



Molecular and immunological studies on honeybee queens *Apis mellifera* (L.) and their antibacterial activity against *Paenibacillus larvae larvae*

A Thesis submitted as a partial fulfillment of the requirements for The award of the Ph.D. degree in Entomology Faculty of Science, Ain Shams University

By Soha Adel Sayed Gomaa

B.Sc. in Entomology, Ain Shams University (2003) M.Sc. in Entomology, Ain Shams University (2009)

SUPERVISORS

Prof. Dr. Mohamed Sayed Salama

Professor of Molecular Biology, Faculty of Science, Ain Shams University

Prof. Dr. Emad Mahmoud Said Barakat

Professor of Insect Physiology, Faculty of Science, Ain Shams University

Dr. El-Gohary El-Saied Attia El-Gohary

Assistant Professor of Molecular Biology, Faculty of Science, Ain Shams University

> Entomology Department Faculty Of Science Ain Shams University

> > 2018





Approval Sheet

Molecular and immunological studies on honeybee queens $Apis\ mellifera\ (L.)$ and their antibacterial activity against $Paenibacillus\ larvae\ larvae$

Thesis submitted for a PH.D. degree in Science (Entomology)

By Soha Adel Sayed Gomaa

B.Sc. in Entomology, Ain Shams University (2003) M.Sc. in Entomology, Ain Shams University (2009)

Supervisors committee:

Examination Date: / /2018	Approval Date: / /2018
Prof. Dr. Emad Mahmoud Said Barakat Professor of Insect Physiology, Faculty of Science, A	-
Professor of Molecular Biology, Faculty of Science,	
Prof. Dr. Mohamed Sayed Salama Professor of Molecular Biology, Faculty of Science	•
Professor of Molecular Biology, Faculty of Science,	•
Prof. Dr. Sobhy EL- Sayed Hassab El - Nabi	· ·
Professor of Insect Physiology, Faculty of Science for	· ·
Prof. Dr. Mostafa Amin Tahaa	Signature
This thesis is for PH.D. degree in Science (En	tomolgy) has approved by:
University	
Assistant Professor of Molecular Biology, Facul	lty of Science, Ain Shams
Dr. El-Gohary El-Saied Attia El-Gohary	_
Professor of Insect Physiology, Faculty of Scien	ice, Am Snams University
	•
Prof. Dr. Emad Mahmoud Said Barakat	Signatura
Professor of Molecular Biology, Faculty of Scie	ence, Ain Shams University
Prof. Dr. Mohamed Sayed Salama	Signature

University Council approval: / /2018





اسم مقدم الرساله: سها عادل سيد جمعه

عنو ان الرساله: دراسات جزيئية ومناعية على ملكات نحل العسل ابيس ميليفرا لينيوس وفاعليتها ضدّ بينيباسيلس لارفي لارفي

الدرجه العلميه: دكتوراه الفلسفه في العلوم (حشرات)

لجنة الإشراف	
ا.د/ محمد سید سلامه	التوقيع:
أستاذ البيولوجيا الجزيئية المتفرغ - قسم علم الحشرات- كا	لية العلوم- جامعة عين شمس
ا.د/ عماد محمود سعید برکات	التوقيع:
أستاذ فسيولوجيا الحشرات قسم علم الحشرات كلية العلو	ِم- جامعة عين شمس
د/ الجوهري السعيد عطيه الجوهري	التوقيع:
أستاذ مساعد البيولوجيا الجزيئية- قسم علم الحشرات- كلية	ة العلوم- جامعة عين شمس
لجنة التحكيم	
ا.د/ مصطفي أمين طه	التوقيع:
أستاذ فسيولوجيا الحشرات- كلية العلوم بنات- جامعة الأز	ز هر
ا.د/ صبحي السيد حسب النبي	التوقيع:
أستاذ البيولوجيا الجزيئيه- كلية العلوم- جامعة المنوفية	
۱.د/ محمد سید سلامه	التوقيع:
أستاذ البيولوجيا الجزيئية المتفرغ - قسم علم الحشرات-كا	لية العلوم- جامعة عين شمس
ا.د/ عماد محمود سعید برکات	التوقيع:
أستاذ فسيولوجيا الحشرات قسم علم الحشرات كلية العلو	ِم- جامعة عين شمس
<u>الدراسات العليا:-</u>	

أجيزت الرساله بتاريخ: / / 2018

تاريخ المناقشة:

ختم الاجازة:

ACKNOWLEDGEMENTS

First of all, gratitude and thanks to **ALLAH** who always helps and guides me to overcome all the problems faced throughout this work. I wish to express my profound, sincere appreciation and supreme gratitude to **Dr. Mohamed Sayed Salama**, Professor of Molecular Biology, Faculty of Science, Ain Shams University for his continuous encouragement, fruitful supervision and criticism of the manuscript, to **Dr. Emad Mahmoud Said Barakat**, Professor of Insect Physiology, Faculty of Science, Ain Shams University, for his direct supervision, suggestion the point of study, designing the plan of work, continuous support throughout this work and for critically reading and correcting the manuscript and to **Dr. El-Gohary El-Saied Attia El-Gohary**, Assistant Professor of Molecular Biology, Faculty of Science, Ain Shams, for his supervision, helpful advice as well as reading and correcting the draft.

Dr. Eslam El-Sayed Mostafa Mikkawye for the Gram negative bacteria stocks.

Deep thanks and gratitude are also due to all the staff members of Research and Training center on vectors of disease and colleagues of Entomology Department, Faculty of Science, Ain Shams University for their help and continuous cooperation.

<u>Dedication</u>

To my Mum spirit God forgive her and give her the supreme paradise, and my Dad God bless him, love and appreciation for their role in my life. They were always the light which illuminates the way for me, encouraged me and supported me.

To my husband for his support and help in this Thesis.

To my sister for her continuous support and encourage.

Also my deep thanks to:

Toline & Yahia

Rozan, Aysel & Mazen

CONTENTS

	Pag
I-INTRODUCTION	1
II-LITERATURE REVIEW	10
1-Honey bee castes	10
2-Queens rearing methods	13
2.1. Natural queen replacement	13
2.2. Artificial queen rearing	15
3-Honey bees and bacteria	21
3.1. American foulbrood	22
3.2. European foulbrood	24
3.3. Powdery scale disease	25
3.4. Half moon disorder	26
3.5. Spiroplasmosis	26
3.6. Septicemia	27
3.7. Rickettsial infections	28
4-Measurements of queen honey bee	28
5-Honey bee immune system	33
5.1. Physical and mechanical barriers	36
5.2.Humoral defense mechanisms	39
6.Antimicrobial activity	41
7. Biochemical and molecular analysis	46
III- MATERIALS AND METHODS	57
1. Source and rearing of honey bee queens	57
2. Preparation of the bacterial pathogen	59
2.1. Source of the bacterial pathogen	59
2.2. Preparation of the bacterial stock suspension	59
2.3. Activation and cultivation of the bacterial pathogen	59
2.4.identification of bacteria	60
2.4.1.Morphological tests	60
2.4.2.Microscopical tests	60
2.5. Biochemical tests	61
2.5.1. Holst milk test	61
2.5.2. Nitrate reduction test	62
2.5.3. Catalase production test	63
2.5.4. Hydrolysis of starch	63
2.5.5. Hydrolysis of casein	63
2.6. Molecular tests.	64
2.6.1. DNA extraction from P. l. larvae cultured	64
colonies	

2.6.2. PCR Primers and reaction mixture	64
2.6.3- The PCR temperature profile	65
2.6. Determination of the bacterial spore concentration	66
3. Susceptibility tests and determination of sub-lethal dose for immunization of honey bee queens	67
4. Morphometric measurements of honey bee queens	68
4.1. Body wet weight	68
4.2. External body measurements:	68
5. Investigation of internal characteristics of honey bee queens	69
5.1. Dissecting of virgin queens' ovaries	69
5.2- Counting of ovarioles	69
6. Preparation of queens samples for analysis	70
6.1- Total body homogenate	70
6.2- Ovary extract.	70
7. Assaying of antibacterial activity	71
8. Estimation of the protein concentration	73
9. Electrophoretic analysis of proteins	76
10. Molecular qualitative study of antibacterial target genes	81
10.1. Extraction of total RNA	81
10.2. Synthesis of first strand cDNA from total RNA	83
10.3. Specific oligonucleotide primer for target genes	83
10.4. PCR amplification of antibacterial genes (cDNAs)	85
10.5. Detection of <i>P. l. larvae</i> DNA in treated queens'	86
ovaries	
11- Molecular quantitative test for target genes using Real-time PCR (RT-PCR)	86
11.1- Normalization of the real-time data and statistical	88
analysis	90
12. Determination of <i>P. l. larvae</i> in honey bee queens ovaries.	89
12.1. Bacterial isolation from the treated queens' ovaries	89 90
12.2. <i>P. l. larvae</i> detection in treated queens' ovaries using PCR technique	90
13.Data analysis	90
IV-RESULTS	91
1. Identification and characterization of <i>P. l. larvae</i>	91
2. Susceptibility of the honey bees queens to <i>P. l. larvae</i>	97
3. Breeding of challenged honey bee queens	99
4. Effects of sub-lethal dose of <i>P. l. larvae</i> on external	,,
characteristics of honey bee queen	104
4.1. The wet body weight	104

4.2. External body characteristics	1
5- Effects of sub-lethal dose of <i>P. l. larvae</i> on internal body	1
characteristics of honey bee queen	
5.1.Ovary length and width	1
5.2- The number of ovarioles	1
6- Effects of sub-lethal dose of <i>P. l. larvae</i> on the production	1
of antibacterial activity in honey bee queen	
6.1. The total body homogenate	1
6.2. The ovary extract	1
7- Effects of sub-lethal dose of <i>P. l. larvae</i> on protein	1
concentrations of honey bee queens	
7.1- The total body homogenate	1
7.2- The ovary extract	1
8- Electrophoretic analysis of total body and ovary proteins for	
the honey bee queens	1
8.1-Separation of total body protein	1
8.2-Separation of ovaries protein	1
9- Molecular qualitative detection of antibacterial gene	1
10- Molecular quantitative of gene expression using Real-time	
PCR (RT-PCR)	
11- Detection of <i>P. l. larvae</i> in treated honey bee queens'	1
ovaries	
11.1. Bacterial isolation from the treated queens' ovaries	1
11.2. P. l. larvae detection in treated queens' ovaries using]
PCR technique.	
V-DISCUSSION AND CONCLUSION]
1- Identification of <i>P. l. larvae</i>	1
2- Susceptibility of honey bee queen larvae to <i>P. l larvae</i>	1
3- Effects of sub-lethal dose of <i>P. l. larvae</i> on external and	1
internal queen body characteristics	
4- Effects of sub-lethal dose of <i>P. l. larvae</i> on the production	1
of antibacterial activity in honey bee queen	
5- Effects of sub-lethal dose of <i>P. l. larvae</i> on honey bee total	1
body proteins	
6- Electrophoretic analysis of body proteins of honey bee	
queens	1
7- Qualitative and quantitative molecular characterization of	
antibacterial genes in P. l. larvae challenged honey bee	
queen	1
8- Presence of <i>P. l. larvae</i> in the ovaries of treated honey bee	
queens]
9-Conclusion	1

10-Recommendations	175
VI-SUMMARY	176
VII- REFERENCES	182
VIII- ARABIC SUMMARY	1

List of Table

Table 1:	Different types of Gram +ve (<i>P. l. larvae</i>) and Gram –ve (<i>E-coli</i> 1 & 2 and <i>Klebsiella pneumonia</i>) bacteria used in determination of antibacterial activity	72
	for healthy, water-treated and bacteriatreated honey bee queens	
Table 2:	Susceptibility test of A. mellifera queens to P. l. larvae by feeding through fourth larval instar.	98
Table 3:	The wet body weight (mg) and external body characteristics (cm) of normal, water-treated (control) and <i>P. l. larvae</i> - treated honey bee queens	107
Table 4:	Ovary size (cm) and number of ovarioles/ovary) of A. mellifera virgin queens in Healthy, Water fed and treated with P. l. larvae	112
Table 5:	Antibacterial zone activity (cm) of total body extract of healthy, water treated and bacterial treated <i>A. mellifera</i> queens with <i>P. l. larvae</i> against <i>P. l. larvae</i> and different multidrug resistant bacteria <i>E-coli</i> 1, <i>E-coli</i> 2 and <i>K. pneumonia</i>	117
Table 6:	Antibacterial zone activity (cm) of healthy, water treated and bacterial treated queens' ovaries against <i>P. l. larvae</i> and different multidrug resistant bacteria <i>E-coli</i> 1, <i>E-coli</i> 2 and <i>K. pneumonia</i>	121
Table 7:	Total body and ovaries protein concentrations (mg/ml) of <i>A. mellifera</i> queens in healthy, +ve control and treated with <i>P. l. larvae</i>	123
Table 8:	Total body protein patterns of healthy, water treated and bacterial treated <i>A. mellifera</i> queens, bands separated using 10% SDS-PAGE technique	128
Table 9:	Comparative analysis of relative concentration (band%) of total body protein patterns of healthy, water treated and bacterial treated <i>A. mellifera</i> queens, bands separated using 10% SDS-PAGE technique.	129
Table 10:	Comparative analysis of rate of flow (Rf) of total body protein bands of healthy, water treated and bacterial treated <i>A. mellifera</i> queens, bands separated using 10% SDS-PAGE technique.	130
Table 11:	Comparative analysis of molecular weight (M.W.) of total ovaries protein bands of healthy, water treated and bacterial treated <i>A. mellifera</i> queens, bands were separated using 10% SDS-PAGE technique.	135
Table 12:	Comparative analysis of relative concentration (band%) of total ovaries protein bands of healthy, water treated and bacterial treated <i>A. mellifera</i> queens, bands were separated using 10% SDS-PAGE technique.	136
Table 13:	Comparative analysis of rate of flow (Rf) of total ovaries protein bands of healthy, water treated and bacterial treated <i>A. mellifera</i> queens, bands were separated using 10% SDS-PAGE technique.	137
Table 14:	The specific primers used in the amplification of Ab, Df and Vg genes from <i>A. mellifera</i> L. queens.	140
Table 15:	Mean values of cycle threshold (ct) of abaecin, defensin and vitellogenin genes expression in control and treated honey bee queens in accordance to the fold change to control	144

List of Figures

Fig. 1:	Standard calibration curve for the estimation of total protein content determined by the dye binding method	75
Fig. 2:	Cultivation of <i>P. l. larvae</i> on j- agar plate showed solitary colonies, whitish to grayish in color and have irregular edges.	93
Fig. 3:	Vegetative and spore cells of <i>P. l. larvae</i> stained with Gram stain showed that <i>P. l. larvae</i> is a Gram positive bacteria	93
Fig. 4:	Vegetative and spore cells of <i>P. l. larvae</i> stained with Malachite green. The spores appeared green and vegetative cells appeared red in color.	93
Fig. 5:	Holst milk test.	94
Fig. 6:	Nitrate reduction test.	94
Fig. 7:	Casein hydrolysis test showed clear zone around the growing <i>P. l. larvae</i> culture.	95
Fig. 8:	Starch hydrolysis test showed inability of <i>P. l. larvae</i> to digest starch, i.e., -ve starch hydrolysis.	95
Fig. 9:	Catalase production test showed no bubbles appearance, i.e., - ve catalase test	95
Fig. 10:	PCR products of 550 bp specific for <i>P. l. larvae</i> cultured colony obtained by using KAT1 and KAT2 primers. (S): bacterial sample and (C): control without bacteria.	96
Fig. 11:	Susceptibility of A. mellifera queens to P. l. larvae by feeding through fourth larval instar.	98
Fig. 12:	Honey bee brood frame containing eggs and 3 different larval instars; arrow show the one day larva selected to grafting	100
Fig. 13:	The queen cups prepared by putting one drop of diluted royal jelly in each cup, to receive the selected larvae.	100
Fig. 14:	The three horizontal wooden bars fixed on a special grafting frame with queens cups, where honey bee workers feed and care about queens.	101
Fig. 15:	The cup cells containing 4th larval instar (3 days later after larval transfer) received the bacterial dose (LD2o) of <i>P. l . l ar v a e</i> or water to the queen's food.	101
Fig. 16:	Six days post grafting (the queen's cells were capped and queens started its pupation period).	102
Fig. 17:	Ten days post grafting (queen cells were carefully separated and transferred individually on honey frame under a half ball cage).	102
Fig. 18:	13 days post grafting the queens were emerged and transferred to a wooden queen bee cages for easy transport	103
Fig. 19:	The emerged honey bee queen after grafting	103
Fig. 20:	The body wet weight (mg) of normal, control and bacterial treated honey bee.	107
Fig. 21:	The physical characteristics (total body length, abdomen length, thorax width, fore wing length and head width (cm) of normal, control and bacterial treated honey bee queens measured to determine the queen quality post treatment with sub-lethal dose of <i>P. l. larvae</i> .	108

Fig. 22:	Stereomicroscopic photograph showing left and right ovaries of honey bee queen dissected via dorsal abdominal midline (magnification 12x). Scale bar 100 µm.	111
Fig. 23:	Stereomicroscopic photograph showing magnified honey bee queen right ovary (magnification 20x). Scale bar 100 µm.	111
Fig. 24:	Length and width of <i>A. mellifera</i> virgin queens right ovary (cm) determined in healthy, control and treated with <i>P. l. larvae</i> .	112
Fig. 25:	Total number of ovarioles in ovary (ovariole/ovary) of <i>A. mellifera</i> virgin queens determined in healthy, control and treated with <i>P. l. larvae</i> .	113
Fig 26:	Antibacterial zone activity (cm) of <i>A. mellifera</i> queens determined for healthy (-ve control) queens, water fed (+ve control) queens, and bacterial fed (treated) queens with <i>P. l. larvae</i> against <i>P. l. larvae</i> , <i>E-coli</i> 1, <i>E-coli</i> 2 and <i>Klebsiella pneumonia</i> .	116
Fig. 27:	Antibacterial zone activity (cm) of healthy, water-treated and bacteria-treated queens total body extract against <i>P. l. larvae</i> and different multidrug resistant bacteria <i>E-coli</i> 1, <i>E-coli</i> 2and <i>K. pneumonia</i> .	117
Fig 28:	Antibacterial zone activity (cm) of <i>A. mellifera</i> queens determined for healthy (-ve control) queens, water fed (+ve control) queens, and bacterial fed (treated) queens' ovaries extracts against <i>P. l. larvae</i> .	120
Fig. 29:	Antibacterial zone activity (cm) of healthy, water-treated and bacteria-treated queens ovary extract against <i>P. l. larvae</i> and different multidrug resistant bacteria <i>E-coli</i> 1, <i>E-coli</i> 2and <i>K. pneumonia</i> .	121
Fig. 30:	Total body protein content (mg/ml) of <i>A. mellifera</i> queens determined in healthy, +ve control and treated with <i>P. l. larvae</i>	123
Fig. 31:	Total protein contents of ovaries (mg/ml) of <i>A. mellifera</i> queens determined in healthy, +ve control and treated with <i>P. l. larvae</i> .	123
Fig. 32:	The total body protein patterns of healthy, control and treated <i>A. mellifera</i> queens separated using 10% SDS-PAGE technique.	127
Fig. 33:	Densitometric scanning of 10% SDS – PAG electrophoresis of protein bands, healthy, water fed and bacterial fed <i>A. mellifera</i> queens. Lane 1: -ve control queens; Lane 2: +ve control queens; Lane3: bacterial fed (treated) queens	131
Fig. 34:	Changes in the ovaries protein banding patterns of healthy, control and treated <i>A. mellifera</i> queens separated using 10% SDS-PAGE technique.	134
Fig. 35:	Denistometric scanning of 10% SDS - PAGE electrophoretesis of ovaries protein bands of healthy, water fed and bacterial fed <i>A. mellifera</i> queens	138
Fig 36:	PCR electrophoresis showing the β-Actin specific PCR product of 151 bp in Healthy (H), Control (C) and Treated (T) queens replicates.	140
Fig 37:	-PCR electrophoresis showing the abaecin gene product of 72 bp in healthy (H), control (C) and treated (T) <i>A. mellifera</i> queens replicates.	141
Fig 38:	PCR electrophoresis showing the defensin gene product of 201 bp in healthy (H), control (C) and treated (T) <i>A. mellifera</i> queens replicates.	141
Fig 39:	-PCR electrophoresis showing the vitellogenin gene product of 63 bp in healthy (H), control (C) and treated (T) <i>A. mellifera</i> queens replicates.	142
Fig. 40:	Histogram represents the comparisons of fold changes of abaecin, defensin and vitellogenin genes expression in treated honey bee queens and control.	144
Fig. 41:	Amplification plot represents the relation between the cycle threshold (ct) and	145

	the fluorescence degree for abaecin gene in control and treated honey bee queens.	
Fig. 42:	Amplification plot represents the relation between the cycle threshold (ct) and the fluorescence degree for defensin gene in control and treated honey bee queens.	145
Fig. 43:	Amplification plot represents the relation between the cycle threshold (ct) and the fluorescence degree for vitellogenin gene in control and treated honey bee queens	146
Fig. 44:	Isolation of <i>P. l. larvae</i> from treated queens' ovaries on J-agar plate showing <i>P. l. larvae</i> colonies.	148
Fig. 45:	Queens' ovaries DNA extraction showed a <i>P. l. larvae</i> specific PCR product of 550 bp from bacterial treated queens (T) obtained by using KAT1 and KAT2 primers, but no bands were detected in healthy (H) and water treated (W) queens.	148

ABREVIATIONS

ABS Absorbency

American foul brood **AFB** :

Ab Abacin

Apis mellifera A. mellifera:

Antimicrobial peptides **AMPs**

Base pair bp

Bovine serum albumin. **BSA CBB** Coomassie brilliant blue **CFU** Colony formed unit :

Centimeter(s) cm Queens fed water +ve control: Healthy queens -ve control: Cycle threshold ct

Df Defensin

Escherichia coli E coli :

DNA Deoxy ribonuclease acid : European foul brood **EFB** : Healthy queens H

HP30 Hypothetical protein 30

hr Hour(s). :

kDa : Kilo dalton(s). LD Lethal dose. :

LDP line Lethal dose probability line

Lipopolysaccharidase LPS

milligram mg Molar \mathbf{M} Microliter ul micromole μM ml Milliliter Min. Minute(s)

Mol. wt. Molecular weight

NNumber of test replicates **PBS** Phosphate buffered saline **PCR** Polymerase chain reaction : Paenibacillus larvae larvae P. l. larvae:

Prophenoloxidase pPo RNA Ribonuclic acid Standard error SE

Sodium duodecyl sulphate-polyacrylamide gel **SDS-PAGE:**

electrophoresis

ssp. T

Sub species Treated queens (queens fed bacteria) Tris-Acetate EDTA buffer Ethylene diamine tetra acetic Acid Ultraviolet

TAE

EDTA :

UV :