCTGGCGGATCCTTATCGATAC	176	323 - 353	[28]
norA	norA F	TTTGTTTTCAGTGTCAGAATTTATGTTTG	140		
	norA R	GGCTTGGTGAAATATCAGCTATTAAAC	140		[29]

The staining was carried out with a 1 µg/mL solution of ethidium bromide. The gels were visualized under a UV lamp, by fluorescence.

2.2.4. Sequencing and Assembly of PCR Products

Eleven (11) PCR fragments of the gyrA, grLA and norA genes from different strains resistant to quinolones/Fluoroquinolones including 3 PCR fragments of the gyrA gene of 223 Pb, from clinical SCN strains (4S9, S51 and S2l); 5 PCR fragments of the grLA gene from 176 Pb, from strains of *S. aureus* (S100, S18, S95, S35, S53) and 3 PCR fragments of the norA gene of 140Pb, from strains of (S55, S3, S79) were purified using the PCR plate NucleoFast 96 (Macherey-Nagel EURL, France) and sequenced using BigDye terminator chemistry on an ABI3730 sequencer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were assembled using the DNA Baser sequence assembler.

2.2.5. *In Silico* Analysis of Resistance Gene Sequences

Sequence analysis 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 translation of the nucleic sequences into protein sequences was carried out on SMS-ORF Finder. Alignment of protein sequences was done on ClustalW. Molecular phylogenetic and evolutionary analyzes were performed using MEGA version 7 [30].

3. Results

3.1. Resistance Phenotypes of Eleven (11) Strains of Staphylococci

Figure 1 shows the different levels of sensitivity and resistance of the strains with respect to the antibiotics tested. (Ciprofloxacin, Ofloxacin, Levofloxacin, Norfloxacin, Moxifloxacin, Nalidixic Acid). Figure 1(a) and Figure 1(b) show that strains 18 and 24 were resistant to Ofloxacin, Levofloxacin, Ofloxacin, Ciprofloxacin, Nalidixic Acid and sensitive to Moxifloxacin.

All strains were tested with antibiotics from the same Quinolones/Fluoroquinolones family (Ofloxacin, Ciprofloxacin, Levofloxacin, Nalidixic Acid, Moxifloxacin

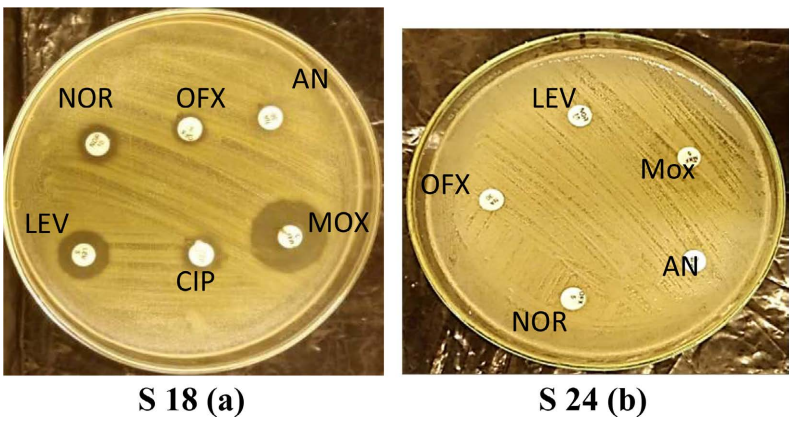

Figure 1. Levels of sensitivity and resistance of the strains according to the tested antibiotics.

and Ofloxacin). Table 2 represents the resistance rates of the strains; it emerges from the analysis of the results of this table that all the strains were sensitive to Moxifloxacin, with a high sensitivity rate of 100% in SCN and resistant to other antibiotics with the high resistance rate of 100% to Nalidixic Acid in both species.

3.2. Identification of Antibiotic Resistance Genes

3.2.1. List of Amplified Genes According to Strains

Table 3 presents the list of strains used for the identification of resistance genes.

3.2.2. Agarose Gel Electrophoresis of Genomic DNA

A total of 34 DNA fragments were extracted from 34 clinical strains (Table 2)

Table 2. Levels of resistance in staphylococci strains.

Staphylococci strains						
Staphylococcus aureus (6)				NCS (5)		
Tested ATBs	R (%)	I (%)	S (%)	R (%)	I (%)	S (%)
CIP	5 (83.33%)	0 (%)	1 (16.16%)	4 (80%)	0 (0%)	1 (30.76%)
NOR	5 (83.33%)	0 (%)	1 (16.16%)	3 (60%)	0 (0%)	2 (38.46%)
LEV	4 (66.66%	0 (%)	2 (33.33%)	4 (80%)	0 (0%)	1 (38.46%)
OFL	4 (66.66%)	0 (%)	2 (33.33%)	4 (80%)	0 (0%)	1 (38.46%)
MOX	1 (16.16%)	0 (%)	5 (83.33%)	0 (0%)	0 (0%)	5 (100%)
A N	6 (100%)	0 (%)	0 (%)	5 (100%)	0 (0%)	0 (0%)

Tested ATBs = tested Antibiotics; n = nomber of strains. NOR: Norfloxacin, CIP: Ciprofloxacin, MXF: Moxifloxacin, LEV: Lévofloxacin.

Table 3. List of the Staphylococci strains used for the identification of Quinolones/Fluoroquinolones resistance genes.

1	**U172 (S16)**	**S.a**	**11**	**PV170 (S53)**	**SCN**	**21**	**PV 15 (S90)**	**NCS**	**31**	**H112 S (24)**	**NCS**
2	LP09 (S8)	S.a	12	PY005 (S54)	S.a	22	U808 (S77)	S.a	32	U44 (S79)	S.a
3	H120 (S39)	S.a	13	PV209 (S15)	S.a	23	R68 (S92)	S.a	33	11-08-20 (S91)	S.a
4	PV175 (S29)	S.a	14	U170 (S55)	S.a	24	PV20 (S84)	S.a	34	PV12 (S96)	S.a
5	R98 (S11)	S.a	15	PV (S35)	S.a	25	PV109 (S89)	S.a			
6	21-08-20 (S49)	NCS	16	PY044 (S18)	S.a	26	U17 (S85)	NCS			
7	PY095 (S3)	S.a	17	H85 (S9)	S.a	27	H20 (S95)	S.a			
8	30-07-20 (S100)	S.a	18	H10 (S60)	S.a	28	21-08-20 (S101)	S.a			
9	H60 (S5)	S.a	19	U9 (S61)	S.a	29	10-06-20 (S97)	S.a			
10	3-07-20 (S51)	NCS	20	PV168 (S34)	S.a	30	H7 (S93)	S.a			

resistant to the antibiotics used (CIP, NOR, LEV, MXF, OFL and AN), including (4) SCN and (30) *S. aureus* to determine genes responsible for resistance.

Figure 2 and Figure 3 show extracts of genomic DNA fragments of staphylococcal strains obtained by electrophoresis on 0.5% agarose gel.

3.2.3. Electrophoresis of PCR Results

1) The norA gene

Figure 4 shows the DNA fragments obtained by PCR amplicon agarose gel electrophoresis of the norA gene from 6 strains of *S. aureus*.

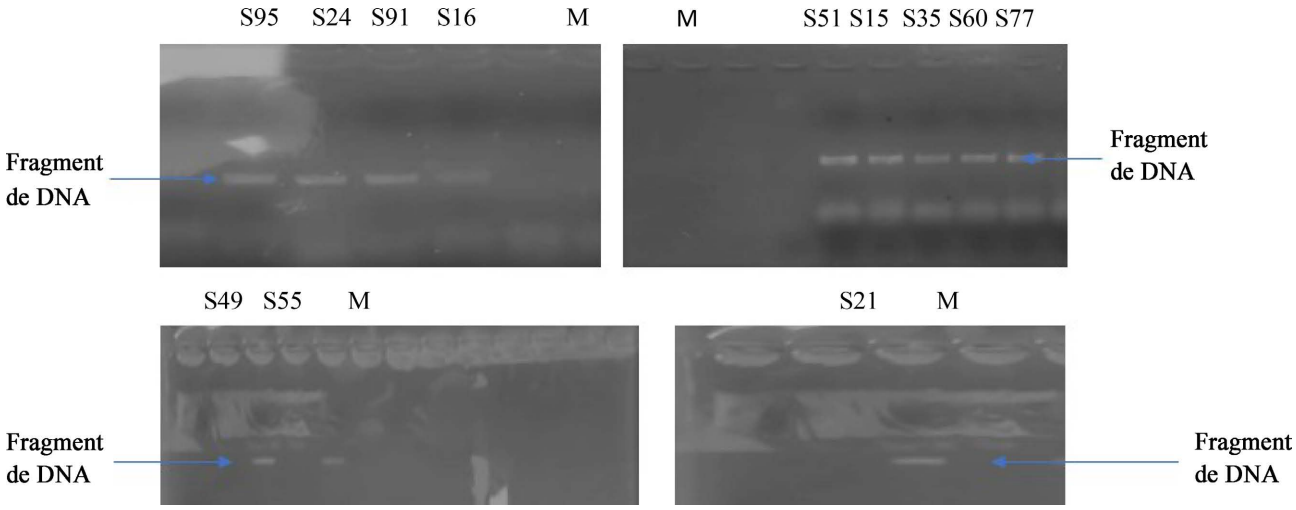

Figure 2. Electrophoresis on 0.5% agarose gel of genomic DNA extracts from Staphylococci strains (S95, S24, S91, S49, S55, S51, S15, S35, S60, S77, S21), M = DNA labder marker (100 Pb).

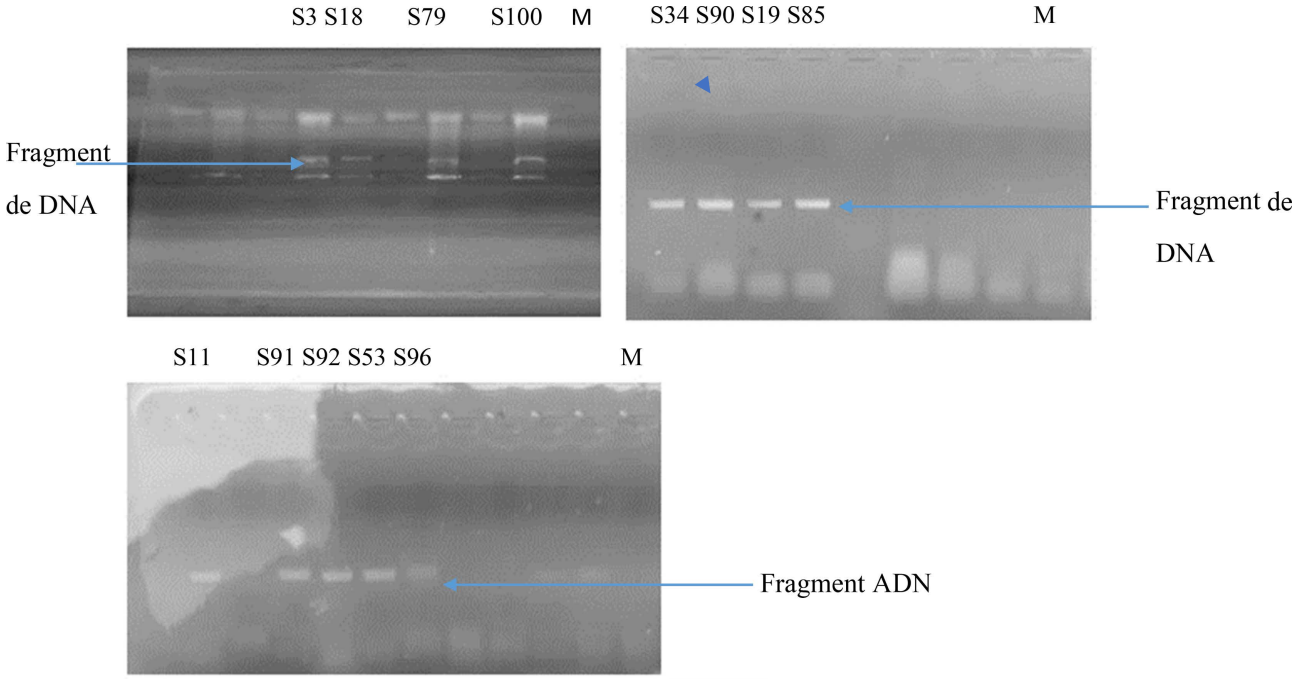

Figure 3. Electrophoresis on 0.5% agarose gel of genomic DNA extracts from Staphylococci strains (S3, S18, S79, S100, S34, S90, S95, S85, S11, S91, S92, S53, S96), M = labder DNA marker (100 Pb).

2) The grLA gene

Figure 5 shows the DNA fragments obtained by PCR amplicon agarose gel electrophoresis of the gyrA gene obtained with 10 strains of *S. aureus* and 1 of SCN.

3.2.4. Synthesis of PCR Results

Analysis of the DNA fragments obtained by PCR amplicon agarose gel electrophoresis of the various genes obtained showed (Table 4) that the gyrA, grLA and norA genes were identified except for the parC gene.

Figure 4. Electrophoresis on agarose gel at 2.5% PCR amplicon of the norA gene obtained with DNA from staphylococci strains. M = labder DNA marker (100 Pb); T = negative control.; S79, SS9, S11, S3, S15 and S55 = *S. aureus* strain fragments.

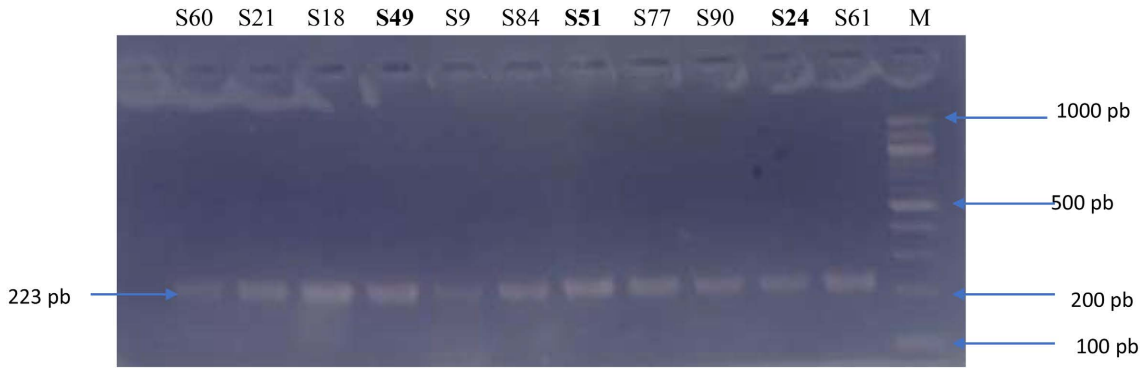

Figure 5. Electrophoresis on agarose gel with 2% PCR amplicon of the gyrA gene obtained with the DNA of strains of staphylococci. M = labder DNA marker (100 Pb). S60, S9, S18, S9, S84, S77 and S61 = *S. aureus* strain fragments. S24, S90, S49 and S51 = NCS strain fragment.

Table 4. Identification of resistance genes according to the species.

Genes	Souches
norA	*S. aureus*
parC	0
gyrA	*S. aureus* SCN
grLA	*S. aureus* SCN

Analysis of DNA fragments obtained by PCR amplicon agarose gel electrophoresis of the various genes obtained showed (Table 5) that one of the three genes (norA, gyrA or grLA) was identified in S16, S100, S55, S9, S34, S92, S97. Two of the three genes (norA and gyrA, norA and grLA, gyrA and grLA) were identified in the strains (S24, S85, S89, S84, S77, S90, S60, S18, S35, S54, S53, S51, S49 and S39), and the three genes at the same time (norA gyrA grLA) in the strains S9, S11, S3, S15, S95, S79. In addition the genes norA, gyrA and grLA were not identified in strains S29, S5, S101, S93, S91, S96. The norA gene was identified with an isolation frequency of 38% only in *S. aureus*.

And that The grLA gene was identified in strains with a frequency of 65%, followed by the gyrA genes at 58.82% and norA at 29.41%.

3.2.5. Bioinformatics Analysis of Amplified Genes

The sequences were analyzed using the BLASTn tool, which confirmed that the strains belonged to the genus *Staphylococcus* and to the species *Staphylococcus aureus*, and Coagulase Negative *Staphylococcus*. The nucleic sequences of the strains were translated into protein sequences by SMS-ORF finder by searching for ORFs in reading frames 1, 2 and 3 on the forward and reverse strand. The proteins chosen for further analysis are the longest, *i.e.* formed by the greatest number of amino acids.

1) The norA gene

a) Multiple alignment of the nucleotide sequences of the strains and the reference sequence downloaded in GenBank.

Figure 6 represents the multiple alignment of the nucleotide sequences of the strains (S79, S3 and S55) and the reference sequence NC_016941.1 using the CLUSTAL W algorithm. These results highlight conserved regions, such as at position 270 - 274, a region highly conserved across all strains due to the T-T-T-T-T motif. Comparing to the reference sequence (NC_0169…), our sequences from the strains (S79, S3, S55) display some substitutions at the positions 244, 258, 261, and 264.

b) Multiple alignment of the protein sequences of the strains and the protein sequence of the reference norA gene uploaded from GenBank.

Figure 7 represents the extract of the multiple alignment of the three protein sequences of the norA genes isolated from *Staphylococcus aureus* and of the protein sequence of the reference norA gene downloaded in GenBank on the number WP_001041324.1 on ClustalW.

Alignment of protein sequences shows conserved regions. However, conservative mutations and one semi-conservative were observed on our sequences in comparison with the reference sequence. The mutations observed are common to all Al (80) Val Ile (93 and 99) Val strains. The mutations do not affect the N-terminal, C-terminal or active site domains of the proteins.

c) Phylogenetic tree

Figure 8 shows the phylogenetic tree of the different norA genes. The analysis of this tree shows 100% at the node level, *i.e.* the distance between the stumps of

Table 5. Involved in resistance against antibiotics genes in this study

strains	Resistances genes		
	gyrA	grLA	norA
S16			x
S9	x	x	x
S39	x	x	
S29			
S11	x	x	x
S49	x	x	
S3	x	x	x
S100		x	
S5			
S51	x	x	
S53	x	x	
S54	x	x	
S15	x	x	x
S55			x
S35	x	x	
S18	x	x	
S19		x	
S60	x	x	
S61		x	
S34		x	
S90	x	x	
S77	x	x	
S92		x	
S84	x	x	
S89	x	x	
S85	x		x
S95	x	x	x
S101			
S97		x	
S93			
S24	x		x
S79	x	x	x
S91			
S96			

```
                                  190       200       210       220       230       240
                                   |         |         |         |         |         |
norAS55                  ----------------------------------------------TTTGTTTTCAGTG
norAS03                  ------------------------------------------------------GTG
norAS79                  ----------------------------------------------TTTGTTTTCAGTG
NC_0169411726977_728137  TTGGCTGACAAACTAGGTAAAAAGTTAATTATTTGTATTGGGTTAATTCTTTTTTCTGCT
                                                  *

                                  250       260       270       280       290       300
                                   |         |         |         |         |         |
norAS55                  TCAGAATTTATGTTTGCAGTTGGCCACAATTTTTCGGTATTGATGTTATCGAGAGTGATT
norAS03                  TCAGAATTTATGTTTGCAGTTGGCCACAATTTTTCGGTATTGATGTTATCGAGAGTGATT
norAS79                  TCAGAATTTATGTTTGCAGTTGGCCACAATTTTTCGGTATTGATGTTATCGAGAGTGATT
NC_0169411726977_728137  TCTGAATTTATGTTTGCCGTAGGACATAATTTTTCAATATTAATGTTATCAAGAATTATT
                         ** ************** ** ** ** ********  **** ******** *** * ***

                                  310       320       330       340       350       360
                                   |         |         |         |         |         |
norAS55                  GGTGGTATGAGTGCTGGTATGGTAATGCCTGGTGTGACAGGTTTAATAGCTGATATTTCA
norAS03                  GGTGGTATGAGTGCTGGTATGGTAATGCCTGGTGTGACAGGTTTAATAGCTGA-------
norAS79                  GGTGGTATGAGTGCTGGTATGGTAATGCCTGGTGTGACAGGTTTAATAGCTGATATTTCA
NC_0169411726977_728137  GGTGGTATGAGTGCTGGCATGGTTATGCCTGGCGTAACAGGCTTAATCGCTGATATTTCG
                         ***************** ***** ******** ** ***** ***** *****
```

Figure 6. multiple alignment of nucleic sequences of strains (S79, S3, S55).

```
                          70        80        90       100       110       120
                           |         |         |         |         |         |
norA55          ----------------LFSVSEFMFAVGHNFSVLMLSRVIGGMSAGMVMPGVTGLIADIS
norAS3          --------------------VSEFMFAVGHNFSVLMLSRVIGGMSAGMVMPGVTGLIA---
norA79          ----------------LFSVSEFMFAVGHNFSVLMLSRVIGGMSAGMVMPGVTGLIADIS
WP_0010413241   LADKLGKKLIICIGLILFSASEFMFAVGHNFSILMLSRIIGGMSAGMVMPGVTGLIADIS
                              .************:*****:******************
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Figure 7. Multiple alignment of protein sequences encoded by norA gene (ClustalW).

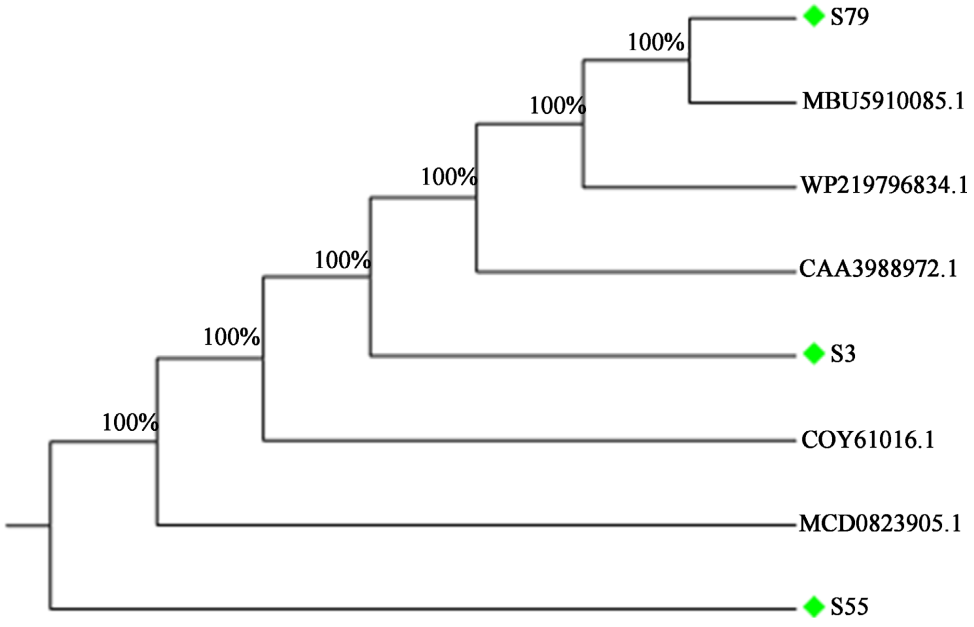

Figure 8. Phylogenetic tree of different norA genes.

0%, and that the stumps have a common ancestor.

The evolutionary history was deduced using the UPGMA method [31]. The optimal tree 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 Poisson correction method [32] and are expressed as the number of amino acid substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each descendant clade is shown next to each internal node in the tree. The analysis focused on 8 amino acid sequences. All positions with gaps and missing data have been eliminated. There were a total of 38 positions in the final dataset. Evolutionary analyzes were carried out in MEGA7 [30].

2) The gyrA gene

a) Multiple alignment of the nucleic acid sequences of the strains and of the reference sequence downloaded in GenBank.

Figure 9 represents the multiple alignment of the nucleic sequences of the strains (S49, S24, S51) and the reference sequence NC_000913.3 using the CLUSTAL W algorithm. These results highlight conserved regions as well as the numerous changes evolutionary, manifested by the various point mutations (substitutions) common to all strains and those which are specific to each strain.

b) Multiple alignment of the protein sequences of the strains and the protein sequence of the reference gyrA gene uploaded in GenBank

Figure 10 represents the extract of the multiple alignment of the 3 protein sequences of the gyrA genes isolated from SCN and the protein sequence of the reference gyrA gene uploaded in GenBank on ClustalW GenBank on number WP_003723770.1.

The analysis of the figure shows conserved regions, conservative mutations, semi-conservative mutations and indels. Certain mutations were common to S49, S24, S51, Ser (60) Pro, Glu (73) Met, Thr (84) Ser, Ala (85) Ser, Val (86) Ile are mentioned. Other mutations were specific to strain S24 and S51: Ala (63) Pro, Val (113) Met and S49: Ala (63) Ser, Gly (108) Asp, Ala (104) Val, Asn (109) Lys, Met (121) Leu, and the mutation that affects the active site Tyr (123) Cys.

c) Phylogenetic tree

Figure 11 shows the phylogenetic tree of the different gyrA genes. The analysis of this tree shows that the distance between the strains is 2% (0.02) and that the strains have a common ancestor.

The evolutionary history was inferred using the maximum likelihood method based on the Poisson correction model [32]. The tree with the highest log probability (−263.42) is displayed. The initial trees for the heuristic search were obtained automatically by applying the Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with a log-likelihood value superior. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The proportion of sites where at least 1 unambiguous base is present in at least 1 sequence for each

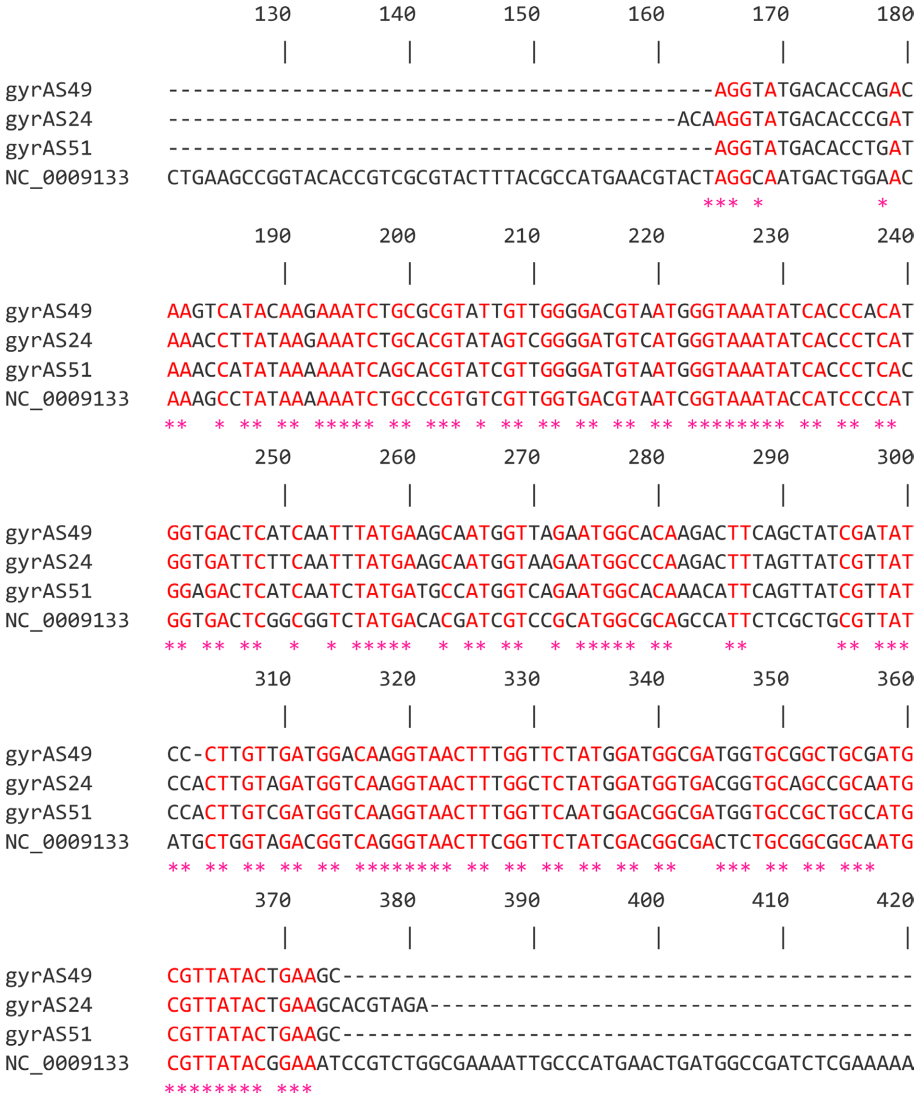

Figure 9. Multiple Alignement of nucleic séquences of the strains (S49, S24, S51).

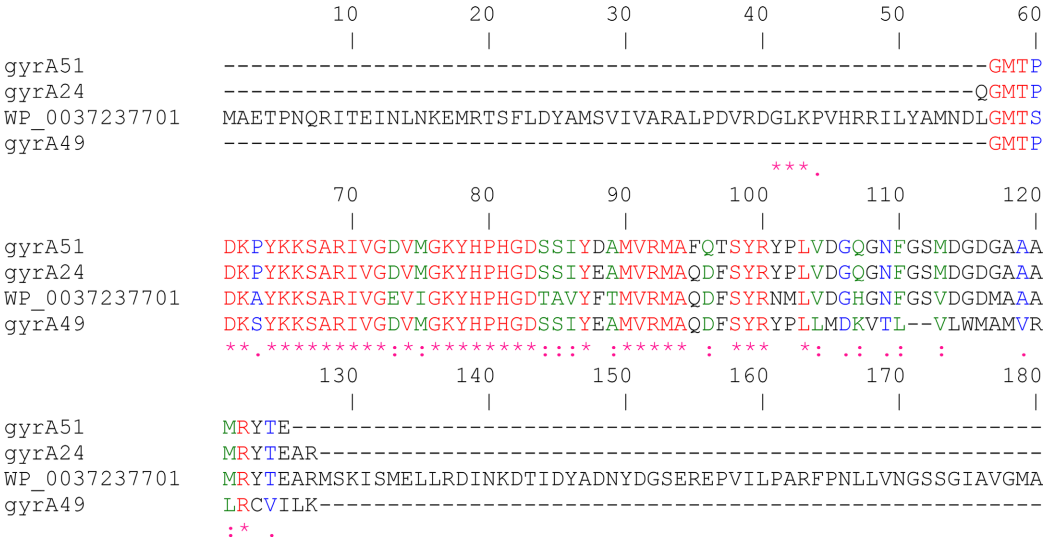

Figure 10. Multiple Alignement of proteines sequences of gyrA genes (ClustalW).

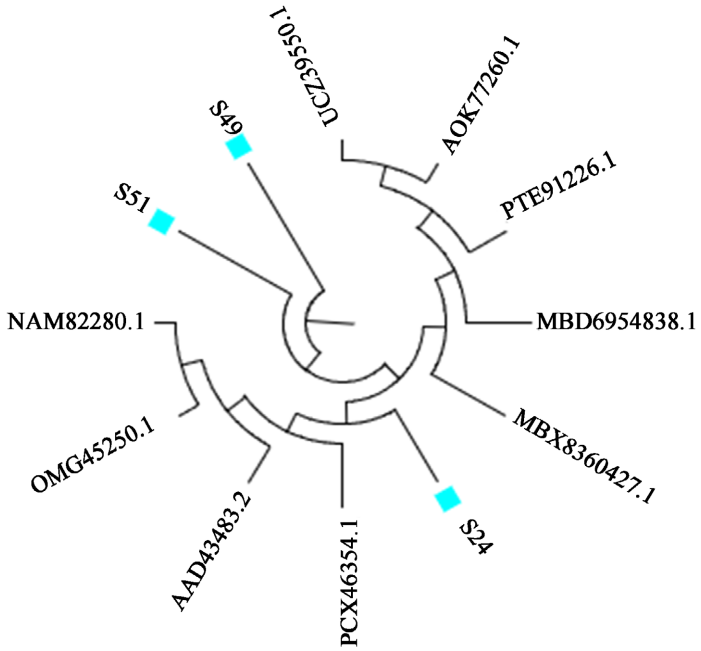

Figure 11. Phylogenetic tree of different gyrA genes.

descendant clade is shown next to each internal node in the tree. The analysis focused on 12 amino acid sequences. All positions with gaps and missing data have been eliminated. There were a total of 60 positions in the final dataset. Evolutionary analyzes were carried out in MEGA7 [30].

3) The grLA gene

a) Multiple alignment of the nucleotide sequences of the strains and the reference sequence downloaded in GenBank.

Figure 12 represents the multiple alignment of the nucleic sequences of the strains (S79, S100, S18, S95, S35, S53) and the reference sequence NC_002695.2 using the CLUSTAL W algorithm. These results highlight regions conserved reflecting a strong similarity between the strains and point mutations at the level of the sequences of the strains. Nucleotide losses were observed at positions 150, 174, 205 - 207, 260 - 269 and nucleotide substitutions

b) Multiple alignment of strain protein sequences and reference grLA gene protein sequence uploaded to GenBank

The analysis of Figure 13 shows that mutations do not affect the N-terminal, C-terminal domains or the active site of the proteins. Conserved regions, conservative mutations, and a semi-conservative mutation are observed. A conservative mutation was observed in all the protein sequences of the Asn (111) Lys strains (S18, S95, S35, S53 and S100). In the protein sequences of the strains S100, S53, S35, the Ser (80) Tyr mutation is observed, while in the protein sequences of the S95, the Glu (84) Asp, Asp (92) Gly and His (103) Gln mutations are observed.

c) Phylogenetic tree

Figure 14 shows the phylogenetic tree of the different grLA genes. The analysis

```
                          130       140       150       160       170       180
                           |         |         |         |         |         |
grLAS100      TTCCGTAAAAGTGCGAAAACAGTCGGTG-ATGTTATTGGTCAATATCATCCAC-ATGGAG
grLAS53       -------AAAGTGCGAAAACAGTCGGTG-ATGTTATTGGTCAATATCATCCAC-ATGGAG
grLAS35       ------AAAAGTGCGAAAACAGTCGGTG-ATGTTATTGGTCAATATCATCCAC-ATGGAG
grLAS95       --------AAGTGCGAAAACAGTCGGTG-ATGTTATTGGTCAATATCATCCAC-ATGGAG
grLAS18       ------AAAAGTGCGAAAACAGTCGGTG-ACGTAATTGGACAGTATCACCCTC-ATGGCG
NC_0026952    CGCAATGATATTGCCGAAGCATTCGGTATAAACCTGAGGAGAGCATCATTTATTATAACT
                        * ***  ** ** *****  *       **  *  ****      **

                          190       200       210       220       230       240
                           |         |         |         |         |         |
grLAS100      ACTACTCAGTGTACGAAGCAATGG---TCCGTTTAAGTCAAGACTGGAAGTTACGACATG
grLAS53       ACTACTCAGTGTACGAAGCAATGG---TCCGTTTAAGTCAAGACTGGAAGTTACGACATG
grLAS35       ACTACTCAGTGTACGAAGCAATGG---TCCGTTTAAGTCAAGACTGGAAGTTACGACATG
grLAS95       ACTCCTCAGTGTACGAAGCAATGG---TCCGTTTAAGTCAAGACTGGAAGTTACGACATG
grLAS18       ACTCATCTGTGTATGATGCTATGG---TAAGACTCAGTCAGGGATGGAAGCTGCGTCATG
NC_0026952    TATATATCGAGAAGAAAAGAAAAAATTTCATTTCGTGTCAGATATGTTAGTTATGGTAAT
                *     * * *  *    *       *        ****    **  ** * *  *  *

                          250       260       270       280       290       300
                           |         |         |         |         |         |
grLAS100      TCTTAATAGAAATGCATGG----------TAATAATGGTAGTATCGATAAGGATCCGCCA
grLAS53       TCTTAATAGAAATGCATGG----------TAATAATGGTAGTATCGATAAGGATCCGCCA
grLAS35       TCTTAATAGAAATGCATGG----------TAATAATGGTAGTATCGATAAGGATCCGCCA
grLAS95       TCTTAATAGAAATGCATGG----------TAATAATGGTAGTATCGATAAGGATCCGCCA
grLAS18       TGCTCATAGAAATGCAAGG----------GAATAACGGTAGTATCGATAAGGATCCGCCA
NC_0026952    TTGCATTATAAGCGCCTTGAGATTTTCATTTATGATGTTAACCTTGAGGCGGTTCCGATA
              *     ** **  **   *            ** * * **   * **   ** **** *

                          310       320       330       340       350       360
                           |         |         |         |         |         |
grLAS100      GCGGCAAGCGGCGCACGAA------------------------------------------
grLAS53       GCGGC------------------------------------------------------
grLAS35       GCGGCAAGCGA------------------------------------------------
grLAS95       GCGTCCGCCAGCGGC---------------------------------------------
grLAS18       GCGG-------------------------------------------------------
NC_0026952    GAAAGTCCTGGAACAACCGGACCAAAAAGAAAAACCTACCGAGTTGGTAATGGTATTGTG
              *
```

Figure 12. Multiple alignment of nucleic sequences of the souches (S100, S18, S95, S35, S53) by ClustalW.

```
                          70        80        90       100       110       120
                           |         |         |         |         |         |
grLAS100          RKSAKTVGDVIGQYHPHGDYSVYEAMVRLSQDWKLRHVLIEMHGNNGSIDKDPPAAS---
grLAS53           --SAKTVGDVIGQYHPHGDYSVYEAMVRLSQDWKLRHVLIEMHGNNGSIDKDPPA-----
grLAS35           -KSAKTVGDVIGQYHPHGDYSVYEAMVRLSQDWKLRHVLIEMHGNNGSIDKDPPAA----
WP_0012895691     RKSAKTVGDVIGQYHPHGDSSVYEAMVRLSQDWKLRHVLIEMHGNNGSIDNDPPAAMRYT
grLAS18           -KSAKTVGDVIGQYHPHGDSSVYDAMVRLSQGWKLRHVLIEMQGNNGSIDKDPP------
grLAS95           -KSAKTVGDVIGQYHPHGDSSVYDAMVRLSQGWKLRHVLIEMQGNNGSIDKDPPA-----
                    ***************** ***:*******.**********:*******:***
```

Figure 13. Multiple alignment of protein sequences encoded by grLA gene (ClustalW).

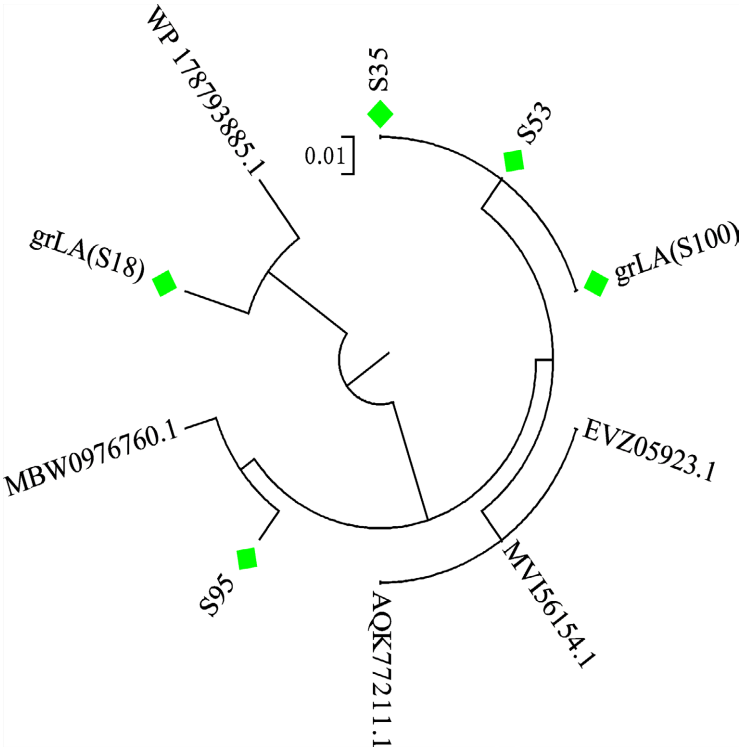

Figure 14. Phylogenetic tree of différent grLA genes.

of this tree shows that the distance between the strains is 1% (0.01) and that the strains have a common ancestor.

The evolutionary history was deduced using the UPGMA method [31]. The optimal tree with sum of branch length = 0.14963294 is displayed. The tree is drawn to scale, with branch lengths (next to the branches) in the same units as the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were calculated using the Poisson correction method [32] and are expressed as the number of amino acid substitutions per site. The analysis focused on 10 amino acid sequences. All positions with gaps and missing data have been eliminated. There were a total of 52 positions in the final dataset. Evolutionary analyzes were carried out in MEGA7 [30].

4. Discussion

We proposed to carry out this study with the aim of identifying genes of resistance to Quinolones/Fluoroquinolones of strains of staphylococci isolated at the hospital and university center of Brazzaville (CHUB). The resistance profile of *S. aureus* and SCN strains to Quinolones/Fluoroquinolones shows that the strains were sensitive to Moxifloxacin, with a sensitivity rate of 100% in SCN and 83.33% in *S. aureus* strains. The resistance rate of 100% to Nalidixic Acid was observed in both species. Strains of Staphylococci presented resistance rates of 66.66% to Ofloxacin and Levofloxacin, 83.33% to Norfloxacin and Ciprofloxacin for strains of *S. aureus*; 80% to Ofloxacin, Levofloxacin and Ciprofloxacin, 60% to Norfloxacin for SCN strains. Moxifloxacin was the most active molecule on the strains

and Nalidixic Acid the most resistant. Variability in the rate of resistance from one antibiotic to another has been observed within the Quinolones/Fluoroquinolones family. The sensitivity of the strains to Moxifloxacin could be explained by the fact that this antibiotic is among the last ATBs synthesized in the Fluoroquinolones family [5]. The strain resistance rates to AN observed are explained by the natural resistance profile of the *Staphylococcus* genus to this ATB [33].

On the other hand, the variability of the rate of resistance from one antibiotic to another within the family could be explained by the impact of the activity of the antibiotic against the germ and is a function of the generation to which belongs the antibiotic [33].

To explain the resistance profile of the strains of Staphylococci to Quinolones/Fluoroquinolones observed in this study, a bibliographic review was carried out for the choice of the gyrA, grLA norA and parC genes [8] [34]. 34 DNA fragments of clinical staphylococci strains were amplified. Analysis of DNA fragments obtained by PCR amplicon agarose gel electrophoresis of the various genes obtained showed that the gyrA, grLA and norA genes were identified except for the parC gene. This result is similar to that [8] on the effects of mutations in the grLA gene for *Staphylococcus aureus* topoisomerase IV, which shows that the ParC gene is not involved in the resistance of *Staphylococcus aureus* strains to Quinolones/Fluoroquinolones. The enzyme or topoisomerase parC encoded by this gene is not the target of quinolones/Fluoroquinolones in *staphylococcus aureus* but for other bacteria such as *E. coli*. The gyrA, grLA and norA genes are involved in the resistance of the isolated strains except the ParC gene.

We also note from the analysis of the results that one of the three genes (norA, gyrA or grLA) was identified in S16, S100, S55, S9, S34, S92, S97. The resistance of these strains to Quinolones/Fluoquinolones is due to the mechanism of alteration of the target enzyme of the antibiotics or that of the alteration which limits the permeation of the antibiotics to the target. Two of the three genes (norA and gyrA, norA and grLA, gyrA and grLA) were identified in the strains (S24, S85, S89, S84, S77, S90, S60, S18, S35, S54, S53, S51, S49 and S39), and the three genes at the same time (norA gyrA grLA) in strains S9, S11, S3, S15, S95, S79. The resistance of the majority of strains to antibiotics is due to both mechanisms. The norA, gyrA and grLA genes were not identified in strains S29, S5, S101, S93, S91, S96. The identified genes are not involved in the resistance of these strains to the molecules used. These results are similar to those of [34] which describes the discovery of a new plasmid-mediated extrachromosomal quinolones/Fluoroquinolones resistance by an as-yet undefined mechanism in clinical isolates of Klebsiella.

We deduce from these results that the resistance of the strains could be due to chromosomal mutations in the grLB and gyrB genes or by the existence of an extrachromosomal mutation (by acquisition of a plasmid).

The norA gene has been identified with an isolation frequency of 38% only in *S. aureus*. These results are close to those reported by [29], with an isolation fre-

quency of 47% of the gene in this work on the identification of this gene in clinical MRSA strains resistant to Ciprofloxacin. The mechanism of resistance by the alteration which limits the permeation of antibiotics to the target was not observed in the SCN strains isolated, this could be explained either by the absence of mutations in the norA gene of the SCN strains isolated or even the nature of the samples used for the isolation of the SCN strains.

The grLA gene was identified with a frequency of 65%, followed by the gyrA genes with 58.82% and norA 29.41%. The identification results for the grLA and gyrA genes are in line with those obtained by [35] when determining quinolones targets in *E. coli*.

The grLA gene was the most identified; the high frequency obtained could be explained by the accumulation of several mutations by the selection pressure of antibiotic therapy due to the excessive use of antibiotics (Quinolones/Fluoroquinolones) in hospitals.

Eleven PCR fragments of the gyrA, grLA and norA genes from different clinical strains, including three of the gyrA gene (223 bp) from SCN strains (S49, S51 and S2l), five of the grLA gene (176 bp) from *S. aureus* strains (S100, S18, S95, S35, S53) and three of the norA gene (140 bp) from strains of *S. aureus* (S55, S3, S79) were sequenced.

The translation on SMS-ORF Finder of the nucleic sequences provided protein sequences of size between 38 and 72 amino acids.

The analysis of the results of the alignment of the nucleic sequences of the clinical *S. aureus* strains (S79, S3 and S55) and of the reference sequence downloaded in GenBank of the norA gene, made it possible to highlight conserved regions, such as that at position 270-274, a region highly conserved in all strains due to the T-T-T-T-T motif, evidence of phylogenetic interference characterized by a function shared by all strains Point mutations (substitutions) in the nucleotide sequences of the strains (S79, S3, S55) by replacements of T-> A (243, 324), T -> C (267); C -> T (318, 333, 342, 258), C -> A (258); A -> T (261), A -> C (264, 348), A -> G (276, 277, 282, 291, 295, 297, 335, 336) common to all strains were observed. These results are close to those of [36], where mutations G4 -> A (491), C -> T (537), and A -> G (585) were observed at the norA gene identified as responsible for the resistance of *S. aureus* strains to CIP. The conserved regions and the nucleotide substitution mutations common to the S79, S3 and S55 strains observed indicate that the strains of the same species have undergone the same nucleotide changes responsible for the resistance of the strains to antibiotics as indicated by the results of the antibiograms where all three strains are resistant to CIP and AN.

For the gyrA gene, the analysis of the results of the alignment of the nucleic sequences of the SCN strains (S49, S24, S51) and of the reference sequence also highlights conserved regions, numerous point mutations (substitutions of nucleotides), mutations common to all strains and specific to each strain. The same nucleotide changes are observed at the level of all the strains and specific nucleo-

tide changes at the level of each strain indicating that the acquired antibiotic resistance is common for the three strains on the one hand and specific to each strain on the other hand. These results also agree with the antibiograms of all the strains which are resistant to A.N and OFL, while specifically the S49 strain (resistant to AN, OFL, CIP, NOR, MOX); S24 resistant to AN, OFL, NOR, LEV and CIP and S51 resistant to AN and OFL.

Finally, the analysis of the multiple alignment of the nucleic sequences of the S.aureus strains (S79, S100, S18, S95, S35, S53) and the reference sequence for the grLA gene highlights next to the nucleotide substitutions common to all strains and those which are specific to each strain losses of nucleotides in position 150, 174, 205 - 207, and 260 - 269 as well as the conserved regions. These results are similar and can be explained as those observed at the level of the gene gyrA with the resistance of S100 to CIP, OFL, MOX and AN; from S18 to CIP, LEV, NOR, OFl and AN; from the S95 to OFLet AN; from the S35 to the CIP, NOR, OFL, MOX, and AN; from S53 to CIP, LEV, OFL and AN, and from all strains to CIP, OFL and AN.

Conservative mutations (conservative substitutions), semi-conservative, indels, several of the conserved regions were observed in the multiple alignment extracts of the protein sequences of the staphylococci strains and the reference protein sequence uploaded in GenBank on ClustalW.

For the norA resistance genes, the mutations do not affect the N-terminal, C-terminal domains or the active site of the proteins. Two conservative mutations and one semi-conservative Al (80) Val, Ile (93 and 99) Val common to all the strains were observed on the protein sequences of the strains S55, S3 and S79 in comparison with the reference protein sequence. These results are similar to those [36] and [37], which respectively identified Asp (315) Gly and Asp to Al substitutions during work on the determination of point mutations responsible for quinolones resistance in *S. aureus* and go hand in hand with those observed from the alignment of the nucleic sequences of these strains with the reference sequence on ClustalW.

For the gyrA resistance genes, conservative and semi-conservative substitutions were also observed. Certain mutations were common to all strains S49, S24, S51, Ser (60) Pro, Glu (73) Met, Thr (84) Ser, Ala (85) Ser, Val (86) Ile are cited. Other mutations were specific to certain strains, such as S24 and S51: Ala (63) Pro, Val (113) Met, strain S49: Ala (63) Ser, Gly (108) Asp, Ala (104) Val, Asn (109) Lys, Met (121) Leu, associated with a substitution which affects the active site Tyr (123) Val.

[28] also observed Ser (84) Leu, Glu (88) Gly and lys, Ser (85) Pro, Ser (84) val substitutions in *S. aureus* in work on detection of gyrA and grLA mutations in *S. aureus*. This partially confirms the results observed in our study.

The mutation profile of the grLA genes is similar to that observed with the norA genes, the mutations do not affect the N-terminal, C-terminal domains or the active site of the protein sequences. Conservative and semi-conservative substitutions have been observed. A conservative mutation in all protein sequences

(S18, S95, S35, S53 and S100) Asn (111) Lys, Glu (84) Asp, Asp (92) Gly and His (104) Gln mutations were observed in protein sequences of strain S95. In the protein sequences of strains S100, S53, S35, the Ser (80) Tyr mutation is observed, a mutation also observed by [28], to which is added the Glu (84) Lys and Gln mutations.

The nature and position of the amino acid during the substitution, and the loss of an amino acid observed reflect the evolutionary changes adopted by different species of Staphylococci studied to be able to resist antibiotics. These evolutionary changes are confirmed by the phylogenetic trees constructed for each gene using the protein sequences of the strains and their homologous sequences in the protein bank. Tree analysis shows distances of 0%, 1% and 2% respectively between norA, grLA and gyrA genes and have a common ancestor. These results clearly reflect that the strains are very close, similar in each gene group confirming their belonging to the same genus (*Staphylococcus*).

5. Conclusion

This study has identified the resistance genes of staphylococci to quinolones/Fluoroquinolones of strains isolated at the University Hospital of Brazzaville, in an attempt to explain the causes of treatment failures. The resistance profile of the strains was characterized by variability in the resistance rate of an antibiotic within the Quinolones/Fluoroquinolones family as well as respectively the sensitivity and resistance to Moxifloxacin and Nalidixic Acid observed. The genomic norA, gyrA and grLA genes were identified by PCR amplification of the fragments of these genes from different strains and are responsible for the resistance of the strains to antibiotics. The results of the bioinformatics analysis of the sequencing products of the fragments PCR of the gyrA, grLA and norA genes have made it possible to highlight, on the one hand, point mutations, substitutions and indels characterized respectively by a replacement and a loss of nucleotides or amino acids at the level of the nucleic or protein sequences of the strains for the gyrA and grLA genes; on the other hand, mutations responsible for the efflux mechanism by substitutions in the norA gene.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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