

CHARACTERIZATION OF SOME BACTERIAL VIRUSES ISOLATED FROM DRAIN WATER AND THEIR APPLICATIONS

By

YASMER SAYED HUSSEIN MUHAMMAD

B.Sc. Agric. Sc. (Agric. Microbiology), Ain Shams Univ., 2003

M.Sc. Agric. Sc. (Agricultural Viruses), Ain Shams Univ., 2010

A Thesis Submitted in Partial Fulfillment

Of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in

**Agricultural Sciences
(Agricultural Viruses)**

**Department of Agricultural Microbiology
Faculty of Agriculture
Ain Shams University**

2018

Approval Sheet

CHARACTERIZATION OF SOME BACTERIAL VIRUSES ISOLATED FROM DRAIN WATER AND THEIR APPLICATIONS

By

YASMER SAYED HUSSEIN MUHAMMAD

B.Sc. Agric. Sc. (Agric. Microbiology), Ain Shams Univ., 2003

M.Sc. Agric. Sc. (Agricultural Viruses), Ain Shams Univ., 2010

This thesis for Ph.D. degree has been approved by:

Dr. Gamal El-Didamony Mohamed
Prof. of Virology, Fac. of Science, Zagazig University.

Dr. Atef Shoukry Sadik
**Prof. Emeritus of Agric. Viruses, Fac. of Agric., Ain Shams
University.**

Dr. Badawi Abd El-Salam Othman
**Prof. Emeritus of Agric. Viruses, Fac. of Agric., Ain Shams
University.**

Dr. Khalid Abdel-Fattah El-DougDoug
**Prof. Emeritus of Agric. Viruses, Fac. of Agric., Ain Shams
University.**

Date of Examination: 19/12 /2017

CHARACTERIZATION OF SOME BACTERIAL VIRUSES ISOLATED FROM DRAIN WATER AND THEIR APPLICATIONS

By

YASMER SAYED HUSSEIN MUHAMMAD

B.Sc. Agric. Sc. (Agric. Microbiology), Ain Shams Univ., 2003

M.Sc. Agric. Sc. (Agricultural Viruses), Ain Shams Univ., 2010

Under the Supervision of:

Dr. Khalid Abdel-Fattah El-DougDoug

Prof. Emeritus of Agricultural Viruses, Dept. of Agric.
Microbiology, Fac. of Agriculture, Ain Shams University
(Principle Supervisor)

Dr. Badawi Abd El-Salam Othman

Prof. Emeritus of Agricultural Viruses, Dept. of Agric.
Microbiology, Fac. of Agriculture, Ain Shams University

ABSTRACT

Yasmer Sayed Hussein Muhammad: Characterization of some Bacterial Viruses Isolated from Drain Water and Their Applications. Unpublished Ph.D. Thesis, Department of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, 2018.

We are here focusing on isolation of some bacterial viruses from sewage water, irrigation water contaminated with sewage and characterization of these viruses biologically and chemically using some modern technologies. Their use as biological control agents against the contaminated bacteria was also aimed. A number of water samples, including water from irrigation and other systems contaminated with sewage, were collected from four different canals in El-Kalubiah and El-Giza Governorates. On microbiological analysis, some pathogenic bacteria, including *Listeria* sp. and *Salmonella* sp., were isolated and then the presence of phages specific to these bacteria was detected by spot test. Six phages for each bacterial host were propagated, and partially purified. The phages were characterized by determining their stability under different factors, and determination of their morphology by electron microscopy. The molecular weight of their proteins was estimated using SDS-PAGE, and finally, RAPD-PCR technique was used to determine the genetic variability between the isolated phages. Samples of green salad, which is not exposed to any thermal transactions, and could be contaminated with undesirable microbes, were tested as previously mentioned. Determination the safety of using phages as a food additive was done by testing the cytotoxicity of phages on liver carcinoma cell line *in vitro*. The results indicated that all phages didn't show any cytotoxic effect on liver carcinoma cell line. Phage cocktails were applied as biological control agents against the pathogenic bacteria of this study (*L. monocytogenes* and *S. Typhimurium*). Promising results were recorded, therefore, the results can be considered as a milestone for the formation of

a bank of bacterial viruses which can be widely used in biological control as well as gene therapy.

Keywords: Phages, Microbiological analysis, Stability, SDS-PAGE, RAPD-PCR, Cytotoxicity on cell line, *Listeria monocytogenes*, *Salmonella* Typhimurium, Biocontrol.

ACKNOWLEDGMENT

Praise and thanks be to ALLAH, the most merciful for assisting and directing me to the right way

I would like to express my deepest gratitude and special respect to **Dr. Khalid Abd El-Fattah El-DougDoug**, Emeritus Professor of Agric. Virology, Agric. Microbial. Dept., Fac. of Agric., Ain Shams Univ., for his sincere, kind supervision and important accurate notices from the beginning to the end of this work.

Thanks to **Dr. Badawi Abd El-Salam Othman**, Emeritus Professor of Agric. Virology, Agric. Microbial. Dept., Fac. of Agric., Ain Shams Univ., for his helpful supervision, valuable guidance, and his encouragement to accomplish this study.

Thanks are also due to **Dr. Samar Sayed Ahmed**, Assistant Professor of Agric. Virology, Agric. Microbial. Dept., Fac. of Agric., Ain Shams Univ., for her kindly and useful help and continuous guidance during the period of this study, especially within preparing the thesis.

I would like to express my great appreciation to all staff members of the Agric. Microbial. Dept., Fac. of Agric., Ain Shams Univ., for their support during this investigation.

CONTENTS

	Page
LIST OF TABLES	iv
LIST OF FIGURES	vi
LIST OF ABBRIVIATION	viii
INTRODUCTION	1
REVIEW OF LITERATURE	4
MATERIALS AND METHODS	37
1. Water samples collection.....	37
2. Microbiological analyses of the water samples.....	38
2.1. Determination of TVBCs.....	38
2.2. Determination of the coliform group.....	39
2.2.1. Determination of the TC count	38
A. Presumptive Phase.....	38
B. Confirmed Phase.....	39
C. Completed Phase.....	39
2.2.2. Determination of the FC count	39
2.3. Determination of the FS count.....	39
2.4. FC/TC percentage	40
2.5. FC/FS ratio	40
2.6. Detection of pathogenic bacteria.....	40
2.6.1. Detection of <i>Salmonella</i> spp.....	40
2.6.2. Detection of <i>Listeria</i> spp.....	40
3. Occurrence of bacteriophages specific for <i>Listeria</i> and <i>Salmonella</i> as a bacterial pathogens in the water	41
3.1. Detection of the phages specific for <i>Listeria</i> and <i>Salmonella</i> in the water	41
3.1.1. Bacterial hosts used for detection and isolation of phages	41
3.1.2. Growing and maintenance of bacterial hosts	41
3.1.3. Detection of the temperate phages in <i>Listeria</i> and <i>Salmonella</i> bacterial cultures	42
3.1.3.1. Temperate phages	42
3.1.3.2. Detection of <i>Listeria</i> and <i>Salmonella</i> lytic phages in the water samples.....	42

3.1.4.1. Detection of the lytic phages qualitatively.....	43
3.1.4.2. Detection of the lytic phages quantitatively.....	43
3.1.5. Isolation and propagation of bacteriophages	43
3.1.6. Purification and concentration of <i>Listeria</i> and <i>Salmonella</i> phage stocks.....	44
3.1.7. Characterization of the isolated bacteriophages.....	44
3.1.7.1. Microscopic examination of <i>Listeria</i> and <i>Salmonella</i> phages.....	44
3.1.7.2. Determination of phages stability.....	45
3.1.7.2.1. Thermal inactivation point	45
3.1.7.2.2. Longevity <i>in vitro</i>	45
3.1.7.2.3. pH stability	45
3.1.7.2.4. UV radiation stability	46
3.1.7.3. Host range pattern.....	46
3.1.8.4. Characteristic of UV spectra.....	47
3.1.8. Chemical properties <i>Listeria</i> and <i>Salmonella</i> phages.....	47
3.1.8.1. Determination of phages protein quantity by Bradford method	47
A. Preparation of the standard curve of protein.....	47
B. Determination of protein contents	48
3.1.8.2. Determination of molecular weights of phages pro- teins by SDS-PAGE.....	48
A. Preparation of SDS-Polyacrylamide Gel.....	48
B. Preparation of phage's proteins.....	49
C. Electrophoresis of protein.....	49
3.1.8.3. Molecular variability among phage isolates	50
3.1.8.3.1. Extraction of total nucleic acids.....	50
3.1.8.3.2. Agarose gel electrophoresis.....	51
3.1.8.3.3. RAPD PCR.....	51
3.1.8.3.3.1. DNA amplification cycles.....	51
3.1.8.3.3.2. Gel electrophoresis.....	52
4. Application of bacteriophages for biological control.....	52
4.1. Experimental design.....	53
4.2. Microbiological analysis.....	53

4.3. Statistical analysis.....	53
5.Studying the cytotoxicity of different concentrations of phage cocktails on tissue culture.....	53
6. Collection of vegetable sample irrigated with polluted water.....	56
7. Collection of green salad samples and detection of the pathogenic bacteria	57
8. Detection and isolation of phages from the collected green salad samples.....	57
9. Host range of the isolated bacteriophages from green salads	58
10. Tested the effect of different concentrations of some food additives to green salad on the phage cocktails.....	58
11.Combating the bacterial pathogens in food by the phages.....	58
11.1.Post-harvest phage application	58
11.1.1. Green salad preparation.....	58
11.1.2. Preparation of the bacterial inoculum.....	59
11.1.3. Preparation of the phage inoculum.....	59
11.1.4. Design of the experiment.....	59
11.1.5. Bacterial pathogens counts.....	60
11.1.6. Assaying of bacteriophages.....	61
11.1.7. Statistical analysis.....	61
12. Media and Buffers.....	62
RESULTS.....	75
DISCUSSION.....	129
SUMMARY.....	146
REFERENCES.....	155
ARABIC SUMMARY.....	

LIST OF TABLES

No.		Page
1	Preparation of separating and stacking gel of SDS-PAGE...	49
2	Program of RAPD-PCR.....	52
3	Microbiological analyses of water samples obtained from different canals	77
4	Detection of <i>Listeria</i> lytic phages in water and sewage water samples.....	79
5	Detection of <i>Salmonella</i> lytic phages in water and sewage water samples.....	80
6	Plaque morphology and concentrations of <i>Listeria</i> phages...	82
7	Plaque morphology and concentrations of <i>Salmonella</i> phages.....	83
8	Virus particles morphology of <i>Listeria</i> phages.....	85
9	Virus particles morphology of <i>Salmonella</i> phages.....	87
10	Determination of thermal inactivation point of the isolated <i>Listeria</i> phages.....	89
11	Determination of thermal inactivation point of the isolated <i>Salmonella</i> phages.....	90
12	Effect of pH values after different periods on the <i>Listeria</i> phages.....	91
13	Effect of pH values after different periods on the <i>Salmonella</i> phages.....	92
14	Effect of UV light on the <i>Listeria</i> phages.....	93
15	Effect of UV light on the <i>Salmonella</i> phages.....	94
16	Host specificity of the isolated <i>Listeria</i> phages.....	95
17	Host specificity of the isolated <i>Salmonella</i> phages.....	96
18	Spectrophotometric data of purified <i>Listeria</i> phages.....	97
19	Spectrophotometric data of purified <i>Salmonella</i> phages.....	100
20	Protein patterns of <i>Listeria</i> phages determined by SDS-PAGE analysis.....	105

21	Protein patterns of <i>Salmonella</i> phages determined by SDS-PAGE analysis.....	107
22	Genetic polymorphoism among amplified DNA of <i>Listeria</i> phages.....	109
23	Genetic polymorphoism among amplified DNA of <i>Salmonella</i> phages.....	111
24	Effect of different phage multiplicity of infection on the growth of <i>Listeria in vitro</i>	113
25	Effect of different phage multiplicity of infection on the growth of <i>Salmonella in vitro</i>	115
26	Microbiological analysis of different green salad samples obtained from different levels of restaurants.....	118
27	Host range of the green salads phages.....	120
28	Plaque morphology of <i>Listeria</i> phages isolated from the green salads.....	120
29	Plaque morphology of <i>Salmonella</i> phages isolated from the green salads.....	121
30	Lytic efficiency of <i>Listeria</i> phages in green salad application.....	124
31	Lytic efficiency of <i>Salmonella</i> phages in green salad application.....	127

LIST OF FIGURES

No.		Page
1	Photographs showing (A) Meet Nama canal as one of the four water sample sources and (B) Removing of the sewage into the canal.....	37
2	Photoplate of spot test shows the bacterial lysis (arrow) caused by virulent phage specific for <i>S. Typhimurum</i>	81
3	(A) Single plaques of <i>L. monocytogenes</i> phage showing identical morphology of the original plaques..... (B) Single plaques of <i>S. Typhimurum</i> phage showing identical morphology of the original plaques.....	81
4	Electron micrographs of the isolated <i>Listeria</i> phages (ØLG, ØLA ØLM, ØLN, ØLD, and ØLP) negatively stained with 2% uranyl acetate	86
5	Electron micrographs of the isolated <i>Salmonella</i> phages (ØSM, ØSF, ØSG, ØSP, ØSA and ØSD) negatively stained with 2% uranyl acetate	88
6	UV spectrum of purified ØLG <i>Listeria</i> phage.....	97
7	UV spectrum of purified ØLN <i>Listeria</i> phage.....	98
8	UV spectrum of purified ØLA <i>Listeria</i> phage.....	98
9	UV spectrum of purified ØLD <i>Listeria</i> phage.....	98
10	UV spectrum of purified ØLM <i>Listeria</i> phage.....	99
11	UV spectrum of purified ØLP <i>Listeria</i> phage.....	99
12	UV spectrum of purified ØSM <i>Salmonella</i> phage.....	100
13	UV spectrum of purified ØSF <i>Salmonella</i> phage.....	101
14	UV spectrum of purified ØSG <i>Salmonella</i> phage.....	101
15	UV spectrum of purified ØSP <i>Salmonella</i> phage.....	101
16	UV spectrum of purified ØSA <i>Salmonella</i> phage.....	102
17	UV spectrum of purified ØSD <i>Salmonella</i> phage.....	102
18	Quantitation of <i>Listeria</i> viral protein using Bradford method: 1, 2,3,4,5 and 6 for ØLG, ØLN, ØLA, ØLD, ØLM and ØLP, respectively.....	103

19	Quantitation of <i>Salmonella</i> viral protein using Bradford method: 1, 2, 3, 4, 5 and 6 for ØSM, ØSF, ØSG, ØSP, ØSA and ØSD.....	103
20	SDS-PAGE protein patterns of <i>Listeria</i> phages.....	106
21	SDS- PAGE protein patterns of <i>Salmonella</i> phages.....	108
22	Electrophoresis of extracted DNA of <i>Listeria</i> (1-6) and <i>Salmonella</i> (7-12) phages in 1% agarose.....	108
23	Agarose gel (1%) shows the DNA polymorphism of the six <i>Listeria</i> phages (ØLG, ØLN, ØLA, ØLD, ØLM and ØLP) amplified by OPA-01 primer. M, DNA marker.....	110
24	Agarose gel (1%) shows the DNA polymorphism of the six <i>Salmonella</i> phages (ØSM, ØSF, ØSG, ØSP, ØSA and ØSD) amplified by OPA-01 primer. M, DNA marker.....	111
25	The increasing and reduction of <i>Listeria</i> log numbers in control and at the different MOI.....	114
26	The increasing and reduction of <i>Salmonella</i> log numbers in control and at the different MOI.....	116
27	Negative control (non-treated livercar cinoma cell line).....	117
28	Positive control (Doxorubicin–HCL with concentration 6 µg/mL).....	117
29	Different pathogenic bacterial colonies growing on different media, (A) BSA; (B) XLD; (C) EMB and (D) PAL-CAM agar.....	119
30	The increasing and reduction of <i>Listeria</i> log numbers in the different treatments.....	125
31	The increasing and reduction of <i>Salmonella</i> log numbers in the different treatments.....	128

LIST OF ABBREVIATIONS

A

Agric.	Agriculture
APHA	American Public Health Association
APS	Ammonium persulfate

B

BCM	Billion Cubic Meter
BHI	Brain Heart Infusion
bp	Base pair
BPB	Bromophenol blue
BPW	Buffer peptone water
BGLBB	Brilliant green lactose bile broth
BSA	Bovine serum albumin
BiSA	Bismuth sulphite agar

C

°C	Centigrade
CaCl ₂	Calcium chloride
CBB	Coomassie brilliant blue
cfu	Colony forming unit
Cm	Centimeter
Cm ²	Square centimeter
CR agar	Congo red agar
CsCl	Cesium chloride

D

Dept.	Department
DLA	Double layer agar
DMSO	Dimethyl sulfoxide
DNA	Deoxy ribonucleic acid
ds-DNA	Double strand deoxy ribonucleic acid

E

<i>e.g.</i>	Exempli gratia (For example)
EDTA	Ethylene diamine tetra acetic acid
EHEC	Enterohemorrhagic <i>E. coli</i>
EM	Electron microscope
EMB	Eosin methylene blue
EPA	Environmental protection agency
<i>et al.</i>	And others (<i>et alii</i>)

F

°F	Fahrenheit
Fac.	Faculty
FC	Fecal coliform
FDA	Food and drug agency
FS	Fecal streptococci

G

<i>g</i>	Gram
<i>g</i>	Gravity
GRAS	Generally recognized as safe