



جامعة الإسكندرية  
ALEXANDRIA  
UNIVERSITY



**Faculty of Veterinary Medicine  
Department of Microbiology**

**"Molecular investigation of some virulence  
genes and antibiotic resistance of  
*Staphylococcus aureus* of animal origin"**

**A Thesis**

**Presented to the Graduate School  
Faculty of Veterinary Medicine, Alexandria University  
In Partial Fulfillment of the Requirements for the Degree**

**Of  
Ph.D. of Veterinary Sciences  
In  
Microbiology  
Specialization**

**(Bacteriology and mycology)**

Presented by

**Amr Mohamed Mohamed Abd El-Rahman**

(B.V.Sc., Fac. Vet. Med. South Valley Univ., 2005)

(M.V.Sc., Fac. Vet. Med. Alexandria Univ., 2015)

**2021**

## LIST OF CONTENTS

Contents		Page
<b>I</b>	<b>INTRODUCTION</b>	1-3
<b>II</b>	<b>REVIEW OF LITERATURE</b>	4-30
2.1.	<i>Staphylococcus aureus</i> .	4
2.2.	<i>S. aureus</i> in Ruminants.	6
2.3.	Antibiotic resistance.	8
2.4.	MRSA.	13
2.5.	Biofilm.	15
2.6.	Integron.	19
2.7.	Virulence.	23
2.8.	ERIC-PCR.	29
<b>III</b>	<b>MATERIAL AND METHODS</b>	31-48
3.1.	<b>MATERIAL</b>	31
3.1.1.	Samples.	31
3.1.2.	Reference strain.	31
3.1.3.	Media used.	32
3.1.4.	Stain used.	33
3.1.5.	Antimicrobial discs.	33
3.1.6.	Material used for extraction of DNA.	33
3.1.7.	PCR Master Mix used for PCR.	33
3.1.8.	Oligonucleotide primers used in PCR.	34
3.1.9.	DNA Molecular weight marker.	35
3.1.10.	Material used for agarose gel electrophoresis.	35
3.1.11.	Materials used for PCR product purification of MG.	36

3.1.12.	Material used for sequencing of the purified PCR product.	36
<b>3.2.</b>	<b>Methods</b>	37
3.2.1.	Collection of Samples.	37
3.2.2.	Isolation and identification of <i>S. aureus</i> .	37
3.2.3.	Identification of <i>S. aureus</i> using specific biochemical tests.	38
3.2.4.	Hemolytic activity of <i>S. aureus</i> isolates.	39
3.2.5.	Antibiotic sensitivity test for <i>S. aureus</i> isolates.	39
3.2.6.	Biofilm (Microtiter plate method).	41
3.2.7.	Extraction of DNA.	42
3.2.8.	Preparation of PCR Master Mix.	43
3.2.9.	Cycling conditions of the primers during PCR.	44
3.2.10.	DNA Molecular weight marker.	44
3.2.11.	Agarose gel electrophoreses.	45
3.2.12.	Methods for purification of the PCR Products.	45
3.2.13.	Sequencing reaction.	46
3.2.14.	Purification of the sequence reaction.	46
3.2.15.	Loading the sequencer machine.	47
3.2.16.	Computer Analysis of The sequence data.	47
3.2.17.	Methods of ERIC Analysis.	48
<b>IV</b>	<b>RESULTS</b>	49-72
<b>V</b>	<b>DISCUSSION</b>	73-86
<b>VI</b>	<b>SUMMARY</b>	87-89
<b>VII</b>	<b>REFERENCES</b>	90-113
<b>ARABIC SUMMARY</b>		٢-١

## LIST OF TABLES

<b>Table</b>	<b>Title</b>	<b>Page</b>
<b>1</b>	Samples	<b>31</b>
<b>2</b>	Oligonucleotide primers sequences	<b>34</b>
<b>3</b>	Zone size interpretation chart of antimicrobial susceptibility.	<b>40</b>
<b>4</b>	Components of PCR mastermix	<b>43</b>
<b>5</b>	Cycling conditions of the different primers during PCR	<b>44</b>
<b>6</b>	Preparation of master mix using Big dye Terminator V3.1 cycle sequencing kit.	<b>46</b>
<b>7</b>	Percentage of isolated <i>S. aureus</i> in examined samples	<b>49</b>
<b>8</b>	Results of biochemical tests of the isolated <i>S. aureus</i>	<b>52</b>
<b>9</b>	Antimicrobial sensitivity of <i>S. aureus</i> isolates.	<b>54</b>
<b>10</b>	Detailed antimicrobial sensitivity of different <i>S. aureus</i> isolates isolated from ruminants.	<b>55</b>
<b>11</b>	Biofilm formation of <i>S. aureus</i> isolates	<b>56</b>
<b>12</b>	Reactions of colonies of <i>S. aureus</i> replicated onto various types of blood-agar plates.	<b>58</b>
<b>13</b>	Antimicrobial resistance, biofilm& hemolysins patterns of <i>S. aureus</i> isolates.	<b>61</b>
<b>14</b>	Molecular detection of integron in MDR <i>S. aureus</i> isolates from ruminants.	<b>62</b>
<b>15</b>	Virulence genes profiles of <i>S. aureus</i> isolated from ruminants and poultry.	<b>64</b>
<b>16</b>	Virulence genes, biofilm, antimicrobial resistance and Eric patterns of <i>S. aureus</i> isolates from ruminants and poultry.	<b>72</b>

## LIST OF FIGURES

<b>Figure</b>		<b>page</b>
1	Gram staining	50
2	Cultural characteristics on different media	51
3	Coagulase test	53
4	Biofilm formation on tissue culture plate	57
5	Beta hemolysis on blood agar with 5% rabbit blood	59
6	Beta hemolysis on blood agar with 5% horse blood	59
7	Beta hemolysis on blood agar with 5% sheep blood	60
8	PCR detection of integron	63
9	PCR detection of class 1 integron cassette	63
10	PCR detection of 23S rRNA gene of <i>S. aureus</i>	65
11	PCR detection of agr I gene of <i>S. aureus</i>	65
12	PCR detection of agr II gene of <i>S. aureus</i>	66
13	PCR detection of agr III gene of <i>S. aureus</i>	66
14	PCR detection of agr IV gene of <i>S. aureus</i>	67
15	PCR detection of lukED gene of <i>S. aureus</i>	67
16	PCR detection of tst gene of <i>S. aureus</i>	68
17	ERIC-PCR profiles of <i>S. aureus</i> isolates isolated from ruminants samples	70
18	ERIC-PCR profiles of <i>S. aureus</i> isolates isolated from poultry arthritis samples	70
19	ERIC-based dendrogram showing genetic relatedness among <i>S. aureus</i> isolates.	71

## VI. SUMMARY

A total number of 164 samples (mastitis milk, nasal swabs, wound swabs, abscesses' contents) were collected from ruminant animals (cattle, sheep, and goat) either individually or from farms in Alexandria governorate and subjected to bacteriological examination.

Seventeen *Staphylococcus aureus* isolates were identified morphologically, biochemically (5 isolates from mastitis milk samples, 6 isolates from nasal swabs and 6 isolates from wound/pus samples).

Antibiogram profile of the isolates was carried out against 11 antimicrobials. 16/17 (94%) of isolates were resistant to at least one antimicrobial, 11/17(64.7) of them were resistant to three or more of antimicrobials (multi-drug resistance), highest sensitivity was observed for vancomycin and amoxicillin/clavulanic acid 17/17 (100%) while the highest resistance was observed for penicillin 14/17 (82.4%), and gentamicin 9/17(53%). Eight out of seventeen isolates (47%) showed resistance to oxacillin/cefoxitin (methicillin resistant *S. aureus*) all of them were multi drug resistant (MDR). i.e. MRSA strains are notorious for being multidrug resistant.

Among 17 clinical isolates of *S. aureus* isolated from collected samples, 1/17 (6%) was non biofilm producer and 16/17(94%) showed biofilm production, of them 7/17(40%) of isolates showed weak biofilm production, and 4/17(24%) showed moderate biofilm production, and 5\17 (30%) showed strong biofilm production.

The MDR *Staphylococcus aureus* isolates11/11(100%) were showing biofilm production, (3strong, 3 moderate and 5 weak) biofilm producers, while 5/6(83%) of the sensitive isolates were showing biofilm production (2 strong, 1 moderate and 2 weak) biofilm producers.

All the 17 isolates were examined for hemolysis by using three types of blood agar sheep blood agar, rabbit blood agar and horse blood agar for estimation of production of  $\alpha$ ,  $\beta$ ,  $\delta$  hemolysins. All the isolates were showing at least one type of hemolysin, 16/17(94%) of isolates were showing  $\beta$  hemolysin, 7/17(41%) were showing  $\alpha$  hemolysin and 12/17(70%) of isolates were showing  $\delta$  hemolysin.

Ten of MDR *S. aureus* isolates were molecularly screened for integron. Class 1 integron cassette were detected in 1/10 of tested isolates which exposed to further sequence

analysis reveals dihydrofolate reductase (*dfrA15*) gene cassette which encodes trimethoprim resistance, the GenBank accession number of the *dfrA15* gene sequences determined in this study is MW036489.

PCR was applied on 10 isolates of *S. aureus* representing all animal recovered isolates, and 2 isolates which were isolated from poultry (arthritis), all the isolates were MDR. The isolates confirmed as *S. aureus* by 23s rRNA PCR in all 12 isolates (100%).

The PCR assays for confirmation of the presence of 6 virulence genes (*agrI*, *agrII*, *agrIII*, *agrIV*, *tst*, *lukED*).

*agrI* gene was detected in all the 12 isolates(100%), *agrIII* gene was detected in 10/12 (83%) of the isolates, *agrII* and *agrIV* genes were absent in all the isolates. *tst* gene encoding (Toxin shock syndrome toxin) was detected in 7/12(58%) of isolates, *lukED* gene was detected in 11/12 (92%) of isolates.

The ERIC-PCR analysis showed that 12 *Staph aureus* isolates grouped into 6 Eric types (A1:A3, B1:B3) at 75% genetic similarity grouped into two main clusters (A & B).

The Eric type A1 contain one isolate(43SM) which were isolated from sheep mastitis, the Eric type A2 contain 2 isolates (71CW&90SN) which were isolated from cattle wound and sheep nasal swab respectively, the dissimilarity between the Eric type A1&A2 was 10%. The Eric type A3 contain isolates (26SW&52SW) which were isolated from sheep wound and cattle wound respectively.

The dissimilarity of the Eric types of cluster (A) was 14%.

The Eric type B1 contain two isolates (78CN&P11) which were isolated from cattle nasal swab and poultry arthritis respectively, the Eric type B2 contain two isolates, the Eric type B2 contain two isolates (02CM& P12) which were isolated from cattle mastitis and poultry arthritis respectively, the dissimilarity between the Eric type B1&B2 was 8%.

The Eric type B3 contain 3 isolates (014CM, 21SW&8SP) which were isolated from cattle mastitis, sheep wound and sheep internal abscess respectively.

The dissimilarity of the Eric types of cluster (B) was 16%.

The dissimilarity between the Eric types of cluster A&B was 25%.

All the five MSSA isolates occur in the ERIC types of cluster (B)

All the isolates(5) in the Eric types of cluster (A) were MRSA, and only 2MRSA isolates occur in the ERIC types of cluster(B)

Various clusters generated in the ERIC-PCR dendrogram showed a 75% similarity of isolates from diverse origin. For example Eric type B2 contain two isolates (02CM& P11) which were isolated from cattle mastitis and poultry arthritis respectively, Thus, cross transmission of *S. aureus* isolates may occur in different animal species (cross-species transmission).