



Phenotypic and Genotypic Characterization of *Staphylococcus aureus* Isolated from Raw Camel Milk Samples from Libya and its Histopathological Effects on Mice Liver

A Thesis

*Submitted for the Degree of doctor of Philosophy
(Ph.D) in Science (Zoology)*

By

Ebtesam Mohamed Abdraba Mohamed

(B.Sc., M. Sc.)

Department of Zoology, Omer Al Mokhtar University (Libya)

Under the Supervision of

Prof. Dr. Nagui Hassan Fares

*Professor of Cell Biology and Histology
Faculty of Science – Ain Shams University
(Cairo – Egypt)*

Prof Dr. Sherif Mousa Hussieny

*Professor of Microbiology
Faculty of Girls – Ain Shams University
(Cairo – Egypt)*

Prof Dr. Yomna Ibrahim Mahmoud

*Professor of Cell Biology and Histology
Faculty of Science – Ain Shams University
(Cairo – Egypt)*

Dr. Nawara Aboelgasim Mohammed Eissa

*Associate Professor of Microbiology
Faculty of Veterinary Medicine – Omar Elmokhtar University
(Libya)*

2019

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

قالوا

سبحانك لا علم لنا
إلا ما علمتنا إنك أنت
العليم العظيم

صدق الله العظيم

سورة البقرة الآية: ٣٢



*All the praises and thanks be to **Allah**, the most beneficent and merciful of all.*

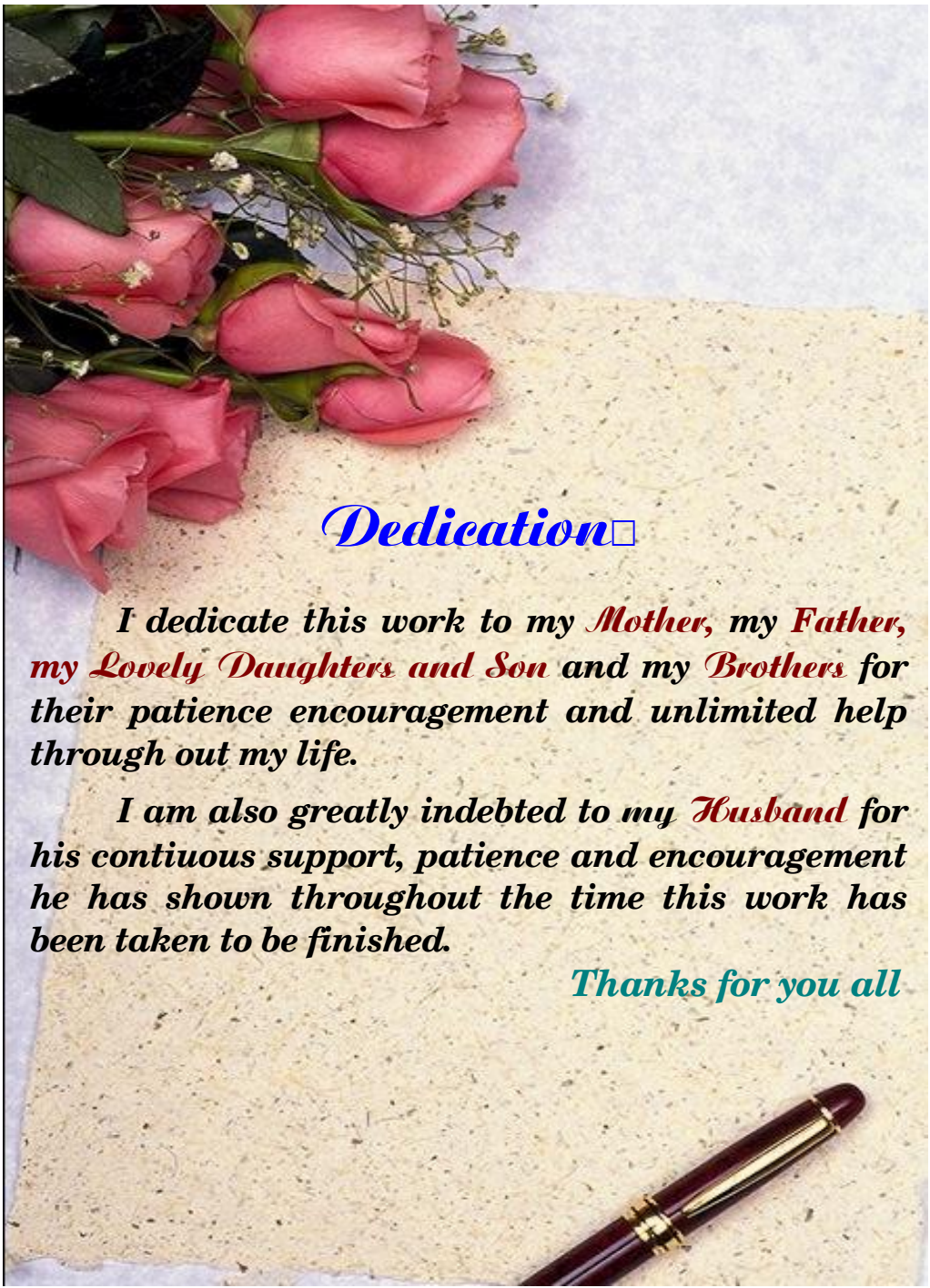
*My profound appreciation is due to **Prof. Dr. Nagui Hassan Fares**, Professor of Cell Biology and Histology, Zoology Department, Faculty of Science, Ain Shams University for suggesting the point, his precious advice, continuous encouragement, and for his critical reading of the manuscript. It is a great honor to work under his supervision. His research experience has added a lot to the integrity of thesis.*

*I would like to express my gratitude to **Prof. Dr. Sherif Mousa Hussieny**, Professor of Bacteriology, Faculty of Girls – Ain Shams University, for his continuous support and valuable efforts during stages of this study.*

*I am greatly indebted to **Prof. Dr. Yomna Ibrahim**, Professor of Histology and Cell Biology, Zoology Department, Faculty of Science, Ain Shams University for her valuable guidance and careful reading of the manuscript, unlimited assistance, instructive guidance and kind support. No word of thanks can equal her contribution.*

*I owe much to **Dr. Nawara Aboelqasim Mohammed**, Associate Professor of Bacteriology, Faculty of Veterinary Medicine- Omar Elmoktar University, El-Beida, Libya, for suggesting the point, reading of the manuscript, continuous encouragement and kind support.*

Ebtesam Mohamed Abdraba

A decorative background featuring a bouquet of pink roses in the upper left corner and a dark red fountain pen with gold accents in the lower right corner. The central text is set against a light-colored, textured paper background.

Dedication□

*I dedicate this work to my **Mother**, my **Father**, my **Lovely Daughters and Son** and my **Brothers** for their patience encouragement and unlimited help through out my life.*

*I am also greatly indebted to my **Husband** for his continuous support, patience and encouragement he has shown throughout the time this work has been taken to be finished.*

Thanks for you all

Content

Title	Page No.
Introduction	i
Aim of Study	12
Review of Literature.....	13
Materials and Methods	36
3.1. Collection of Milk Samples	30
3.2. Primary isolation and identification procedures.....	33
3.2.1. Visual Examination of cultures	34
3.2.1.1 Nutrient agar	34
3.2.1.2 Sheep blood Agar	35
3.2.1.3 MacConkey agar.....	35
3.3 Selective and differential media	36
3.3.1 Mannitol Salt Agar	36
3.3.2 Baird-Parker Agar Base.....	36
3.3.3 Deoxyribonuclease (DNase) medium.....	37
3.4 Identification of <i>S. aureus</i>	38
3.4.1 Microscopic examination	38
3.4.2 Biochemical tests.....	39
3.4.2.1 AVIPATH Staph (CERTIFIED, UK).....	39
3.4.2.3.1 Test procedure	39
3.4.2.2 Coagulase test.....	40
3.4.2.2.1 Collection of Plasma.....	40
3.4.2.2.2 Test procedure	40
3.4.2.3 Catalase test	41
3.5 Molecular detection the <i>S. aureus</i> DNA by Qualitative PCR.....	41
3.5.1 Materials and reagent used for Qualitative PCR.....	42
3.5.1.1 Chemicals	42
3.5.1.2 Kits	43
3.5.1.3 Supplies	43

Content

Title	Page No.
3.5.1.4 Reagents and Solutions.....	43
3.5.2 Extraction of DNA from Bacteria samples	46
3.5.3 PCR Amplification:	48
3.5.4 Analysis of amplified DNA	49
3.6 Histological Experiments	50
3.6.1 Animals.....	50
3.6.2 Bacterial strain.....	50
3.6.3 Final experimental design.....	51
3.6.4 Gross morphology	51
3.6.5 Sample collection	51
3.6.6 Histological Procedures.....	52
Results	59
Discussion	108
Summary	117
References	122
List of Tables.....	i
List of Figures.....	ii
List of Abbreviations.....	vi
Abstract	6
Arabic Summary	-

List of Tables

Table No.	Title	Page No.
Table (1):	Locations, coordinates and numbers of milk samples	38
Table (2):	History and relevant information on she-camels from which milk samples were collected	39
Table (3):	List of used primers, thermocycler-programs and amplicon sizes of the applied PCR reactions in the present study	48
Table (4):	Prevalence of <i>S. aureus</i> isolated from she- camels milk in Libya	59
Table (5):	Cultural and staining characteristics of <i>S. aureus</i> isolated from She- Camels Milk.....	64
Table (6):	Identification tests of six microbial isolates from she- camels milk.....	66
Table (7):	The body weight and relative liver weight of the control, <i>sec</i> -, and <i>tst</i> – infected groups	71

List of Figures

Figure No.	Title	Page No.
Figure (1):	Culture plate of β -hemolysis on 5% sheep blood agar by microbial isolate.	60
Figure (2):	Culture plate fermentation of MSA by <i>S. aureus</i>	61
Figure (3):	<i>S. aureus</i> on Barid parker agar.....	62
Figure (4):	<i>S. aureus</i> on DNase Agar.....	63
Figure (5):	Coagulase test.....	66
Figure (6):	Agarose gel electrophoresis patterns showing PCR-amplified products in qualitative PCR for indentifying <i>S. aureus 23s rRNA</i> gene.....	68
Figure (7):	Agarose gel electrophoresis patterns showing PCR-amplified products in qualitative PCR for indentifying <i>tst</i> enterotoxin gene for <i>S. aureus</i>	68
Figure (8):	Agarose gel electrophoresis patterns showing PCR-amplified products in qualitative PCR for indentifying <i>sec</i> enterotoxin gene for <i>S. aureus</i>	69
Figure (9):	Histogram showing the body weight, liver weight and relative liver weight of the control, <i>sec</i> -, and <i>tst</i> -infected groups.	71
Figure (10):	Liver of control group	73
Figure (11):	Liver of control group	73
Figure (12):	Liver of <i>S. aureus</i> (<i>sec</i>)-infected group	73
Figure (13):	Liver of <i>S. aureus</i> (<i>sec</i>)-infected group.....	73
Figure (14):	Liver of <i>S. aureus</i> (<i>tst</i>)-infected group	73
Figure (15):	Liver of <i>S. aureus</i> (<i>tst</i>)-infected group.....	73
Figure (16):	Photomicrograph of C.S. of mouse liver of control group showing its normal architecture	76
Figure (17):	High power photomicrograph of C.S. of mouse liver from the control group	78

List of Figures Cont...

Figure No.	Title	Page No.
Figure (18):	High power photomicrograph of C.S. of mouse liver from the control group	80
Figure (19):	High power photomicrograph of C.S. of mouse liver from the control group	82
Figure (20):	Photomicrograph of C.S. of liver from <i>S. aureus</i> (sec)-infected group	84
Figure (21):	High power photomicrograph of C.S. of liver from <i>S. aureus</i> (sec)-infected group	86
Figure (22):	High power photomicrograph of C.S. of liver from <i>S. aureus</i> (sec)-infected group	88
Figure (23):	High power photomicrograph of C.S. of liver from <i>S. aureus</i> (sec)-infected group	90
Figure (24):	High power photomicrograph of C.S. of liver from <i>S. aureus</i> (sec)-infected group	92
Figure (25):	Photomicrograph of C.S. of liver from <i>S. aureus</i> (tst)-infected group	94
Figure (26):	Photomicrograph of C.S. of liver of <i>S. aureus</i> (tst)-infected group	96
Figure (27):	Photomicrograph of C.S. of liver of <i>S. aureus</i> (tst)-infected group	98
Figure (28):	High power photomicrograph of C.S. of liver from <i>S. aureus</i> (tst)-infected group	100
Figure (29):	High power photomicrograph of C.S. of liver from <i>S. aureus</i> (tst)-infected group	102
Figure (30):	High power photomicrograph of mice liver of <i>S. aureus</i> (tst)-infected group	104
Figure (31):	High power photomicrograph of mice liver of group <i>S. aureus</i> (tst)-infected group	106

List of Abbreviations

Abb.	Meaning
C.S.	Cross Section
CFU.....	Colony forming units
Chisam	Chloroform-iso-amyl alcohol
CMT.....	California mastitis test
CNS	Cogulase nagtive staphylococci
CPS.....	Coagulase positive staphylococci
DNA	Deoxyribonucleic acid
Dnase.....	Deoxyribouuclease
EDTA.....	Etheline diamine tetra acetic acid
GST	Glutathione-S-transferase
Hx. & E.	Hematoxylene and Eosin
LAB.....	Lactic acid bacteria
LB Medium	Lauria-Bertani medium
LF	Left-fore
LH	Left-hind
MSA	Mannitol salt agar
PBS.....	Phosphate buffer saline
PCR.....	Polymerase chain reaction
PTS Ags	Pyrogenic toxin superantigens
RF	Right-fore
RH	Right-hind
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rRNA.....	Ribosomal RNA
<i>S. aureus</i>	<i>Staphylococcus aureus</i>

List of Abbreviations Cont...

Abb.	Meaning
SCC	Somatic cell count
<i>sec</i>	<i>S. aureus</i> gene that produces SEC
SEC	<i>Staphylococcus</i> enterotoxin C
β-toxin	beta toxin
Tris	Tris (hydroxymethyl) aminomethane
TSST	Toxic shock syndrome toxin
<i>tst</i>	<i>S. aureus</i> gene that produces TSST
U.A.E.....	United Arab Emirates
UV	Ultraviolet light
Y-toxin	Gamma toxin
α - toxin	Alpha toxin

ABSTRACT

The present study was conducted to determine the phenotypic and hereditary description of *Staphylococcus aureus* (*S. aureus*) isolated from raw camel milk from different regions in Libya (Tubrak, Shahat, Alsafsaf, Omar Al Mokhtar, Makailie, Labrag, and Gardas) as well as to identify the different toxin genes of these bacteria. Out of the total 220 milk samples collected from 55 teats of apparently healthy lactating she-camels, 6 coagulase positive *Staphylococcus spp* were obtained. These 6 (2.7 %) isolates were identified as *S. aureus* based on cultural and biochemical properties. All of the 6 isolates showed β -hemolysis on blood agar media enriched with 5% sheep blood. Gram-stained smears of the pure cultures exhibited clusters of Gram-positive cocci. The isolates also fermented mannitol with the color change of MSA and production of small yellow colonies. Isolates were positive for catalase and coagulase tests. The species identity of all 6 isolates could be confirmed by PCR amplification of the *S. aureus*-specific chromosomal DNA fragment using *23s rRNA* primer for *23s rRNA* gene. The ability to synthesize classical enterotoxins was found in 3 of 6 (50%) isolates by using the qualitative PCR technique. The enterotoxin gene (*sec*) was identified in two isolates (33.3%), while the enterotoxin gene (*tst*) was identified in only one isolate (16.7%).

The study was also planned to investigate the histopathological changes of the liver of two groups of mice after intraperitoneal injection of one dose (0.1ml) of *S. aureus* (*sec*) and *S. aureus* (*tst*) aqueous solutions at a concentration of 5×10^8 / 0.1ml. The relative weight of the liver of *S. aureus* (*tst*)-infected mice was significantly increased as compared with the control group and the *S. aureus* (*sec*)-infected. Liquid-filled abscesses appeared on the liver surfaces of the infected groups. Histopathological studies showed several microscopic changes in the liver of infected groups including inflammatory cells infiltration, hepatic cell degeneration, preiductal fibrosis, and the appearance of black spots thought to be colonies of *S. aureus* bacteria in association with inflammatory cells.

1. INTRODUCTION

Camels are important source for milk production in nomadic societies. Camel milk is supposed to have medicinal properties, as it contains insulin-like protein, so it has hypoglycemic effect. Milk is an excellent source of nutrients for human (*Abrhaley and Leta, 2018*) and, yet in a different context, it provides a suitable medium for microbial growth and metabolism. In raw milk, bacteria can affect the quality, safety and consumer acceptance of dairy products. Nonpathogenic bacteria may affect milk and milk products quality (*Samaržija et al., 2012*). Thus, many countries have milk quality regulations, including limits on the total number of bacteria in raw milk, to ensure the quality and safety of the final product. The number and types of microorganisms in milk, immediately after milking, are affected by several factors such as animal health, equipment cleanliness, season, and food. It is hypothesized that differences in feeding and housing strategies of cows may influence the microbial quality of milk (*Swai and Schoonman, 2011*).

Staphylococcus spp (*S. spp*) are microorganisms that are naturally present in milk and dairy products. *Gabriela et al. (2009)* reported that they are often associated with food-borne disease outbreaks due to the ability of some strains to produce a thermostable enterotoxins. Diseases are usually associated with coagulase and thermonuclease positive *Staphylococcus aureus*

(*S. aureus*). Milk is a good substrate for *S. aureus* growth and enterotoxin production. Enterotoxins are thermostable to heat retaining some biological activity even after 28 minutes at 121°C. The bacterium is also capable of producing several pathological conditions in human. Pathogens can invade the teat canal ascending toward the mammary parenchyma, then colonize, multiply and produce their toxins, and finally predispose to mastitis. So, teat skin should be free from microbial contamination lesions to maintain the health of animals (**Ahmed et al., 2010**). A high percentage of subclinical mastitis in camels is reported by several authors (**Barbour et al., 1985; Abdurahman et al., 1995; Obeid et al., 1996; Almaw and Molla, 2000**). The pathogenic bacteria reported by different scientific groups in camels are similar to those associated with mastitis in cows or other animals kept in traditional nomadic environments or camel farms (**Barbour et al., 1985; Almaw and Molla, 2000**).

In 1880, *S. aureus* was first discovered by a surgeon named Sir Clifton Smith in pus from surgical abscesses in Aberdeen, Scotland (**Ogston, 1984**). It is a nonmotile, non-sporeforming, Gram positive, aerobic facultative anaerobic coccus, with the appearance of grape-like clusters when viewed through a microscope. *S. aureus* cells are spherical and are about 1µm in diameter. They form a cluster arrangement because of their special division way. These cells divide in

three dimensional axis, and the new cells remain attached to each other followed by each division successively.

S. aureus is catalase positive and oxidase negative. The catalase test is an important, yet simple, method to distinguish staphylococci from streptococci, which are catalase negative (**Raus and Love, 1983**). Typical *S. aureus* has large, round, creamy smooth colonies with golden yellow color. Most strains have beta or alpha hemolysis when growing on blood agar plates (**Kloos and Schleifer, 1975**). *S. aureus* can survive for several hours on dry environmental surfaces and grow at a temperature range of 7 to 48^oC (**Neely and Maley, 2000**). There are about 32 described species of *Staphylococcus* (**Kloos and Bannerman, 1994**), but *S. aureus* is the only pathogen of increasing importance due to the rise in antibiotic resistance (**Lowy, 1998**).

Infectious agents have caused epidemic and endemic diseases involved in the deaths of hundreds of millions of humans, as well as significant animal morbidity and mortality throughout history (**Musser and DeLeo, 2015**). *S. aureus* is a widespread Gram-positive coccus that is both a human commensal bacterium and pathogen. Approximately 50% to 60% of individuals are intermittently or permanently colonized with *S. aureus* and, thus, there is relatively high potential for infections (**Wertheim et al., 2005; Gorwitz et al., 2008**). A relatively high percentage of healthy people are asymptotically colonized with *S. aureus* in the anterior nares