Alexandria University

Faculty of Veterinary Medicine

Effects of silver and zinc oxide nanoparticles incorporated with chitosan hydrogel beads against some MDR and biofilm forming bacteria affecting cultured shrimp

A Thesis

Presented to the Graduate School,

Faculty of Veterinary Medicine, Alexandria University

In Partial Fulfillment of the Requirement for the degree

of

Ph.D. of Veterinary Science

Specialization

(Bacteriology – Mycology)

By

Fatma Ali Zaref Moustafa Sanhoury

(B.V. Sc., Fac. Vet. Med. Alexandria University, 2011)

(M.V. Sc., Fac. Vet. Med. Alexandria University, 2016)

(2021)

LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PHOTOS

LIST OF ABBREVIATIONS

A. hydrophila	***Aeromonas hydrophila***
α- hemolysis	**Alpha hemolysis**
AMC30µg	**Amoxicillin/clavulanic acid**
AgNO	**Silver nitrite**
Ag NPs	**Silver Nanoparticles**
β hemolysis	**Beta hemolysis**
cfu	**Colony Forming Unit**
CTX 30µg	**Cefotaxime**
CIN Agar	**Cefsulodin-Irgasan-Novobiocin Agar**
CL30µg	**Cephalexin**
CN 10µg	**Gentamicin**
C 30µg	**Chloramphenicol**
CIP 5µg	**Ciprofloxacin**
CIT	**Citrate utilization**
conc.	**Concentration**
CS	**Chitosan**
DW	**Distilled water.**
DO 30µg	**Doxycycline**
GIT	**Gastrointestinal tract**
g	**Gram**
GN card	**Gram negative card**
hr/s	**hour/s.**

H_2S	**Hydrogen sulphide production**
I	**Intermediate**
ID	**Identification number**
MALDI-TOF MS	**Matrix-assisted laser desorption/ionization time-off light Mass Spectrometry**
MAR	**Multiple Antibiotic Resistance**
MDR	**Multi Drug Resistance**
mg	**Milligram**
MHA	**Mullar Hinton Agar**
MHB	**Mullar Hinton Broth**
ml	**Milliliter**
min.	**Minute(s)**
µg	**Microgram**
µl	**Microliter**
MSA	**Mannitol Salt Agar**
MR	**Methyl red**
NaCl	**Sodium Chloride**
NaOH	**Sodium hydroxide**
no.	**Number**
O.D	**Optical Density**
ODC	**Optical Density cut-off value**
P 10 µg	**Penicillin**
P. aeruginosa	***Pseudomonas aeruginosa***
ppm	**Part per million**
R	**Resistant**

SAM20 µg	**Ampicillin/sulbactam**
SXT 25µg	**Trimthoprim /Sulpha-methoxazole**
S	**Sensitive**
sec.	**Second**
SEM	**Scanning Electron Microscope**
SD	**standard deviation**
S. algae	***Shewanella alga*e**
S. ***putrefaciens***	***Shewanella putrefaciens***
S. aureus	***Staphylococcus aureus***
TCBS	**Thiosulphate citrate bile salt sucrose agar**
TCP	**Tissue culture plate**
TSA	**Trypticase soya agar**
TSB	**Trypticase soya broth**
TL15µg	**Tylosin**
V. alginolyticus	***Vibrio alginolyticus***
ZnO NPs	**Zinc Oxide Nanoparticles**

List of Abbreviations of GN card of VITEK system

Well	Test	Mnemonic	Amount/Well
2	Ala-Phe-Ppro-ARYLAMIDASE	APPA	**0.0384 mg**
3	ADONITOL	ADO	**0.1875 mg**
4	L-Pyrrolydonyl-ARYLAMIDASE	PyrA	**0.018 mg**
5	L-ARABITOL	IARL	**0.3 mg**
7	D-CELLOBIOSE	dCEL	**0.3 mg**
9	BETA-GALACTOSIDASE	BGAL	**0.036 mg**
10	H2S PRODUCTION	H2S	**0.0024 mg**
11	BETA-N-ACETYL-GLUCOSAMINIDASE	BNAG	**0.0408 mg**
12	Glutamyl Arylamidase pNA	AGLTp	**0.0324 mg**
13	D-GLUCOSE	dGLU	**0.3 mg**
14	GAMMA-GLUTAMYL-TRANSFERASE	GGT	**0.0228 mg**
15	FERMENATATION/GLUCOSE	OFF	**0.45 mg**
17	BETA-GLUCOSIDASE	BGLU	**0.036 mg**
18	D-MALTOSE	dMAL	**0.3 mg**
19	D-MANNITOL	dMAN	**0.1845 mg**
20	D-MANNOSE	dMNE	**0.3 mg**
21	BETA-XYLOSIDASE	BXYL	**0.0324 mg**
22	BETA-Alanine arylamidase pNA	BAlap	**0.0174 mg**
23	L-Proline ARYLAMIDASE	ProA	**0.0234 mg**
26	LIPASE	LIP	**0.0192 mg**
27	PALATINOSE	PLE	**0.3 mg**

29	Tyrosine ARYLAMIDASE	TyrA	**0.0276 mg**
31	UREASE	URE	**0.15 mg**
32	D-SORBITOL	dSOR	**0.1875 mg**
33	SACCHAROSE/SUCROSE	SAC	**0.3 mg**
34	D-TAGATOSE	dTAG	**0.3 mg**
35	D-TREHALOSE	dTRE	**0.3 mg**
36	CITRATE (SODIUM)	CIT	**0.054 mg**
37	MALONATE	MNT	**0.15 mg**
39	5-KETO-D-GLUCONATE	5KG	**0.3 mg**
40	L-LACTATE alkalinisation	ILATk	**0.15 mg**
41	ALPHA-GLUCOCSIDASE	AGLU	**0.036 mg**
42	SUCCINATE alkalinisation	SUCT	**0.15 mg**
43	Beta-N-ACYTYL-GALACTOSAMINIDASE	NAGA	**0.0306 mg**
44	ALPHA-GALACTOSIDASE	AGAL	**0.036 mg**
45	PHOSPHATASE	PHOS	**0.0504 mg**
46	Glycine ARYLAMIDASE	GlyA	**0.012 mg**
47	ORNITHINE DECARBOXYLASE	ODC	**0.3 mg**
48	LYSINE DECARBOXYLASE	LDC	**0.15 mg**
52	DECARBOXYLASE BASE	ODEC	**N/A**
53	L-HISTIINE assimilation	IHISa	**0.0378 mg**
56	COUMARATE	CMT	**0.126 mg**
57	Beta- GLUCORONIDASE	BGUR	**0.0378 mg**
58	O/129 RESISTANCE (comp.vibrio)	O129R	**0.0105 mg**

59	GLU-Gly-Arg- ARYLAMIDASE	GGAA	**0.0576 mg**
61	L-MALATE assimilation	IMLTa	**0.042 mg**
62	ELLMAN	ELLM	**0.03 mg**
64	**L-LACTATE assimilation**	**ILATa**	**0.186 mg**

6. SUMMARY

Shrimp covers a major part of the aquaculture industry and as demanding seafood; it comprises 15-20% of total fishery products worldwide. Seafood act as a vehicle for all important species of foodborne pathogens to human and cause serious human disturbances which sometimes leads to death. Therefore, this study aimed to demonstrate the drug-resistant pathogenic bacteria that affected the cultured shrimp, and considered as important human disease factors associated with seafood consumption and try to find an antibacterial alternative to face the worsening problem of antibiotic resistance.

A total number of 250 cultured shrimp samples were collected from different private shrimp farms in Alexandria Governorate, Borg Al-Arab city and Kafr El-Sheikh during the period from May (2018) till June (2019).

A total of 62 bacterial isolates were recovered from cultured shrimp samples including 53 isolates (85.5%) of Gram-negative bacteria and 9 isolates (14.5%) of Gram-positive bacteria. After the biochemical characterization, using conventional phenotypic characteristics and automated VITEK 2 system and then confirmation by MALDI-TOF MS, the results identified the isolated bacteria as following, the most common isolated bacteria among shrimp samples were *V. alginolyticus* constituting 15 isolates (24.2%) followed by *P. aeruginosa* and *A. hydrophila*, 13 isolates (21%) and 12 isolates (19.4%); respectively. Then *Shewanella algae* and *Shewanella putrefaciens* showed moderate to low prevalence constituting 9 isolates (14.5 %) and 4 isolates (6.4%); respectively. On the other hand, *Staphylococcus aureus* was the only isolated Gram-positive bacterial species and it was 9 isolates with a percentage of (14.5%).

On 5% sheep blood agar medium most of the isolated bacteria gave beta hemolysis and some of them gave alpha hemolysis, but few isolates didn't show any hemolytic activity.

Antibiogram sensitivity test and multi-drug resistant bacteria detection showed that some of the tested isolates had a strong resistance to the antibiotic as; *P. aeruginosa* (2 isolates), *Staph. aureus* (2 isolates), *S. algae* (one isolate) *S. putrefaciens* (one isolate) and *A. hydrophila* (one isolate) and some of them showed moderate resistance to the antibiotic as; *S. algae* (2 isolates), *S. putrefaciens* (2 isolates), *V. alginolyticus* (2 isolates), *A. hydrophila* (one isolate), *P. aeruginosa* (one isolate) and *Staph. aureus* (one

isolate). I tried to find out the mechanism of this resistance by uncovering the biofilm formation ability of these MDR bacteria. Biofilm formation was observed in all tested isolates but with different degrees. I found that; Out of 24 isolates, 16 isolates (66.6%) were strong to moderate biofilm-forming 7 isolates and 9 isolates; respectively. But the other 8 isolates (33.3%) had weak biofilm formation. Also, I found a strong relationship between MDR ability and biofilm formation ability.

I tried to apply alternative anti-bacterial components to counteract antibiotic resistance and biofilm formation. The antibacterial effects of pure chitosan against sensitive, intermediate and strong antibiotic-resistant isolated bacteria were tested, also, I incorporated silver NPS and zinc oxide NPs into chitosan polymer and test the antibacterial effects of them. Blank chitosan beads at 2% concentration showed acceptable inhibitory effects against all the drug-sensitive bacteria and moderately drug-resistant bacteria, but they didn't show any antibacterial effect against the MDR, strong biofilm-forming bacteria. Also, ZnO NPs- CS beads in their low concentration (10- 20 ppm) had a very good antibacterial effect on sensitive and moderately resistant isolates but the antibacterial effect on MDR isolates is low, at high concentrations (50-100 ppm) ZnO NPs- CS beads showed a significant antibacterial effect on all isolated bacteria. Referring to Ag NPs- CS beads, they showed magnificent antibacterial effects on all isolated bacteria at all their tested concentrations.

From the aforementioned results, I found that many antibiotic resistant bacteria were present and were isolated from cultured shrimp. Bacterial diseases affecting shrimp cause significant economic losses in farms, and the presence of antibiotic resistance reduce the efficacy of antibiotic treatment for their infections. Also, there is a strong relationship between MDR ability and biofilm formation ability, as well as increasing antibiotic use and its concentrations to overcome antibiotic resistance and biofilm, makes the condition get worse. So trying to find alternatives to antibiotics has become an urgent necessity. After my experiments on the antibacterial activity of chitosan hydrogel beads and on ZnO NPs and Ag NPs incorporated into chitosan beads, I recommend chitosan as a natural and prophylactic antibacterial alternative to reduce the rate of antibiotic use, I also recommend ZnO NPs (50 ppm) and Ag NPs (10 ppm) which were incorporated into chitosan beads in case of recurrent diseases precedence due to MDR bacteria.