



# FACULTY OF VETERINARY MEDICINE DEPARTMENT OF MICROBIOLOGY

# *Salmonella* species isolated from different sources with special reference to biofilm formation

### 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

# **Bacteriology and Mycology**

## By

# Samir Ibrahim Abd El-Aziz Abd El-Fattah

(B.V.Sc. Faculty of Veterinary Medicine, Beni-Suef University, 2010)

(M.V.Sc. Faculty of Veterinary Medicine, Damanhour University, 2017)

## (2020)





## TABLE OF CONTENTS

Title	Page
1-INTRODUCTION	1
2-REVIEW OF LITERATURE	4
2. 1. Salmonella classification and characterization	4
2.2. Virulence of <i>Salmonella</i>	5
2.3. Incidence of <i>Salmonella</i> infections in different species:	6
2.3.1. Cattle	6
2.3.2. Poultry	8
2.4.Diagnosis of Salmonella infections	9
2.4.1. Phenotypic diagnosis	9
2.4.1.1. Isolation of Salmonella	9
2.4.1.2. Biochemical identification	11
2.4.1.3. Serological identification	11
2.4.1.4. Antimicrobial sensitivity of <i>Salmonella</i>	13
24.1.5. Microbial biofilm	15
2.4.1.5.1. Historical aspect	15
2.4.1.5.2. Composition	17
2.4.1.5.3. Steps in biofilm formation	18
2.4.1.5.3.1. Initial contact/attachment to the surface	18
2.4.1.5.3.2. Micro-colony formation	18
2.4.1.5.3.3. Maturation and architecture	19
2.4.1.5.3.4. Detachment/dispersion of biofilm	19
2.4.1.5.4. Genetic control of biofilm	19
2.4.1.5. 5. Strategies to overcome microbial biofilms:	21
2.4.1.5. 5.1. Inhibition of quorum sensing	21
2.4.1.5. 5.2. Dispersion of extracellular polysaccharide substance of biofilm by	21
enzymes	22
2.4.1.5. 5.3. Cleavage of peptidoglycan	22
2.4.1.5. 5.4. Inhibition through biofilm disassembly	22
2.4.1.5. 5.5. Neutralization or disassembly of lipopolysaccharides	22
2.4.1.5. 5.6. Alteration of membrane potential or membrane permeabilization	23
2.4.1.5. 5.7. Nanotechnology	23





=

2.4.1.5. 6. Relationship between biofilm formation and antimicrobial resistance	23		
2.4.1.5. 7. Biofilm formation of <i>Salmonella</i>			
	20		
2.4.2. Genotypic diagnosis	27		
2.4.2.1. PCR	27		
2.4.2.2. Gene cassettes, integrons and sequencing	29		
2.4.2.3. Eric PCR	32		
3. Material and Methods	34		
3.1-Material	34		
3.1.2. Media used	34		
3.1.2.1. Solid media	34		
3.1.2.2. Semi-solid media	35		
3.1.2.3. Fluid media:	35		
3.1.2.4. Media used for biochemical reactions	35		
3.1.3. Reagents and chemicals	36		
3.1.4. Stain used	36		
3.1.5. Antibiotic sensitivity discs	36		
3.1.7. Material used for detection of biofilm	37		
3.1.8. PCR technique (conventional PCR)	37		
3.1.8.1 Material used for extraction of DNA	37		
3.1.8.2. Equipment and apparatuses used for extraction of nucleic acids	38		
3.1.8.3. PCR Master Mix used for cPCR	38		
	50		
3.1.8.4. Oligonucleotide primers used in cPCR	38		
3.1.8.5. DNA Molecular weight marker	39		
3.1.8.6. Material used for agarose gel electrophoresis	40		
3.1.8.7. Equipment and apparatuses used in PCR	41		
3.1.9. Materials used for DNA sequencing	41		
3.2. Methods	42		
3.2.1. Phenotypic identification	42		
3.2.1. 1. Samples collection	42		
3.2.1.2. Isolation of Salmonella organisms	42		
3.2.1.3. Biochemical identification	43		
3.2.1.4. Serological identification	45		



\_



3.2.1.5. Antimicrobial sensitivity testing	45
3.2.1. 6. Detection of biofilm formation of the isolated Salmonella	46
3.2.1.6.1 Tissue culture plate method	46
3.2.1.6.2. Removal of formed biofilm	47
3.2.2.Genotyoic identification of Salmonella serovars	48
3.2.2.1. PCR and ERIC (enterobacterial repetitive intergenic consensus) PCR	48
3.2.2.1.2. Preparation of PCR Master Mix	49
3.2.2.1.3. Cycling conditions of the primers during cPCR	50
3.2.2.1.4. DNA Molecular weight marker	50
3.2.2.1.5.Agarose gel electrophoreses	51
3.2.2.2. Sequencing method	52
4- RESULTS	56
4.1. Phenotypic identification	56
4.1.1. Prevalence of <i>Salmonella</i> among the calves and chickens samples	56
4.1.2. Colony morphology on different agar media	57
4.1.3. Biochemical identification	57
4.1.4: Serological identification	57
4.1.5. Results of Antimicrobial susceptibility testing	58
4.1.6. Results of biofilm binding activity of <i>Salmonella</i>	60
4.2. Genotypic identification	63
4.2.1. PCR results	63
4.2.2. Integron and sequencing	66
5-DISCUSSION	70
6- ENGLISH SUMMARY	80
7-REFERANCE	80
Arabic summary	2-1



=



#### List of Tables

Table No.	Title	Page	
1	Samples taken for <i>Salmonella</i> isolation.	34	
2	Digonucleotide primers sequences.		
3	Biochemical tests of the isolated Salmonellae	44	
4	Preparation of PCR Master Mix	49	
5	Cycling conditions of the different primers during PCR	50	
6	Preparation of master mix using Big dye Terminator V3.1 cycle sequencing kit.	53	
7	Prevalence of Salmonella among the calves and Chickens samples	56	
8	Serovars of Salmonella isolated from chickens	57	
9	Serovars of Salmonella isolated from calves	58	
10	Antimicrobial sensitivity of the isolated Salmonellae	59	
11	Phenotypic antimicrobial resistance of isolated Salmonellae	59	
12	MDR serovars in chickens	60	
13	MDR serovars in calves	60	
14	Degree of biofilm formation among the isolated <i>Salmonellae</i> from chickens before and after addition of lactic acid 2% (antibiofilm).	61	
15	Degree of biofilm formation among the isolated <i>Salmonellae</i> from calves before and after addition of lactic acid 2% (antibiofilm)	61	
16	Classification of the 14 strains of <i>Salmonella spp.</i> according to the degree of biofilm forming ability.	62	





=

17	Chicken and Calves serovars examined by PCR	66
18	Results of gene cassettes sequencing	68
19	Relationshipbetweenbiofilmformation,Antimicrobialresistance,Resistance genes and ERIC PCR of isolatedSalmonella serovars	69



=



### List of Figures

Figure	Title	Page
No		No
1	Colony morphology on different solid media	57
2	Use of EIISA reader to read the results of biofilm of the samples on 96 wells microtiter plate before and after addition of lactic acid 2% (antibiofilm) at optical density 490 Nabajit (2014).	62
3	Tissue culture plate wells after incubation, staining and adding 30% glacial acetic acid. Right: Strong biofilm binding activity, dark staining of the well. Middle: Moderate biofilm binding activity, moderate staining of the well. Left:	63
4	Weak biofilm binding activity, weak staining of the well Ethidium bromide stained agarose gel electrophoresis containing the PCR products along with 100bp DNA ladder (lane L). Pos: positive control; Neg: negative control which is (Nuclease free water). PCR was carried out using primers specific for <i>inv</i> A (284bp) virulence gene. The ten isolates were positive for this gene.	64
5	Ethidium bromide stained 1.5% agarose gel electrophoresis containing the PCR products along with 100bp DNA ladder (lane L). P: positive control; N: negative control which is (Nuclease free water). PCR was carried out using primers specific for $csgD$ (651bp) virulence gene. The ten isolates were positive for this gene	64
6	ERIC PCR showing different bands in each isolate	65
7	Eric bands (measured by BioDocAnalyze software for installation and image acquistion on computer). Isolates from right to left are (4, 5, 6 and 7). As shown, the number of bands are seven to eight.	65





		•	-	
FACULTY	0 F	VETERINARY	MEDICINE	

\_

8	Ethidium bromide stained agarose gel electrophoresis containing the PCR products along with 100bp DNA ladder (lane L). P: positive control; N: negative control which is (Nuclease free water). PCR was carried out using primers specific for <b>integron</b> (491bp) gene.	66
9	Ethidium bromide stained agarose gel electrophoresis containing the PCR products along with 100bp DNA ladder (lane L). P: positive control; N: negative control which is (Nuclease free water). PCR was carried out using primers specific for <b>Class 1 integron (280 bp)</b> gene. The eight isolates were positive for this gene.	67
10	Bands of gene cassettes. Sizes of cassettes are shown in Table. (19).	68

#### **ENGLISH SUMMARY**

The aim of this work was to study the distribution of *Salmonella* serovars among diseased chickens and diarrheic calves, also to determine the genotypic and phenotypic causes of resistance of *Salmonella* to antimicrobials. To achieve this aim, two hundred samples (100 diseased chicken samples and 100 fecal swabs from diarrheic calves) were directed to bacteriological examination according to ISO 6579: 2002 A1: 2007.

Confirmation was done by biochemical reactions, serotyping and molecular characterization which revealed the isolation of fourteen (14) Salmonella spp. isolates (9 from calves and 5 from chickens) with a percentage of 9% and 5% respectively. Phenotypic antimicrobial susceptibility testing of all isolates was done which revealed high sensitivity to Cefipime, Cefotaxime, Levofloxacin and Norofloxacin and high resistance to Sulphatrimethoprim, Chloramphenicol, Neomycin, Colistin, Amoxycillin and Spectinomycin. Eight isolated were multidrug resistant, five of them were from chickens (Three S. Gallinarum, one S. Enteritidis and one S. Typhimurium) representing all the isolated Salmonellae while the other three isolates were isolated calves which were S. Enteritidis and two S. from one Typhimurium.

Biofilm binding activity of the 14 *Salmonella* isolates showed that three isolates had strong binding activity was and 11 isolates showed moderate binding. With addition of lactic acid 2%, the binding ability had been decreased. Three from the eight multi-drug resistant *Salmonellae* were strong biofilm producers which means that there is a relation between biofilm formation of *Salmonella* and its resistance to antimicrobials.

Genotypically, isolated *Salmonellae* were positive for class 1 integron and gene cassettes. Sequencing of gene cassettes revealed the presence of resistant genes to the following antimicrobials, Trimethoprim, Streptomycin, Spectinomycin, streptomycin

and Ampicillin in addition to a resistant gene to a disinfectant which is quaternary ammonium compound.

Enterobacterial repetitive intergenic consensus PCR (ERIC PCR) is important to identify and differentiate *Salmonella* isolates through the production of reproducible and complex fingerprints.

•