ISOLATION, MOLECULAR CHARACTERIZATION AND ANTIBIOGRAM RESPONSE OF BACTERIA FROM DEAD-IN-SHELL CHICKS

A THESIS

BY

RABEYA SULTANA

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SEMESTER: JULY– DECEMBER, 2017

SESSION: 2016

MASTER OF SCIENCE (MS)

IN

MICROBIOLOGY

DEPARTMENT OF MICROBIOLOGY

FACULTY OF POST GRADUATE STUDIES

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY, DINAJPUR-5200

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DECEMBER, 2017

DEDICATED
TO MY
BELOVED PARENTS
AND SISTERS

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The Author

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ABSTRACT

The present study was conducted for isolation, identification and molecular characterization of bacteria causing dead-in-shell chicks in different hatcheries with determination of their antibiogram profiles. For this purpose a total of 60 samples were collected randomly from dead-in-shell chicks. Bacteria were investigated using standard bacteriological techniques. The identified isolates were characterized by molecular techniques like PCR, Electropherogram and phylogenetic analysis. The identified isolates were studied for their in vitro antibiotic sensitivity by agar disk diffusion method against commonly used antibiotics. The number of bacteria isolated from dead-in-shell chicks were *E. coli* 29 (32.2%), *Salmonella* spp. 31 (34.5%), *Staphylococcus* spp. 17 (18.9%), *Pseudomonas* spp. 11 (12.2%) and *Aeromonas hydrophila* 2 (2.2%). The highest number of bacteria were recovered from lungs (44) followed by yolk (25) and intestine (21). Antibiogram profile revealed that, *E. coli* were sensitive to Levofloxacin and Azithromycin, *Salmonella* spp. were sensitive to Ciprofloxacin and Gentamicin, *Staphylococcus* spp. were sensitive to Levofloxacin, Ciprofloxacin, Gentamicin and Chloramphenicol and resistant to Penicillin, *Pseudomonas* spp. were sensitive to Levofloxacin and Gentamicin and *Aeromonas hydrophila* were sensitive to Levofloxacin and Ciprofloxacin. In addition, Multidrug Resistant Organisms (MDROs) were also found which suggest that appropriate antibiotic should be selected before antibiotic therapy to chickens. From this study it was concluded that dead-in-shell chicks are quite common in hatcheries in our country and that bacterial contamination in the hatcheries constitute an important threat to the poultry industry in the area. The results of this study would be helpful for prevention and control of bacterial causes of dead-in-shell chicks.

CONTENTS

CHAPTER	TITLE	PAGE NO.
	ACKNOWLEDGEMENTS	**i**
	ABSTRACT	**ii**
	CONTENTS	**iii-v**
	LIST OF TABLES	**vi**
	LIST OF FIGURES	**vii**
	LIST OF PLATES	**viii-x**
	LIST OF ABBREVIATIONS AND SYMBOLS	**xi**
CHAPTER 1	**INTRODUCTION**	**01-03**
CHAPTER 2	**REVIEW OF LITERATURE**	**04-16**
CHAPTER 3	**MATERIALS AND METHODS**	**17-37**
3.1	Materials	17-20
3.1.1	Study area and period	17
3.1.2	Collection and preparation of samples for bacterial isolation	17
3.1.3	Glassware and other appliances	18
3.1.4	Media for Culture	19
3.1.4.1	Solid media	19
3.1.4.1	Liquid media	19
3.1.5	Media for biochemical test	19
3.1.6	Reagents	20
3.1.7	Materials used for bacterial genomic DNA isolation	20
3.1.8	Materials used for Polymerase Chain Reaction	20
3.2	Methods	21-37
3.2.1	Laboratory preparations	21
3.2.2	Experimental layout	22-23
3.2.3	Preparation of Culture Media	24-26
3.2.3.1	Nutrient broth media	24
3.2.3.2	Nutrient agar (NA) media	24
3.2.3.3	Eosin Methylene Blue (EMB) agar	24
3.2.3.4	MacConkey agar media	24-25
3.2.3.5	Salmonella Shigella (SS) Agar media	25
3.2.3.6	Mannitol Salt Agar (MSA) media	25
3.2.3.7	Staphylococcus Agar No. 110 media	25-26
3.2.3.8	Cetrimide Agar media	26
3.2.3.9	Mueller Hinton Agar media	26
3.2.4	Preparation of reagents	26-27

CONTENTS (Contd.)

3.2.4.1	Methyl- Red solution	26
3.2.4.2	Methyl Red	26
3.2.4.3	Alpha- naphthol solution	26
3.2.4.4	Potassium hydroxide solution	27
3.2.4.5	Phosphate Buffered Saline solution	27
3.2.4.6	Indole reagent (Kovac's reagent)	27
3.2.5	Cultivation and isolation of organisms	27
3.2.6	Morphological characterization by Gram's staining method	27-28
3.2.6.1	Preparation of Gram's staining solution	28
3.2.6.2	Gram's staining procedure	28
3.2.7	Biochemical examination	28-30
3.2.7.1	Indole test	28-29
3.2.7.2	Methyl Red test (MR)	29
3.2.7.3	Voges-Proskauer test (VP)	29
3.2.7.4	Simmon's Citrate Agar (SCA)	29
3.2.7.5	Triple Sugar Iron Agar (TSI)	29
3.2.7.6	Mortility Indole Urease (MIU) test	30
3.2.8	Antibiotic sensitivity test against isolated microbes	30
3.2.8.1	Recording and interpretating results of antibiogram study	31-34
3.2.9	PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Aeromonas hydrophila*	34-37
3.2.9.1	Basic protocol of bacterial genomic DNA isolation	34-35
3.2.9.2	PCR amplification and sequencing of 16S rRNA	35
3.2.9.3	Electrophoresis	36
3.2.9.4	Nucleotide sequence accession number and BLAST analysis	36
3.2.9.4.1	Chain-termination methods (Sanger sequencing)	37
3.2.9.5	Maintenance of stock culture	37
CHAPTER 4	**RESULTS**	**38-63**
4.1	Results of Isolation of organism	38
4.2	Isolation and identification of bacteria by different bacteriological methods	39-63
4.2.1	Results of Cultural Examination	39
4.2.1.1	Nutrient Agar (NA)	40
4.2.1.2	Mac Conkey Agar	40
4.2.1.3	Eosin Methylene Blue Agar (EMB)	41
4.2.1.4	Salmonella-Shigella Agar (SS)	42
4.2.1.5	Manitol Salt Agar (MSA)	43
4.2.1.6	Staphylococcus Agar No. 110	43

CONTENTS (Contd.)

4.2.1.7	Cetrimide Agar	44
4.2.2	Results of Gram's staining	44
4.2.2.1	Microscopic examination	45
4.2.3	Results of Biochemical Test	46
4.2.3.1	Indole Test	47
4.2.3.2	Methyl Red test	48
4.2.3.3	Voges-Proskauer Test	49
4.2.3.4	MIU Test	50
4.2.3.5	Triple Sugar Iron (TSI) Test	51
4.2.3.6	Simmons Citrate test	52
4.2.3.7	Selenite Broth	53
4.2.4	Results of Antibiotic Sensitivity Test	54-60
4.2.5	Result of PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Aeromonas hydrophila*	61
4.2.5.1	Electropherogram	62
4.2.5.2	Phylogenic tree analysis of *Aeromonas hydrophila*	62
4.2.5.3	Contig Sequence of *Aeromonas hydrophila*	63
CHAPTER 5	**DISCUSSION**	**64-67**
CHAPTER 6	**CONCLUSION**	**68**
	REFERENCES	**69-77**
	APPENDICES	**78-83**

LIST OF TABLES

TABLE NO.	TITLE	PAGE NO.
1	Summary of samples collected from dead-in-shell chicks	18
2	PCR Reaction Mixture for 16s rRNA.	20
3	Antimicrobial agents with their disc concentration	31
4	Zone diameter interpretative standards for *Escherichia coli*	32
5	Zone diameter interpretative standards for *Salmonella* spp.	32
6	Zone diameter interpretative standards for *Staphylococcus* spp.	33
7	Zone diameter interpretative standards for *Pseudomonas* spp.	33
8	Zone diameter interpretative standards for *Aeromonas hydrophila*	34
9	Condition of PCR.	35
10	Summary of isolation of bacteria from different samples of dead-in-shell chicks	38
11	The result of cultural characteristics of the isolated organisms	39
12	Result of biochemical test for the representative isolates	46
13	Antimicrobial profile of *Escherichia coli*	54
14	Antimicrobial profile of *Salmonella* spp.	54
15	Antimicrobial profile of *Staphylococcus* spp.	55
16	Antimicrobial profile of *Pseudomonas* spp.	55
17	Zone diameter interpretative standards for *Aeromonas hydrophila*	56

LIST OF FIGURES

FIGURE NO.	TITLE	PAGE NO.
1	Egg samples	17
2	Dead-in-shell chick samples	17
3	Collection and grinding of parts of lungs and intestine	18
4	Serial dilution of samples	18
5	Schematic illustration of the experimental layout	23
6	Steps of Sanger sequencing using ABI 3130 Genetic analyzer.	37
7	Percentage of bacterial species isolated from yolk, lungs and intestine of dead-in-shell chicks	39
8	Result of amplification of 16S rRNA gene region of *Aeromonas hydrophila* by PCR.	61
9	Electropherogram of 16s rRNA Gene Sequence of *Aeromonas hydrophila*	62
10	Phylogenic tree analysis of *Aeromonas hydrophila*	62

LIST OF PLATES

PLATE NO.	TITLE	PAGE NO.
1	Growth on Nutrient agar (left) and control (right)	40
2	Growth on MacConkey agar (left) and control (right)	40
3	Growth on MacConkey agar (left) and control (right)	41
4	Growth of E. coli on EMB agar (left) and control (right)	41
5	Growth of *Salmonella* spp. on SS agar (left) and control (right)	42
6	Growth of *Aeromonas hydrophila*. on SS agar (left) and control (right)	42
7	Growth of *Staphylococcus* spp. on MSA (left) and control (right)	43
8	Growth of *Staphylococcus* spp. on Staphylococcus agar 110 (left) and control (right)	43
9	Growth of *Pseudomonas* spp. on Cetrimide agar (left) and control (right)	44
10	Microscopic studies and staining properties of the bacterial isolates under alight microscope (100x)	45
11	Indole test for *E. coli* showing positive result by red coloration of the medium (right) and control (left).	47
12	Indole test for *Salmonella* spp.showing negative result by no colour change of the medium (right) and control (left).	47
13	Indole test for *Staphylococcus spp.* showing negative result by no colour change of the medium (right) and control (left).	47
14	Indole test for *Pseudomonas spp.* showing negative result by no colour change of the medium (right) and control (left).	47
15	Indole test for *Aeromonas hydrophila* showing positive result by red coloration of the medium (right) and control (left).	47
16	Methyl-Red test for *E. coli* indicated positive by changing the medium into bright red colour (right) and control (left).	48
17	Methyl-Red test for *Salmonella spp.* indicated positive by no colour change of the medium (right) and control (left).	48
18	Methyl-Red test for *Staphylococcus* spp. indicated positive by changing the medium into bright red colour (right) and control (left).	48

LIST OF PLATES (Contd.)

19	Methyl-Red test for *Pseudomonas* spp. indicated negative by no colour change of the medium (right) and control (left).	48
20	Methyl-Red test for *Aeromonas hydrophila* indicated negative by no colour change of the medium (right) and control (left).	20
21	Voges-Proskauer test for *E. coli* showing negative result by no change of the medium (right) and control (left).	49
22	Voges-Proskauer test for *Salmonella* spp. showing negative result by no change of the medium (right) and control (left).	49
23	Voges-Proskauer test for *Staphylococcus spp.* showing positive result by changing the medium into rose red colour (right) and control (left).	49
24	Voges-Proskauer test for *Pseudomonas* spp. showing negative result by no change of the medium (right) and control (left).	49
25	Voges-Proskauer test for *Aeromonas hydrophila* showing positive result by changing the medium into rose red colour (left) and control (right)	49
26	MIU test for *E. coli* showing positive result by the diffuse, hazzy growth and slightly opaque media.	50
27	MIU test for *Salmonella* spp. showing positive result by the diffuse, hazzy growth and slightly opaque media.	50
28	MIU test for *Staphylococcus* spp. showing negative result by no colour change of the media.	50
29	MIU test for *Pseudomonas* spp. showing positive result by the diffuse, hazzy growth and slightly opaque media.	50
30	Triple Sugar Iron test for *E. coli* showing yellow colour butt & yellow colour slant inoculated (right) and control (left).	51
31	Triple Sugar Iron test for *Salmonella* spp. showing yellow colour butt & red colour slant with gas and H_2S production inoculated (right) and control (left).	51
32	Triple Sugar Irontest for *Staphylococcus s*pp. showing yellow colour butt & yellow colour slant inoculated (right) and control (left)	51

LIST OF PLATES (Contd.)

33	Triple Sugar Iron test for *Pseudomonas* spp. showing red colour butt & red colour slant inoculated (right) and control (left)	51
34	Triple Sugar Iron test for *Aeromonas hydrophila* showing yellow colour butt & red colour slant inoculated (left) and control (right)	51
35	Simmons Citrate test for *E. coli* showing negative result by no change of themedium (right) and control (left).	52
36	Simmons Citrate test for *Salmonella* spp. showing negative result by no change of the medium (right) and control (left).	52
37	Simmons Citrate test for *Staphylococcus* spp. showing positive result by colour change of the medium into blue colour (right) and control (left).	52
38	Simmons Citrate test for *Pseudomonas* spp. showing positive result by colour change of the medium into blue colour (left) and control (right).	52
39	Selenite Broth test for *E. coli* showing positive result by the change of the medium to brick red colour (right) and control (left).	53
40	Selenite Broth test for *Salmonella spp.* showing positive result by the change of the medium to brick red colour (right) and control (left).	53
41	Selenite Broth test for *Staphylococcus spp.* showing negative result by no change of the medium (right) and control (left).	53
42	Selenite Broth test for *Pseudomonas spp.* showing negative result by no change of the medium (right) and control (left).	53
43 (A)	Antibiogram profile of *Aeromonas hydrophila*	56
43 (B)	Antibiogram profile of *Aeromonas hydrophila*	56
44 (A)	Antibiogram profile of *E. coli*	57
44 (B)	Antibiogram profile of *E. coli*	57
45 (A)	Antibiogram profile of *Salmonella* spp.	58
45 (B)	Antibiogram profile of *Salmonella* spp.	58
45 (C)	Antibiogram profile of *Salmonella* spp.	58
46 (A)	Antibiogram profile of *Staphylococcus* spp.	59
46 (B)	Antibiogram profile of *Staphylococcus* spp.	59
47 (A)	Antibiogram profile of *Pseudomonas* spp.	60
47 (B)	Antibiogram profile of *Pseudomonas* spp.	60

LIST OF ABBREVIATIONS AND SYMBOLS

- = Negative
% = Percentage
& = And
+ = Positive
≤ = Less-than or equal to
≥ = Greater-than or equal to
°C = Degree Celsius
MDROs = Multi Drug Resistant Organisms
mg = Milligram
ml = Millilitre
MR = Methyl Red
n = Number of isolates from each source
NA = Nutrient Agar
NB = Nutrient Broth
OIE = Office International Des Epizooties
PBS = Phosphate Buffer Saline
R = Resistant
S = Sensitive
Sl. No. = Serial Number
spp. = Species
SS = Salmonella-Shigella Agar
TE = Tetracycline
V-P = Voges-Proskauer
WHO = World Health Organization
µg = Microgram
µl = Microlitre
CLSI = Clinical and Laboratory Standards Institute
Contd. = Continuation
E = Erythromycin
E. coli = *Escherichia coli*
e.g. = Example
EMB = Eosine Methylene Blue
et al. = Et alia (associates)
etc. = Etcetra
FAO = Food and Agricultural Organization
Fig. = Figure
gm = Gram
GN = Gentamicin
H_2O_2 = Hydrogen peroxide
H_2S = Hydrogen Sulphide
I = Intermediate
K = Kanamycin
KOH = Potassium hydroxide
HSTU =Hajee Mohammad Danesh Science and Technology University
i.e. =That is
M.S =Master of Science
MSA =Mannitol Salt Agar
Prof. = Professor
PCR= Polymerase Chain Reaction
kb = kilo base
bp = base pair

CHAPTER 1

INTRODUCTION

Poultry industry in Bangladesh is developing rapidly since 1980. It plays an important role in poverty alleviation and economic development of the country. Government of the People's Republic of Bangladesh has given priority to this potential sector. The current poultry population is approximately 304.17 million including 48.86 million ducks and 255.31 million chickens (DLS, 2015). Poultry industry includes commercial and family farms of broiler and layer chicken, duck and hatcheries or both. There are about one thousand (1000) hatcheries in Government and private sector (Farm Poultry and Livestock Survey 2015). The number of day old chicks (DOC) produced in Government farm stands at about 125 Lac (12.5 million) to 250 Lac (25 million) numbers per month whereas that of private farms is about 80 Lac (8 million) to one corer (10 million) per week. This is being the situation of hatcheries, both the Government and Non- Government sectors requires attention in respect of hygienic and managemental factors. As regards hygienic status of hatcheries and bio-security, any omissions may cause great loss to the hatchery owners and the rising industry too. The dead-in-shell chicks are mostly associated with embryo malpositions in the egg. In flocks with an increased incidence of dead-in-shell chicks are often associated with bacterial contamination in the egg, presence of exudates in the air sacs and lungs of embryos, leg deformities, and unhealed navels. Additional factors associated with an increased incidence of dead-in-shell chicks include inadequate breeder nutrition, thin shelled eggs, delayed transfer of eggs to hatchers, too high temperature setting in the hatchery, too low humidity in the hatchery, too high carbon dioxide level in the hatchery and inadequate air exchange in the hatchery. Bacterial infection of poultry represents a worldwide important factor in terms of their economic losses and public health significance. Some organisms decrease egg production and lead to high embryonic mortalities, others are widely distributed in hatcheries and thus eggs remain as a source of spreading the infection (Safwat *et al*., 1984 and Choudhury *et al*., 1993). Numerous bacterial pathogens that contaminate hatcheries had been isolated from egg shell, egg content as well as from dead in shell chicken embryos. These included *Salmonella* spp., *Escherichia coli, Staphylococcus aureus, Klebsiella* spp*., Proteus* spp. and *Pseudomonas* spp. (Cox *et al.*, 1990; Cason *et al.*, 1994; El-Atrebe *et al.,* 1993; Shawabkeh *et al.,* 1993; El-Latif, 1995; Ibraheem *et al.,* 1997; Walker *et al.*, 2001; Northcutt*et al.*, 2004 and Kim *et al.*, 2007). As soon as eggs are laid by the chicken, bacteria penetrate the egg shell

and other shell barriers (Berrang *et al.,* 1999; Cook *et al.,* 2003). Moreover, there is possibility of transovarian transmission for pathogenic bacteria, including *Salmonella enteritidis*. Contamination of table eggs by *Salmonella* is essential for public health risk associated with consumption of infected eggs (Berrang *et al.,* 1999). In contrast, contamination of hatching eggs may implicate invasion of these bacteria into egg contents and chicken embryoes (Montgomery *et al*., 2005). There is limited number of reports in the literature indicating such route of infection of chicken embryos and newly hatched chickens (Rosario *et al*., 2004). Egg contaminated with microorganisms play a significant role in poultry production, pathology and in the spreading of diseases. Microorganisms cause increased mortality of embryos, lower hatchability and increased early chick mortality. Infections of humans are also common. The penetration of egg shells and egg contents has been shown in table eggs at the level of 9.2% and inhatching eggs at the level of 16.5% in the floor position of hens. The main reason of egg contamination with various microorganisms is wet and soiled litter (Smeltzer *et al.,* 1979).

The moisture content in newly laid eggs diminishes the ability of cuticle to protect the egg contents, with so-called bed wet eggs, drops of water penetrate the cuticle, change its structure and enable microorganisms to enter the egg contents immediately after laying. The infection of eggs is possible at the time of laying and also after washing (Sparks and Board, 1985). The knowledge of basic biology of chicken production under farming conditions is low. So the number of dead-in-shell chicks is increased during hatching process in hatchery. Evaluation of dead-in-shell embryos is often conducted to determine the incidence of specific problems such as Mycoplasmosis associated with exudates in the air sacs and lungs, Salmonellosis associated with liver lesions and opportunistic bacterial contamination associated with heart lesions. An analysis of dead-in-shell chicks to determine significance of *Mycoplasma gallisepticum* (MG) infection is routinely conducted in integrations where MG infection is present (Butcher and Halabi, 2010). For effective management and disease control, poultry production can be divided into the stages of incubation and hatching, brooding, rearing and laying. All of these stages are affected by diseases and need equal attention for a profitable poultry production (Nesheim *et al.,*1979). Hatchery is one of the greatest areas of disease risk in the whole cycle of poultry operations. A large proportion of embryos die at different stages of incubation (Jordan, 1990).

Among the available methods for the control of the pathogens, the one most widely practiced is the use of various antibiotics. Never the less, it is well known that the extended and

continuing use of a range of antimicrobial agents in animals' food has been an important factor in promoting the emergence of resistant strains of Gram-positive and Gram-negative bacteria (Papadopoulou *et al*., 1997; Aarestrup *et al*., 2000). Resistant organisms can spread from chicken to chicken and from chicken to man (Levy *et al*., 1976). Antibiotic resistance in enteric bacteria (*E. coli, Salmonella* spp., *Campylobacter* spp., and *Enterococci* spp.) can be transferred from birds to humans via the food chain or by direct contact. For instance, colonization of the intestinal tract with resistant *E. coli* from chickens has been observed in human volunteers (Linton *et al.,* 1977, Ojeniyi, 1989). Poultry has also been described as a source of antibiotic resistance in humans in northern India (Singh *et al.,* 1992). Polymerase chain reaction have been developed for detection of *Aeromonas* spp. from water samples and food products (Dorsch *et al.,* 1994 and Cascon *et al.,* 1996). Similarly DNA/RNA probes are also in use for this purpose (Dorsch *et al.,* 1994 and Ludwig *et al.,* 1994). *Aeromonas hydrophila*is also responsible for dead-in-shell chicks (Lin *et al*., 1996).

Bacterial and fungal contamination of hatching eggs are important as they not only prevent more than 10 percent of fertile eggs from hatching but might also gave rise to cross contamination and subsequent mortality or poor performance of chicks in the subsequent stages of production (Anonymous, 1996).

Keeping in view the above facts, the present research was undertaken with the following objectives-

1. Isolation and identification of bacteria associated with the dead-in-shell chicks.

2. Molecular characterization of isolated *Aeromonas hydrophila* by using PCR.

3. Determination of the antibiotic sensitivity of bacteria against commonly used antimicrobial agents.

CHAPTER 2

REVIEW OF LITERATURE

These parts of the manuscript containing a review of scientific publication related with the topic of the write-up.

Kalita *et al.*, (2017) studied the etiopathology of dead-in-shell embryos of chicken egg collected from the Kamrupa and Dahlem Red bird being maintained at the experimental poultry farm of "All India Co-ordinated Research Project on Poultry Breeding", Guwahati-22 during the period from February, 2014 to March, 2014. In total 2270 numbers of egg were incubated of which 1250 nos. from Kamrupa and 1020 nos. from Dahlem Red bird. Out of which 184 (58.97 %) and 143 (47.35 %) nos. of egg were found dead-in-shell in case of Kamrupa and Dahlem Red bird, respectively. Various abnormalities detected in the dead-in-shell embryos of Kamrupa and Dahlem Red bird were malposition, malformation, dehydration, adhesion, pathological condition. Out of 37 nos. and 33 nos. of dead-in-shell embryos of Kamrupa and Dahlem Red bird, respectively 13 nos. of embryos in each of the breeds showed septicemia. The organism isolated from the embryos of both the variety was *Streptococcus* spp. *Staphylococcus* spp. and *Escherichia coli*.

Amer *et al*., (2017) collected a total of 360 samples (160 dead in shell and 200 day old chicks) from 10 commercial hatcheries were subjected to microbiological analyses for detection of bacterial contamination. A total bacterial species were isolated from dead in shell and one day old chicks in rate of 21.67% (78/360) including 23.12% from dead in shell and 20.5% from one day old chick. isolation of 9 bacterial species including 2 gram positive *Streptococcus* and *Staphylococcus* and 7 gram negative including *Salmonella spp., E. coli, Citrobacter spp., Proteus spp., Campylobacter spp., Pseudomonas spp.* and *Klebsiella spp.*. The isolated bacterial spp. has been reported to be associated with infection of yolk sac and death of chicken embryos. Isolates of *S. enteritidis, P. vulgaris, C. frundii, K. pneumonia, C. jejuni, Staph. aureus, Streptococcus* and *S. scuiri* are sensitive to Cefatoxaime , Enrofloxacin, Kanamycin and Gentamicin with rate 50- 100%. *P.aeruginosa* was generally resistant to all tested antibacterial, while *S. haemolyticus* and *S. xylosus* are sensitive only to Oxytetracycline. Control usage of antibacterial agents to get good effect and avoid drug resistance.

Razmyar *et al.*, (2016) studied that yolk sac infection (YSI) and dead-in-shell mortality caused by Enterobacteriaceae in birds are not a rare phenomenon, however there are only a few reports indicating the association between these conditions and *Klebsiella* spp. among canary chicks (*Serinus canaria*). There have been reports of high mortality among 1-3 day old canary chicks in an indoor flock of canaries. In order to study the causative agent, yolk sac samples from dead-in-shell and day-old canary chicks were cultured. *Klebsiella pneumonia* was isolated and identified based on biochemical tests and using genus and species-specific multiplex PCR and later tested for their susceptibility to 13 antimicrobial agents. The isolates showed susceptibility to Gentamycin, Chloramphenicol, Florfenicol and Streptomycin.

Cristina *et al.*, (2015) addressed the role of bacterial infection in hatching failure of wild geese, on the Arctic Coastal Plain of Alaska. During 2013, they observed mortality of normally developing embryos and collected 36 addled eggs for analysis. They also collected 17 infertile eggs for comparison. Using standard culture methods and gene sequencing to identify bacteria within collected eggs, they identified a potentially novel species of *Neisseria* in 33 eggs, *Macrococcus caseolyticus* in 6 eggs, and *Streptococcus uberis* and *Rothia nasimurium* in 4 eggs each. They detected seven other bacterial species at lower frequencies. Sequences of the 16S rRNA gene from the *Neisseria* isolates most closely matched sequences from *N. animaloris* and *N. canis* (96-97% identity), but phylogenetic analysis suggests substantial genetic differentiation between egg isolates and known *Neisseria* species. Although definitive sources of the bacteria remain unknown, they detected *Neisseria* DNA from swabs of egg shells, nest contents, and cloacae of nesting females. To assess the pathogenicity of bacteria identified in contents of addled eggs, they inoculated isolates of *Neisseria*, *Macrococcus*, *Streptococcus*, and *Rothia* of varying concentrations into developing chicken eggs. Seven-day mortality rates varied from 70-100%, depending on bacterial species and inoculation dose. Their results provide evidence of bacterial induced embryo mortality in wild geese and in the Arctic.

Kizerwetter *et al*., (2015) examined the presence of bacteria in chicken embryos and newly hatched chicks. The bacteria were determined quantitatively in caecal contents, liver and yolk sac of 25 embryos at 18 day of incubation, 25 embryos at 20 day incubation, and 25 newly hatched chicks. Gram-positive cocci belonging to the Enterococcus genus dominated in all samples examined and their population number amounted from 102 CFU/g. The results

obtained indicate that the profile of gut microflora becomes more differentiated and their population number of particular groups higher with the age of embryos. Moreover, potentially pathogenic bacteria, including anaerobic *Clostridium tertium* may be isolated already during hatching period, suggesting the possibility of infection of embryos and newly hatched chicks.

Heidy *et al.,* (2015) examined the presence of bacteria in ostrich embryo. A total of 141 egg samples (5 non-fertile eggs, 41 dead in shell embryos at 39 days of age , 4 eggs contain dead embryos at 28 days of age) was subjected to bacteriological examination in a trial to detect the actual bacterial causes of embryonic death in ostrich eggs in Ismailia Governorate. 61.7 % of the examined egg samples were positive for bacterial isolation. The result of bacterial isolation revealed that *Klebsiella spp.* was the most prevalent organism isolated from dead-in-shell embryos and infertile eggs with rates of 23.57 % and 20 %, respectively. *Proteus spp.* was isolated from dead-in-shell embryos with a rate of 18.69 %. The only organism isolated from infertile eggs was *Klebsiella spp.* with a rate of 20 %. *Enterococcus spp.* was isolated from dead-in-shell embryos with a rate of 15.44%. The occurrence of *Escherichia coli* in dead-in-shell embryos was (8.94 %) and *Providencia spp*. was (3.25%). The lowest incidence of the recovered bacterial species from dead-in- shell embryos was *Salmonella spp.* and *Serratia marcescens* with the same isolation rate (2.43%). From six isolates of enterococci examined using polymerase chain reaction technique, one isolate was mixed and contained both *E. faecalis* and *E. faecium*, three isolate of *E. faecium* and two isolate of *E. faecalis.*

Ali Ghorbani *et al.,* (2015) studied the pattern of drug resistance of bacteria. The egg shell is purified with Ethanol 70%, and then the contents of fifty eggs are mixed in a dish and incubated with soab in Selenite-f. After 24 hour incubation at 37ºC the Selenite-f sample was transferred to *Salmonella* spp and after incubation at 37ºC was analyzed in terms of suspected colonies to Salmonella. The suspected colonies were inoculated into lysine decarboxylase broth and TSI agar environment. The bacteria that had reactions were related to salmonella, and were analyzed by the PCR test, with special primer for *Salmonella spp.* such as *S. enteritidis* and *S. typhimurium*. The results of this study indicated five samples of mixed eggs (at least 0/33% of eggs) were contaminated with *Salmonella* of serotype *S. enteritidis*. Among the separated *Salmonella* 85.9 % were resistant to Ampicillin, 14.5% to Tetracycline, and 42.9% to Kanamycin, but all of them were sensitive to Norfloxacin in this antibiotic resistance test.

Babaca *et al., (*2014) observed the deployment of dead-in-shell embryos, the bacterial etiology of the condition, and the epidemiology of the dead embryos in three local hatcheries in Erbil province were investigated using standard bacteriologic techniques. Deployment of the condition in the three hatcheries was found to be 37%, 21.6% and 40.5% respectively. Bacteria that were isolated arranged in order of decreasing frequency, included *Escherichia coli, Staphylococcus spp., Streptococcus spp., Pseudomonas spp*. The number of dead-in-shell embryos in association with the various bacteria isolated from the three hatcheries were 18 (*Escherichia. coli),* 8 *(Staphylococcus spp.),* 2 *(Streptococcus spp.),* 9 *(Pseudomonas spp.)*. Epidemiologically from this study, it was concluded that dead-in-shell embryos are quite common in hatcheries in Erbil Province and that bacterial contamination in the hatcheries constitute an important threat to the poultry industry in the area.

James *et al.,* (2014) studied to determine microbial contaminations in hatching eggs and predict the effect on hatchability in Butaleja district of Uganda. Experimental and descriptive survey tools were employed. Results reveal that, important microbial contaminants in hatching eggs included *Escherichia coli, Proteus, Pseudomonas aerogenous, Staphylococcus aureus* and fungal microbes. Prevalence evaluation of the microbes showed the following; *Escherichia coli* (19%), fungi (3%), *Proteus* (2%), *Pseudomonas aerogenous* (9%) and *Staphylococcus aureus* (18%) on outer shell surface and *Pseudomonas aerogenous* (4%) and *Staphylococcus aureus* (4%) inside the egg. The key risk factors identified were associated with location of the farm, breed type, poor farm hygiene, prolonged egg storage days, lack of laying nests and predominance of free-range system. It is important to implement farmers education campaigns to disseminate knowledge and skills on modern poultry production and management practices together with improvement of local breed to adopt the new innovation.

Rezaei *et al.,* (2013) examined the bacterial contamination status, with emphasis on *Escherichia coli*, of ostrich hatcheries and the antimicrobial resistance profile of isolated *Escherichia coli*. A total of 120 ostrich eggs with dead embryos, at weekly intervals, were collected from three ostrich hatcheries. Different types of bacteria were isolated from 56 eggs (46.7%). Twenty-four ostrich eggs were shown to carry *E. coli*. In some eggs, in addition to yolk sac, *E. coli* was also isolated from meconium, liver, or heart blood which increased the total number of *E. coli* isolates to 32. All *E. coli* isolates were susceptible to trimethoprim + sulphamethoxazole, danofloxacin, and flumequine, whereas all were resistant to carbenicillin and erythromycin. Resistance to other agents was variable. Multi-drug resistance pattern was

found among all *E. coli* isolates and included 2 to 12 drugs. Thirty-two *E. coli* isolates generated 30 different resistance profiles against 27 antimicrobial drugs. This was the first comprehensive report regarding the bacterial, particularly *Escherichia coli*, contamination of dead-in-shell ostrich embryos and antimicrobial resistance status of the *Escherichia coli* isolates from ostrich eggs in Iran.

Kalita *et al.*, (2013) studied the etiopathology of dead-in-shell embryos of PB-2 male x Indigenous female crossbred chicken egg. A total of 1377 eggs were incubated which was collected from a flock of crossbreed bird (PB-2x Indigenous) chicken. Out of which 568 (41.25 %) egg failed to pip out, were utilized for further study. All the dead in shell embryos were examined for different anomalies and pathological condition thorough necropsy examination. For bacteriological isolation a piece of liver, lung and yolk sac contents were collected from 25 nos. of dead in shell embryos and send to the Department of Microbiology for further examination. A total of 241 (42.42 %) egg were recorded as dead-in-shell embryos out of 568 eggs which were fail to pip out. The percentage of dead-in-shell was higher on 21 day (61.34%) than 18 day (38.66 %) of incubation. Out of 241 nos. of dead in shell embryos, 47 (19.50%) cases showed malpositions, 19 (7.88%) malformation, 6 (2.49%) adhesion, 4 (1.66%) dehydration, 67 (27.80%) pathological condition and 98 (40.66%) cases showed no definite abnormalities and 327 (57.57%) numbers of egg were found as infertile.

Mazengia *et al.*, (2012) studied on embryo mortality and isolated *E. coli* from dead-in shell chick embryos and first week chicks at Andassa Poultry Farm, Ethiopia. *Escherichia coli* were isolated at highest frequency from dead-in-shell embryos in early rainy 18/20 (90.00%) followed by dry 37 (89.19%), rainy 7 (44.67%), and late rainy 14 (73.68%), seasons with significant difference ($p<0.05$) in frequency of isolation among seasons. The study indicated need of hygienic egg selection and hygienic hatchery management. Comprehensive study to identify factors contributing for death of in shell chick embryos and first week chicks is suggested.

Kalita *et al.*, (2011) undertaken a study to identify the etiopathology of dead-in-shell embryos of chicken egg procured from the indigenous chicken. In total, 1336 egg were incubated, out of which 416 (63.90%) were found with dead-in-shell embryos and 235 (36.09%) were infertile. Various abnormalities detected in the dead-in-shell embryos were malposition (43), malformation (3), dehydrated (21), adhesion (10), pathological condition (128) and undiagnosed (211). Out of 128 dead-in-shell embryos having pathological

condition, 40 dead-in-shell embryos were sent for cultural examination from which 25 embryos showed septicemia. The organisms isolated from such embryos were *Streptococcus* species and *Escherichia coli.* From that study they concluded that there may be several reasons for getting poor hatchability. Therefore, every attempt have to be made to enhance the hatchability of eggs in a breeding flock.

Jahantigh, (2010) studied the bacterial flora of dead-in-shell ostrich chicks, twelve unhatched eggs which did not have external pipping during the hatching period were transferred to the laboratory of microbiology. The egg shells were accurately disinfected and the embryos were removed and placed in a sterile plate. The surface of each embryo was swabbed with a sterile swab which was also plunged through the yolk sac and the embryo contents and the swab were inoculated into tryptic soy broth (TSB) or nutrient broth. To enrich *Salmonella* spp., another swab was prepared as above and inoculated into Selenite-F broth. These media were incubated at 37°C for 24 h and then sub cultured by streak plate method on solid media. Different bacterial colonies on solid media were isolated in pure cultures for further identification. The results of this study showed that the predominant bacterial flora of dead-in-shell embryos of ostrich were *Bacillus* spp. (45%) and *Staphylococcus* spp. (25%).

Al-khalaf *et al.,* (2010) examined a total of 850 swabs from egg shell surfaces (230), egg room (80), setter (115), hatcheries (115), unhatched eggs (liver, heart, yolk of dead in shell embryos) (160) and newly hatched chicks (150) were examined microbiologically. The following bacteria were isolated and identified: *Escherichia coli, Salmonella spp.*, *Klebsiella pneumoniae*, *Proteus vulgaris, Pseudomonas aeruginosa, Staphylococcus aureus, Citobacter diversus and Enterobacter cloacae.* The serological typing of E. coli isolates (n = 88) from hatchery showed 19 were O126:B7 (21.6%), 13 were O111:B4 (14.8%), 11 were O26:B7 (12.5%), 11 were O119:B14 (12.5%), 8 were O125:B15 (9.1%), 8 were O55:B5 (9.1%) and 18 were untypable strains (20.5%). The recovered 11 *Salmonella* isolates were *S. pullorum* (5) (45.5%), *S. typhimurium* (2) (18.2%), *S. anatum* (1) (9.1%) and untypable strains (3) (27.3%). The polymerase chain reaction (PCR) was used for the confirmation of *E. coli* and *S. pullorum* isolates.

Azmy *et al.,* (2010) studied the prevalence of bacterial isolates among native and imported dead-in-shell chicken breeds. The examination of late stage dead chicken embryos revealed high incidence of *E. coli* 44 (25.88%), 28 (21.54%) followed by *Salmonella*

species 44 (25.55%), 15 (11.54%); {including *S. enteritidis*16 (9.41%), 6 (4.61%); *S. gallinarum*8 (4.7%), *S. pullorum*3 (2.30%); *S. dublin*4 (2.35%), 3 (2.30%); Untypable 16 (9.41%), 3 (2.30%)}; *Klebsiella* 42 (24.7%), 15 (11.53%); *Pseudomonas aeruginosa*34 (20.0%), 8 (6.15%); *Proteus Vulgaris* 18 (10.59%), 7 (5.38%); *Staph. aureus*16 (9.41%), 5 (3.85%) and *Streptococcus fecalis*8 (4.7%), 3 (2.3%) in native and imported breeds respectively.

Rezk, (2010) examined a total of 267 samples of fertile eggs containing pipped chicken embryos collected from different hatcheries located in Ismailia Governorate, Egypt. The percentage of positive samples was (58.8%). *E. coli* isolates were isolated with an incidence of 10.8%), *Salmonella* spp. (4.4%). They were serologically identified. *Pseudomonas* spp. also were isolated (15.9%), *Klebsiella spp*. (10.8%), *Proteus* spp. (18.4%), *Staphylococci* spp. (20.3%), S*treptococci* species (12.7%) and *B. anthracoid* (6.3%). These isolates were biochemically identified. The penetration ability of *Salmonella enterica* serotype *enteritidis* to the intact and cracked egg shells was studied. The results revealed that it could penetrate the intact egg shells after 72 hours but it could penetrate the cracked ones after only 24 hours from artificial contamination.

Maryam *et al.*, (2010) investigated the prevalence of *Salmonella* associated mortality at hatching in three hatcheries in Jos, central Nigeria. Their susceptibility to antimicrobial agents was also evaluated. *S. kentucky* and *S. hadar* were isolated. While half of the isolates were from internal organs, 26.7% came from meconial swabs of dead-in-shell embryos, 17.8% from intestinal samples and 4.4% from egg shells. *S. hadar* is known to colonise only the gut and is classified as non-invasive, but in this study 82% were obtained from internal organs which suggests that infections with this serotype may also cause invasive disease. Antimicrobial susceptibility tests showed a high prevalence of antimicrobial resistance in the study area with complete resistance to Gentamycin, Enrofloxacin, Nalidixic acid, Tetracycline and Streptomycin and substantial resistance to triple Sulphur and Ciprofloxacin. Six multiple resistance profiles were recorded with a high level of multiple resistance to quinolones. Quinolone resistance has implications for veterinary and human therapy as their misuse in poultry could lead to the emergence of resistant animal and zoonotic pathogens.

Mamman *et al.*, (2008) isolated and identified the bacteria from 600 dead-in-shell chick embryos from 4 commercial hatcheries in Kaduna state, Nigeria, during 20 April-13 September 2006. A total of 113 Gram positive and Gram negative isolates were recovered. 35

isolates were obtained from dead-in-shell embryos during the 18thday of incubation, while 78 were obtained on the day of hatching. The isolates consisted of 62 *E. coli*, 21 *Proteus spp*., 6 *Pseudomonas spp.,* 11 *Staphylococcus aureus*, 8 *staphylococcus spp.* and 5 *micrococcus spp.* Two *Corynebacterium spp.* and one *Bacillus spp*. were also obtained. Hatchery D had the highest number of isolates, while Hatchery A had the lowest. These results show that bacterial infection occurs during the incubation period and appropriate hatchery sanitation and hygiene is recommended to reduce dead-in-shell embryos**.**

Raji *et al.,* (2007) determined the isolation rate, serovars and biochemical profiles of E. coli from cases of colibacillosis and dead-in shell embryos in Zaria-Northern Nigeria. The isolation rate of *E. coli* from hatcheries studied were 4.67% and 7.50% from farms of Simtu Agricultural Company and National Animal Production Research Institute (NAPRI) Shika Zaria, Nigeria respectively. Twenty *E. coli* isolates from clinical cases of colibacillosis were also used for this study. The results of carbohydrate fermentation are variable without specific character, except for *E. coli* isolates from clinical cases of colibaccillosis that showed 100% fermentation especially for lactose, ducitol, rhamnose and xylose. The antibiotic susceptibility testing showed that most of the isolated were resistant to more than one antibiotic. The majority of the isolates were sensitive to ciprofloxacin 85% and 100% of each of the simtu and the NAPRE isolates. In conclusion, this study confirms the involvement of several *E. coli* serotypes in colibacillosis and dead in shell embryos in poultry.

Nadir *et al.,* (2007) studied to isolate and identify aerobic bacteria associated with dead-in-shell chick embryo, in (ACOLID) with respect to age of embryo, source of fertile eggs, and different seasons of the year. A total of 450 dead-in-shell chick embryos were collected from the hatcheries in the poultry farm of the Arab company for livestock development (ACOLID) in Khartoum State. A total of 103 isolates were recovered, they consisted of 19 (18.5%) Gram-positive and 84 (81.5%) Gram negative organism. Gram-positive bacteria genera included *Staphylococcus* (7.8%), *Bacillus* (3.9%), *Micrococcus* (2.9%), *Corynebacterium* (2.9%), *Enterococcus* (1.9%), Gram-negative bacteria included *Proteus* (18.5%), *Escherichia* (11.7%), *Klebsiella* (9.7%), *Citrobacter* (8.7%), *Pseudomonas* (7.8%), *Janthinobacterium* (6.8%), *Yersinia* (5.8%), *Salmonella* (3.9%), *Flavobacterium* (3.9%) *Enterobacter* (2.9%) and *Morganella* (1.9%). Possible sources of infection in the farm were investigated and included the mothers, incubators and hatcheries environment.

Nazer *et al.,* (2006) were examined the presence of bacteria in day-old chicks. The most frequently isolated organisms in decreasing order were: *Streptococcus spp.*, *Bacillus spp.*, *Staphylococcus spp.*, *Klebsiella spp.*, *Enterobacter spp.* and *Escherichia coli* followed by *Citrobacter spp.*, *Proteus spp.* and *Pseudomonas spp.* from the eggs and *E. coli*, *Enterobacter spp.* and *Citrobacter spp.* followed by *Klebsiella spp.* and *Bacillus spp.* from the chicks. Different detection methods were evaluated which use various enrichment and plating media for bacteria in eggs and day-old chicks. Sensitivity tests showed the presence of antibacterial resistant strains of bacteria. In comparison, resistance to all antibiotics in chicks' isolated bacteria were more frequent than eggs' isolates, but statistically no significant differences between patterns of antibacterial resistance were seen ($P \leq 0.05$). Twenty-three, 54, 55, 60, 24 and 10% of chicks' isolates were resistant to chloramphenicol, enrofloxacin, erythromycin, furazolidone, trimethoprim and tylosin, respectively. Our findings stress the need for increased implementation of hazard analysis of critical control points (HACCP) and consumer food safety education efforts.

Parimal *et al.,* (2004) reported the reduced hatchability in a commercial hatchery in Chennai, India and found that *E. coli* were isolated in pure culture from dead in shell embryos, fluff samples and dead breeder hens. The isolates were serogrouped into O08, O15, O22, O88, O102, O106, O162 and a few were untypable. Based on in vitro drug sensitivity assay, Chloramphenicol was found to be the most sensitive (50%) drug.

Walker *et al.,* (2001) suggested that *Pseudomonas aeruginosa* is an opportunistic pathogen that can invade fertile eggs causing death of embryos and virulent strains can cause diarrhea, dehydration, dyspnea, septicemia and death to newly hatched chicks.

Al-Sadi *et al.,* (2000) stated that the prevalence of dead-in-shell embryos, the bacterial etiology of the condition, and the pathology of the dead embryos in three local hatcheries in Nenevha province were investigated using standard bacteriological and pathological techniques. Potential pathogenic bacteria were isolated from the three hatcheries and they were in the order of 38, 26, and 25 isolates, respectively. Bacteria that were isolated, arranged in order of decreasing frequency, included *Escherichia coli, Pseudomonas spp., Shigella spp., Staphylococcus spp., Proteus spp., Streptococcus spp*. and *Salmonella spp*. Mixed infections were also encountered. The numbers of dead-in-shell embryos in association with the various bacteria isolated from the three hatcheries were 12 *(E. coli*), 6 (*Streptococcus spp.),* 8 (*Staphylococcus spp.),* 3 (*Pseudomonas spp.),* 3 (*Shigella spp*.), 2 (*Salmonella spp*.), and 10

(mixed infections). Histopathological lesions in dead embryos were mainly in the liver and intestines (caecum). Other lesions included hyperaemia and haemorrhages in the spleen and lungs. From this study, it was concluded that dead-in-shell embryos are quite common in hatcheries in Nenevha Province and that bacterial contamination in the hatcheries constitute an important threat to the poultry industry in the area.

Tran-Quang and Dao-Thi, (2000) found that *Salmonella gallinarum* infection reduced laying capacity by 17.85% and hatchability by 14.96%. Embryo mortality increased by 16.18%, which mainly occurred between 7 and 14 days of incubation. The viability of chickens from infected hens during the first 30 days of life was 83.41%, which was 12.39% lower than the control group. Salmonella was isolated from 8.33% of chickens that died at a young age, 41.85% of seropositive hens and from all seropositive hens that died.

Berrang *et al*., (1999) stated that Bacteria including the human pathogen salmonellae can readily penetrate the shell and membranes of an intact hatching egg. The result of this penetration is contamination of not only the embryo within but many other chicks during hatch in the commercial hatching cabinet. Such contamination can be carried onto the grow-out farm and pose a significant food safety hazard.

Kabilika *et al*., (1999) pooled 300 samples containing 3000 in shell dead chicken embryos were cultured. 383 bacterial cultures comprising 17 bacterial species were isolated. 150 samples (50%) were positive for bacterial cultures. Enterobacteriaceae accounted for over 50% of the total isolates. Other isolates included 54 *Staphylococcus* spp. (14.10%), 34 *Pseudomonas spp.* (11.75%), 34 *Salmonella enteritidis* 8.87 (36%) *Klebsiella spp.* 9 (40%), 26 *Acinetobacter spp.* (6.79%) and 24 *Proteus spp*. (6.26%)

Sharada *et al.*, (1999) investigated 105 dead in shell embryos randomly selected from 3 local hatcheries of Faisalbord and were bacteriologically examined. The occurrence of dead in shell was recorded to be 9.91% (8.04% to 12.03%) positive liver samples of dead in shell embryos. The relative occurrences of different bacterial species were, *Escherichia coli* 52.54%. Paratyphoid Salmonella 12.7%, *S. gallinarum* 11.86%, *S. pullorum* 5.93%, *Streptococcus faecalis* 5.93%, *Bacillus subtilis* 4.2%, *Pseudomonas aeruginosa* 3.39% and *Proteus mirabilis* 3.39%. 28.26% positive samples yield mixed growth of two different bacterial species and *E. coli* was found in all of these combination.

Awad *et al.,* (1997) carried out a survey on Salmonella infection in chickens, dead-in-shell embryos and non-fertile eggs. *S. pullorum* infection which was at sometimes one of the most prevalent one could not be isolated neither from diseased chicks, living chicks, apparently healthy chicks nor from dead in shell embryos or infertile eggs. The same author made an experimental study on *S. pullorum* and *S. enteritidis* infection in one-day-old chicks. From the results the author concluded that the incidence of *S. enteritidis* in Kafr ElSheikh province 0.39%. The incidence of *S. pullorum* in Kafr El-Sheikh province 0.07%. Although *S. pullorum* was uncommon as a cause of paratyphoid infection among chickens but they succeed to isolated the organism from a moribund chick. The pathogenicity test revealed that *S. pullorum* is more pathogenic than *S. enteritidis* causing mortality in infected groups as 61% and 47% respectively.

Tuchili *et al.,* (1996) carried out a polymerase chain reaction (PCR) using a pair of primers specific for *Salmonella* phoE gene a 365-bp specific gene fragment could be amplified from yolk of infertile eggs and dead-in-shell chicken embryos, and from environmental samples. Out of 45 dead-in-shell embryo samples, 20 (44.4%) were found positive for Salmonella DNA by PCR compared to 11 (24.4%) by bacteria isolation. Salmonella DNA could also be detected from infertile eggs, chicken faeces, floor litter and chick fluff, which incidence was higher than that by bacteria isolation.

Lin *et al.,* (1996) studied to identify the dead-in-shell chicken embryos, non-hatched eggs and newly hatched chicks from two farms for detection of gram-negative bacterial flora. Among 349 isolates, twelve genera of gram-negative bacteria were classified as *Escherichia coli (E. coli), Enterobacter spp., Klebsiella spp., Proteus spp., Citrobacter spp., Yersinia spp., Pseudomonas spp., Aeromonas spp., Cedecea spp., Acinetobacter spp., Flavobacterium spp., Moraxella spp.,* with *E. coli* being the most frequent isolate. The *E. coli* infection was proven by the vertical transmission from breeder chickens.

Tubu *et al*., (1996) denoted that dead embryos and dead chicks were found during and after hatching on a poultry farm in Hohhot city between March and April 1995; the death rates were 20.57% and 13.75% respectively. The deaths were confirmed to be caused by *Salmonella pullorum* and *S. derby* infection through epidemiological investigation, clinical and postmortem examination, pathogen isolation and identification and pathogenicity experiments. Among 25 isolates, 17 belonged to *S. pullorum* and 8 to S. derby. Pathogens

were detected in 8 to 15 dead embryos; 5 were infected with *S. pullorum*, 1 with *S. derby* and 2 had mixed infections with the two organisms.

El-Latif *et al.,* (1995) recorded that total of 662 samples including infertile eggs and pipped chicken embryos were collected from different hatcheries located in Dakahlia Governorate. The samples were examined for detection the actual bacterial causes of hatching problems. The percentage of positive samples from infertile eggs and from dead in shell chickens embryos were 18.1 and 32.9%, respectively. *E. coli* isolates were the most prevalent organism isolated from infertile eggs with an incidence of (26.4%) and from dead in shell embryos (21.9%). E. coli isolates were serologically identified. *Proteus, Pseudomonas, Staph. aureus, Klebsiella, Citrobacter* and Enterobacter organisms were successively isolated from infertile eggs with an incidence of 22.6%, 13.2%, 9.4%, 11.3%, 7.5% and 7.5% respectively. The same organisms except Proteus were recovered from dead chicken embryos at rate of 10.4%, 17.1% 11.6%, 11.6% and 5.5% respectively. Salmonella was the lowest organism isolated from infertile eggs (1.8%) and from dead embryos (7.3%) and they were serologically identified.

Das *et al.,* (1994) reported that in Bhubaneswar, where reduced hatching percentages of eggs had been observed, particularly in April- June for 3-4 years, 100 developing chick embryos were collected for examination on days 6, 10, 14, 18 and 21. The cause of death was recorded for all dead embryos. The major causes were salmonellosis (35 eggs), adhesion (30), temperature variation incubator faults (15), *Aspergillus niger* infection (7), 8 were infertile. The cases of salmonellosis were found in the younger embryos. Fumigation of the premises, thorough cleaning of eggs and correction of incubator defects improved hatching performance.

Nazer *et al*., (1994) studied dead-in-shell chicken embryos from two commercial hatcheries in Shiraz (a south province of Iran) for isolation of aerobic and anaerobic bacteria. One hundred pooled samples containing 1000 dead-in-shell chicken embryos were cultured for the presence of bacteria comprising *Escherichia coli* (32 isolates), *Staphylococcus aureus* (15 isolates), *Klebsiella spp.* (12 isolates), *Salmonella typhimurium* (10 isoalates), *Arizona spp.* (7 isolates), *Pseudomonas spp.* (5 isolates) and 1 isolate each of *Streptococcus spp*, *Bacillus cereus*, *Bacillus subtils* and *Proteus spp.* were identified. No anaerobic bacterium was isolated. High incidence of pathogenic strains of bacteria from dead-in-shell chicken embryos

was recorded. It suggested that the isolates might have contributed to the embryonic mortality and reduced hatchability.

Lecoanet *et al.,* (1992a) reported that 15-20% embryonic mortalities, 3-5% in shell mortalities and 10-20% newly hatched chicks' mortality are strongly related to the contamination of eggs with *E. coli.*

Lecoanet *et al.,* (1992b) mentioned that the presence of *Salmonella* species in the fertile eggs may result in embryonic deaths (at the 6th day, but especially after the 15th day of incubation) and abnormal hatching.

Orajaka *et al.,* (1985) performed experiment for isolation of aerobic bacteria from dead-in-shell chicken embryos from two commercial hatcheries in Anambra State of Nigeria. For this purpose, 79 pooled samples containing 632 dead-in-shell chicken embryos were cultured. From these samples, 23 isolates of *Escherichia coli* and 25 isolates of *Staphylococcus aureus* were recorded. Other bacterial species isolated included *Micrococcus spp. (*fifteen isolates), *Klebsiella spp.* (thirteen isolates), *Pseudomonas spp.* (nine isolates), and *Proteus spp.* (seven isolates). *Salmonella, Streptococcus, and Mycoplasma spp.* could not be isolated. A high incidence of pathogenic strains of bacteria from dead-in-shell chicken embryos was observed. This suggests that the isolates may have contributed to the embryonic mortality and reduced hatchability recorded in the farms investigated.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Study area and period

The present research work was carried out on different hatcheries in Bogra district. The laboratory works were conducted in the Department of Microbiology, Faculty of Veterinary and Animal Science, Hajee Mohammad Danesh science & Technology University (HSTU), Dinajpur, during the period from January to June, 2017.

3.1.2 Collection and preparation of samples for bacterial isolation

A total of 60 dead-in-shell chick egg samples were collected from different hatcheries of Bogra district with variable levels of sanitary conditions and transported to the Bacteriology laboratory of the Department of Microbiology, Faculty of Veterinary and Animal Science, HSTU, Dinajpur. The egg shells were disinfected with 70% ethylalcohol, left for 15 minutes on the disinfected rack. With the aid of sterile scissors, a sufficient area around the air sac was removed, the egg fluid as well as samples from lungs, intestine and yolk of dead embryos were collected in a sterile petridish. Then grinding the parts of intestine and lungs with pestle and morter. All cracked eggs or contaminated samples were excluded.

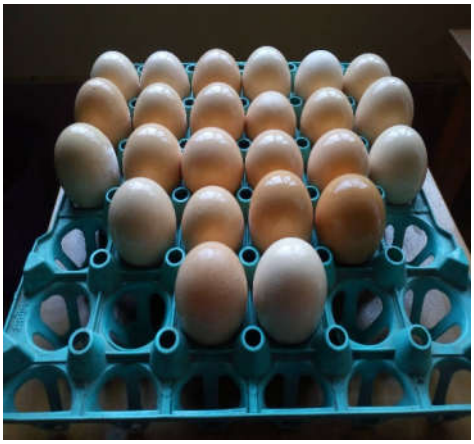

Fig 1: Egg samples

Fig 2: Dead-in-shell chick samples

Fig 3: Collection and grinding of parts of lungs and intestine

Fig 4: Serial dilution of samples

The detailed information of samples collected from dead-in-shell chicks which is shown in Table 1:

Table 1: Summary of samples collected from dead-in-shell chicks

Samples	Number of samples	Grand total
Yolk	20	60
Lungs	20	
Intestine	20	

3.1.3 Glassware and other appliances

Glass wares and appliances used during the course of the experiment were as follows-

- Postmortem tray
- Postmortem sets containing-scissors, scalpel, forceps
- Test tubes (with or without Durham's fermentation tube and stopper)
- Hand gloves and musks
- Petridishes (medium and large size)
- Conical flasks
- Pipette
- Glasses
- Cotton swabs
- Bacteriological loop
- Microscope
- Incubator etc

3.1.4 Media for Culture

The media and reagents that have been used for the isolation and identification of the bacteria are mentioned below.

3.1.4.1 Solid media

- Nutrient Agar Medium, (HI-MEDIA, India)
- Eosin Methylene Blue, (EMB) (HI-MEDIA, India)
- Mueller Hinton Agar (Difco)
- MacConkey Agar medium, (HI-MEDIA, India)
- Salmonella and Shigella Agar, (HI-MEDIA, India)
- Simmons Citrate Agar Medium, (HI-MEDIA, India)

3.1.4.2 Liquid media

- Nutrient broth, (HI-MEDIA, India)
- Methyl Red-Voges Proskauer (MR-VP) broth, (HI-MEDIA, India)
- 1% Pepton Water, (HI-MEDIA, India)
- Tetrathionate broth, (HI-MEDIA, India)
- Lactose broth, (HI-MEDIA, India)
- Pepton broth, (HI-MEDIA, India)

3.1.5 Media for Biochemical test

- Selenite broth
- Indole Broth
- Methyl Red Broth
- Voges-proskauer Broth
- Simmon's citrate Agar
- Triple sugar iron agar
- Motility Indole Ureas (MIU)

3.1.6 Reagents

The chemicals and reagents used during the study were-

- Gram's staining reagents (Crystal violet, Gram's iodine, Acetone alcohol, Safranin)
- Potassium- di-hydrogen phosphate (0.2M, KH_2PO_4 $2H_2O$)
- Dehydrated sodium citrate
- Phosphate Buffered Saline (PBS)
- Physiological Saline Solution (PSS)
- Methylene Blue stain
- Di-sodium hydrogen phosphate (0.2M, $Na_2HPO_4 12H_2O$)
- Sugar media (Dextrose, Maltose, Lactose, Sucrose, and Mannitol) and other chemicals and reagents as when required during the experiment.
- Indole test
- Methyl Red test

3.1.7 Materials used for bacterial genomic DNA isolation

- TE buffer
- 10% (w\v) Sodium dodecyl sulfate (SDD)
- 20 mg\ml protinase k (stored in small single-use aliquots at -20^{0}C)
- 3 M Sodium Acetate, pH 5.2
- 25:24:1 Phenol/Chloroform/Isoamyl alcohol
- Isopropanol
- 70% Ethanol
- 95% Ethanol
- 1.5 ml micro centrifuge tubes

3.1.8 Materials used for Polymerase Chain Reaction

Table 2: PCR reaction mixture for 16s rRNA.

Buffer	2.5 µl
dNTP	2.5 µl
$MgCl_2$	2.5 µl
Forward Primer (27F)	1.0 µl
Reverse Primer (1492R)	1.0 µl
Nano Pure Water	12.5 µl
DNA	2.0 µl
Taq DNA Polymerase	1.0 µl
Final Volume	25 µl

- Primers used for PCR:

  16S rRNA gene region was amplified with the universal primers.

  - Forward primer- 27F (5'AGAGTTTGATCCTGGCTCAG 3')
  - Reverse primer- 1492R (5' TACCTTGTTACGACTT 3')
- Product size: 1500bp
- Thermal Cycler (Thermocycler, ASTEC, Japan)
- 2% agarose gel
- Gel casting tray with gel comb
- TAE buffer
- Microwave oven
- Conical flask
- Electrophoresis apparatus (Biometra standard power pack P 2T)
- 100 bp DNA size marker
- Bromphenicol blue of loading bufter.
- Ethidium bromide (0.5 μg/ml)
- Distilled water
- UV trans-illuminator

3.2 Methods

The following methods were used for the isolation and identification of bacteria.

3.2.1 Laboratory preparations

All items of required glassware including test tubes, pipettes plate, slides, cylinder, flasks, conical flasks, glass and vials soaked in a household dishwashing detergent solution ('Trix' Recket and Colman Bangladesh Ltd.) overnight. Contaminated glassware was disinfected with 2% sodium hypochloride solution prior to cleaning. The glassware were then cleaned by brushing, washed thoroughly in running tape water, rinsed within distilled water and finally sterilized either by dry heat at 160˚C for 2 hours or by autoclaving for 15 minutes at 121˚C under 15 lbs pressure per sq inch. Autoclaved items were dried in a hot air oven over at 50˚C. Disposable plastic (items e.g. micropipette tips) was sterilized by autoclaving. All the glassware was kept in oven at 50˚C for future use.

3.2.2 Experimental layout

The experimental work was divided into three steps: The first step was performed for the isolation and identification of the organisms of the collected sample using cultural, staining and biochemical characteristics. The second step was conducted for the determination of antibiotic sensitivity and resistant pattern of isolated organisms of various samples by using different antibiotic discs available in the market. And finally molecular characterization of isolated organism by using PCR. The layout of the diagrammatic illustration of the present study is shown in figure 5.

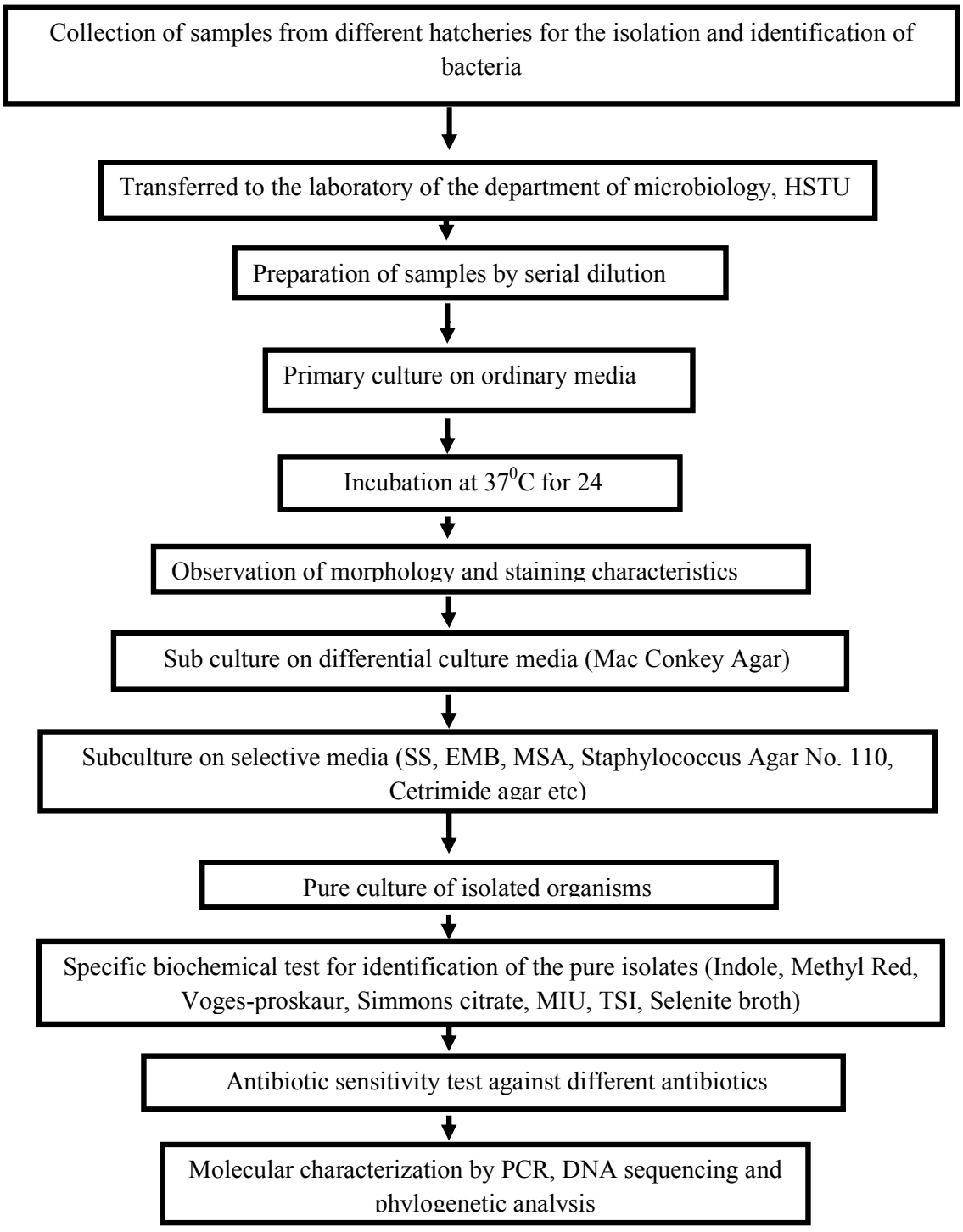

Fig. 5: Schematic illustration of the experimental layout

3.2.3 Preparation of Culture Media

All the media, broth and reagents used in this experiment were prepared according to instruction of the manufacturer.

3.2.3.1 Nutrient broth media

Thirteen grams of dehydrated nutrient broth was suspended into 1000 ml of distilled water and boil to dissolve it completely. The solution was then distributed in tubes, stopper with cotton plugs and sterilized in autoclaving at 121° C and 1.2 kg/cm^2 pressure for 15 minutes. The sterility of the medium was checked by incubating at 37° C for overnight and stored at 4° C in aerator for further use (Cowan 1985).

3.2.3.2 Nutrient agar (NA) media

28 grams of nutrient agar powder was dissolved in 1000 ml of cold distilled water in a flask. The medium was then sterilized by autoclaving. After autoclaving, the medium was poured into each sterile petri dishes and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37°C for overnight to check their sterility and used for cultural characterization or stored at 4°C refrigerator for future use (Cowan 1985).

3.2.3.3 Eosin Methylene Blue (EMB) agar media

Thirty six grams of EMB agar base was added to 1000 ml of water in a flask and boil to dissolve the medium completely. The medium was sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121°C for 15 minutes and shake the medium in order to oxidize the methylene blue (i.e. to restore its blue colour). Then 10 ml of medium was poured into each sterile petri dish sized and allowed to solidify. After solidification of the medium in the petri dishes, these were incubated at 37° C for overnight to check their sterility and petri dishes without contamination were used for cultural characterization or stored at 4°C in refrigerator for future use (Cowan 1985).

3.2.3.4 MacConkey agar media

51.5 grams MacConkey agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^0C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass petri dishes (large sized) to form thick layer there in. To accomplish the

surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. (Cowan 1985).

3.2.3.5 Salmonella Shigella (SS) Agar media

Suspend 50g in 1 liter of distilled water. Bring to the boil to dissolve completely. Sterilize by boiling for 5 minutes. After boiling the medium was put into water bath at 45^0- 50^0C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. (Cowan 1985).

3.2.3.6 Mannitol Salt Agar (MSA) media

111 grams Mannitol Salt Agar base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^0C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass petri dishes (large sized) to form thick layer there in. To accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. (Cowan 1985).

3.2.3.7 Staphylococcus Agar No. 110 media

149.5 grams Staphylococcus Agar No. 110 base powder was added to 1000 ml of distilled water in a flask and heated until boiling to dissolve the medium completely. The medium was then sterilized by autoclaving at 1.2 kg/cm^2 pressure and 121° C for 15 minutes. After autoclaving the medium was put into water bath at 45^0- 50^0C to decrease the temperature. Then medium was poured in 10 ml quantities in sterile glass petri dishes (medium sized) and in 15 ml quantities in sterile glass petri dishes (large sized) to form thick layer there in. To

accomplish the surface be quite dry, the medium was allowed to solidify for about 2 hours with the covers of the petri dishes partially removed. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. (Cowan 1985).

3.2.3.8 Cetrimide Agar media

Suspend 45.3 g of the medium and 10 ml of glycerol in one liter of purified water. Heat with frequent agitation and boil for one minute to completely dissolve the medium. Autoclave at 121°C for 15 minutes. Mix well and pour into sterile petri plates. The sterility of the medium was checked by incubating at 37°C for overnight. The sterile medium was used for cultural characterization or stored at 4°C in refrigerator for future use. (Cowan 1985).

3.2.3.9 Mueller Hinton Agar media

Mueller Hinton Agar is used in antimicrobial susceptibility testing by the disk diffusion method. 38 grams of Mueller Hinton agar powder was suspended in 1000 ml of distilled water. It was mixed well. It was heated agitating frequently and boiled for about one minute. It was dispensed and sterilized in autoclave at 116 - 121°C (15 lbs. sp) for 15 minutes. It was cooled to 45 or 50°C.

3.2.4 Preparation of reagents

3.2.4.1 Methyl- Red solution

The indicator MR solution was prepared by dissolving 0.1 gm of Bacto methyl- red in 300 ml of 95% alcohol and diluted to 500 ml with the addition of distilled water.

3.2.4.2 Methyl Red

A quantity of 17 gms of MR-VP medium was dissolved in 1000 ml of distilled water, dispensed in 2 ml amount in each tube and the tubes were autoclaved. After autoclaving, the tubes containing medium were incubated at 37^{o}C for overnight to check their sterility and then kept at 4^{0}C in refrigerator for future use.

3.2.4.3 Alpha- naphthol solution

Alpha- naphthol solution was prepared by dissolving 5 gm of Alpha- naphthol in 100 ml of 95% ethyl alcohol.

3.2.4.4 Potassium hydroxide solution

Potassium hydroxide (KOH) solution was prepared by adding 40 grams of Potassium hydroxide crystals in100 ml of cooled water.

3.2.4.5 Phosphate Buffered Saline solution

Eight grams of sodium chloride (NaCl), 2.89 grams of di-sodium hydrogen phosphate (Na_2HPO_4, $12H_2O$), 0.2 gram of potassium chloride (KC1) and 0.2 gram of potassium hydrogen phosphate (KH_2PO_4) were suspended in 1000 ml of distilled water for the preparation of phosphate buffered saline solution. The solution was heated to dissolve completely. Then the solution was sterilized by autoclaving at 1.2 kg / cm^2 pressure and 121° C for 15 minutes and stored for future use.

3.2.4.6 Indole reagent (Kovac's reagent)

This solution was prepared by dissolving 25 ml of concentrated Hydrochloride acid in 75 ml of amyl alcohol and to the mixture 5 grams of paradimethyl –amino- benzyldehyde crystals were added. This was then kept in a flask equipped with rubber cork for future use.

3.2.5 Cultivation and isolation of organisms

Samples were collected and each of the samples diluted with distilled water as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} and inoculated into nutrient agar. Then the petri dishes were marked properly and incubated at 37℃ for 24hours aerobically in bacteriological incubator. Then sub-cultured onto the Mac Conkey, SS agar, MSA agar EMB agar, Staphylococcus agar 110 and Cetrimide agar by streak plate method (Cheesbrough, 1985) to observe the colony characteristic of *E. coli, Salmonella* spp, *Staphylococcus* spp*., Pseudomonas* spp. and *Aeromonas hydrophila.* It was repeatedly sub-cultured onto Mac-Conkey, SS agar, MSA agar, EMB agar, Staphylococcus agar 110 and Cetrimide agar until the pure culture were obtained (shape, size, surface texture, edge and elevation, color, opacity etc). The organisms showing with homogenous colonies.

3.2.6 Morphological characterization by Gram's staining method

The most widely used staining procedure in microbiology is the Gram stain discovered by the Danish scientist and physician Hans Christian Joachim Gram in 1884,Gram staining is a differential staining technique that differentiates bacteria into two groups :gram- positives and gram-negatives. The procedure is based on the ability of microorganisms to retain color of the stains used during the gram stain reaction. Gram-negative bacteria are decolorized by

the alcohol, losing the color of the primary stain, purple .Gram-positive bacteria are not decolorized by alcohol and will remain as purple. After decolorization step, and a counterstain is used to impart a pink color to the decolorized gram- negative organisms.

3.2.6.1 Preparation of Gram's staining solution

The four solutions needed for the Gram's staining procedure.

- Crystal violet
- Gram's iodine
- 95% alcohol
- Safranin

3.2.6.2 Gram's staining procedure

1. Clean glass slides were taken.
2. Using sterile technique, prepared a smears of each of the organisms. Did this by placing a drop of water on the slide, and then transferring each organisms separately to the drop water with a sterile, cooled loop .Mixed and speeded organism by means of a circular motion of the inoculating loop.
3. Allowed smears to air dry and then heat fixed in the usual manner.
4. Gently flooded smears with crystal violet and let stood for 2 minutes. Gently washed with tap water.
5. Gently flooded smears with Grams iodine mordant and let stood for 1 min .Gently washed with tap water.
6. Decolorized with 95% ethyl alcohol for 15 second .Gently washed with tap water.
7. Counter stain with safranin for 1 minute.
8. Gently washed with tap water.
9. Examined under oil immersion.

3.2.7 Biochemical examination

Isolated organism with supporting growth characteristics of suspected identified by biochemical test are performed Indole test , MR Test, Voges-proskauer test, Simmon's citrate, Triple sugar iron agar(TSI), Mortility Indole Urease **(**MIU) test, Selenite broth.

3.2.7.1 Indole test

Two milliliter of peptone water was inoculated with the 5 ml of bacterial culture and incubated at 37°C for 48 hours. Kovac's reagents (0.5ml) were added, shake well and

examined after 1 minute. A red color in the reagent layer indicated Indole test positive. In negative case there is no development of red color (Cheesbrough, 1985).

3.2.7.2 Methyl Red test (MR)

Sterile MR-VP broth was inoculated with the test organism and following incubation at 37°C for 24 hours. If the organism would ferment glucose via the mixed acid fermentation pathway like lactic, acetic, which decreases the P^H ,hence upon the addition of the indicator methyl red the broth becomes red in color and yellow color indicated a negative result (Cheesbrough, 1985).

3.2.7.3 Voges-Proskauer test (VP)

If the organism would ferment glucose via the butylenes glycol pathway, an intermediate product, acetyl methyl carbinol or acetone which is neutral is converted to diacetyl upon the addition of the VP – Reagent –B (40% KOH with 0.3% creatine) in the presence of VP – Reagent – A (5% alpha- naphthol in abs. methyl alcohol). Diacetyl is red in color. Negative is yellow color (Cheesbrough, 1985).

3.2.7.4 Simmon's Citrate Agar (SCA)

This tube medium is used to identify Gram negative enteric bacilli based on the ability of the organisms to utilize citrate as the sole source of carbon. (Citrate utilization test).The organism which utilizes citrate as its source of carbon degrades it to ammonia and subsequently converts it to ammonium hydroxide. The pH of the medium is then increased and this is indicated by a change in color from green to blue (Cheesbrough, 1985).

3.2.7.5 Triple Sugar Iron Agar (TSI)

This tube medium is used to identify Gram negative enteric bacilli based on the following biochemical characteristics (Cheesbrough, 1985):

- Glucose fermentation – indicated by yellow butt
- Lactose fermentation – indicated by yellow slant
- Hydrogen sulfide production – indicated by blackening of the medium
- Gas production – indicated by presence of a crack, bubble or gas space
- pH indicator – phenol red
- Hydrogen sulfide indicator – ferric ammonium citrate with sodium thiosulfate.

3.2.7.6 Mortility Indole Urease (MIU) test

MIU medium is a semisolid medium used in the qualitative determination of motility, production of indole and ornithine decarboxylase. MIU medium is used for the differentiation of the Family Enterobacteriaceae. The organisms tested must ferment glucose for proper performance of the medium. MIU medium contains dextrose as fermentable sugar, ornithine as an amino acid, bromcresol purple as pH indicator, casein peptone as a source of tryptophan, and other essential nutrients for growth. The medium contains a small amount of agar allowing for detection of motility (Cheesbrough, 1985).

3.2.8 Antibiotic sensitivity test against isolated microbes

To determine the drug Sensitivity and resistance patterns of isolated organisms used different types of commercially available antimicrobial discs, (Mast diagnostics Mersey side, UK.) which were showed in table no 3. The antibiotic resistance was determined by Kirby-Bauer disc diffusion technique using Mueller-Hinton agar (Difco), according to the recommendations of National Committee for Clinical Laboratory Standards (CLSI 2013). After overnight incubation at 37°C, the diameter in millimeters of the zones of inhibition around each of the antimicrobial discs was recorded and categorized as resistant, sensitive and intermediate in accordance with company recommendations (Cappuccino 2005*). E. coli, Salmonella* spp*., Staphylococcus* spp. and *Pseudomonas* spp. isolates were tested for sensitivity to different antibiotics such as Levofloxacin (5µg), Penicillin G (10units), Amoxicillin (30 **µ**g), Cefxime (5**µ**g), Cephradine (25**µ**g), Chloramphenicol (30 **µ**g), Gentamicin (10 **µ**g), Vancomycine (30 **µ**g), Azithromycin (15 **µ**g), Eryhromycine (15 **µ**g) Tetracycline (30 **µ**g), Ciprofloxacin (5 **µ**g), Amikacin (30 **µ**g), Ampicillin (10 **µ**g), Kanamycin (30 **µ**g) and Neomycin (30 **µ**g). The disks were purchased from national company. The results were interpreted by special manufacturer's tables.

Table 3: Antimicrobial agents with their disc concentration

Antimicrobial agents	Symbol	Disc concentration (µg/disc)
Levofloxacin	LE	5 µg
Penicillin G	P	10 unites
Amoxicillin	AMX	30 µg
Chloramphenicol	C	30 µg
Gentamicin	GEN	10 µg
Ciprofloxacin	CIP	5 µg
Azithromycine	AZM	15 µg
Erythromycine	E	15 µg
Tetracycline	TE	30 µg
Cephradine	CH	25 µg
Cefixime	CFM	5 µg
Vancomycin	VA	25 µg
Neomycin	N	30 µg
Ampicillin	AMP	10 µg
Kanamycin	K	30 µg
Amikacin	AK	30 µg

Notes: µg =microgram.

3.2.8.1 Recording and interpretating results of antibiogram study

The zones of growth inhibition was compared with the zone-size interpretative table no 4, 5, 6, 7, 8 standard for *E. coli, Salmonella* spp., *Staphylococcus* spp*., Pseudomonas* spp. and *Aeromonas hydrophila.*

Table 4: Zone diameter interpretative standards for *Escherichia coli*

Antimicrobial agents	Zone diameter		
	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Levofloxacin	≤13	14-16	≥17
Amoxicillin	≤13	14-17	≥18
Neomycin	≤12	13-16	≥17
Cephradine	≤14	15-17	≥18
Chloramphenicol	≤12	13-17	≥18
Penicillin G	≤14	-	≥15
Cefalexin	≤14	-	≥14
Azithromycin	≤12	-	≥13
Erythromycin	≤13	14-22	≥23
Cloxacillin	≤18	-	≥30

(Source: CLSI, 2013)

[Legand: LE=Levofloxacin; AMX= Amoxicillin; N= Neomycin; CH= Cephradine; C= Chloramphenicol; P= Penicillin G; CN= Cefalexin; AZM= Azithromycin; E= Erythromycin; COX= Cloxacillin]

Table 5: Zone diameter interpretative standards for *Salmonella* spp.

Antimicrobial agents	Zone diameter		
	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Ciprofloxacin	≤15	16-20	≥21
Amoxicillin	≤13	14-17	≥18
Ampicillin	≤13	14-16	≥17
Amikacin	≤14	15-16	≥17
Chloramphenicol	≤12	13-17	≥18
Gentamicin	≤12	13-14	≥15
Vancomycin	≤12	-	≥12
Kanamycin	≤13	14-17	≥18
Erythromycin	≤13	14-22	≥23
Cephradine	≤14	15-17	≥18
Penicillin G	≤17	-	≥17
Piperacillin-Tazobactam	≤17	18-20	≥21

(Source: CLSI, 2013)

[**Legand:** CIP= Ciprofloxacin; AMX= Amoxicillin; AMP= Ampicillin; AK= Amikacin; C= Chloramphenicol; GEN= Gentamicin; VA= Vancomycin; K= Kanamycin; E= Erythromycin; CH= Cephradine; P= Penicillin G; TZP= Piperacillin-Tazobactam]

Table 6: Zone diameter interpretative standards for *Staphylococcus* spp.

Antimicrobial agents	Zone diameter		
	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Levofloxacin	≤15	16-18	≥19
Kanamycin	≤12	14-17	≥18
Ciprofloxacin	≤15	16-20	≥21
Cefixime	≤15	16-18	≥19
Cloxacillin	≤14	15-17	≥18
Gentamicin	≤12	13-14	≥15
Chloramphenicol	≤12	13-17	≥18
Penicillin G	≤28	-	≥29
Azithromycin	≤13	14-17	≥18
Amoxicillin	≤19	-	≥20

(Source: CLSI, 2013)

[Legand: LE=Levofloxacin; K= Kanamycin; CIP= Ciprofloxacin; CFM= Cefixime; CN= Cefalexin; N= Neomycin; TE= Tetracycline; CH= Cephradine; AZM= Azithromycin; E= Erythromycin]

Table 7: Zone diameter interpretative standards for *Pseudomonas* spp.

Antimicrobial agents	Zone diameter		
	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Levofloxacin	≤13	14-16	≥17
Gentamicin	≤12	13-14	≥15
Ciprofloxacin	≤15	16-20	≥21
Cefixime	≤13	14-20	≥21
Cefalexin	≤14	-	≥14
Cephradine	≤13	14-18	≥19
Chloramphenicol	≤20	-	≥21
Amoxicillin	≤19	-	≥20
Erythromycin	≤19	20-21	≥22
Tetracycline	≤14	15-18	≥19

(Source: CLSI,2013)

[Legand: LE= Levofloxacin; GEN= Gentamicin; CIP= Ciprofloxacin; CFM= Cefixime; CN=Cefalexin; CH= Cephradin; C= Chloramphenicol; AMX= Amoxicillin; E= Erythromycin; TE= Tetracyclin**]**

Table 8: Zone diameter interpretative standards for *Aeromonas hydrophila*

Antimicrobial agents	Zone diameter		
	Resistant (mm)	Intermediate (mm)	Sensitive (mm)
Levofloxacin	≤23	17-21	≥22
Gentamicin	≤14	10-13	≥15
Ciprofloxacin	≤16	12-15	≥17
Azithromycin	≤16	11-15	≥17
Ampicillin	≤22	16-21	≥23
Penicillin	≤14	10-13	≥15
Kanamycin	≤16	11-14	≥17
Erythromycin	≤15	10-13	≥18
Chloramphenicol	≤13	-	≥15
Tetracycline	≤14	09-13	≥15

(Source: CLSI,2013)

[Legand: LE= Levofloxacin; GEN= Gentamicin; CIP= Ciprofloxacin; AZM= Azithromycin; P= Penicillin; AMP= Ampicillin; C= Chloramphenicol; K= Kanamycin; E= Erythromycin; TE= Tetracyclin**]**

3.2.9 PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Aeromonas hydrophila*

3.2.9.1 Basic protocol of bacterial genomic DNA isolation

Bacteria from saturated liquid nculture are lysed and protins are removed by digestion with protinase-k. Cell wall debris, polysaccharides and remaining proteins are removed by Phenol-chloroform extraction and high-molecular-weight DNA is recovered from the resulting supernatant by isopropanol precipitation.

Procedure

- Inoculate a 25 ml of liquid culture with *Aeromonas hydrophila.* Grow in conditions appropriate for *Aeromonas hydrophila* until the culture is saturated.
- Spin 1.0 ml of the overnight culture in a micro centrifuge tube for 5 minutes at 10000 rpm.
- Discard the supernatant.
- Repeat this step. Drain well onto a kimwipe.
- Re-suspend the pellet in 467 µl TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ ml Proteinase k to give a final concentration of 100 µg/mg Proteinase k in 0.5% SDS. Mix thoroughly and incubate 30 min for 1 hr at 37^0C.

- Add an approximately equal volume (500 µl) of Phenol/Chloroform/Isoamyl alcohol. Mix thoroughly but very gently to avoid shearing the DNA, by inverting the tube until the phase are completely mixed.
- Then centrifuge the tubes at 12000 rpm for 10 minutes.
- Remove aqueous, viscous supernatant (≈ 400 µl) to a fresh microcentrifuge tube , leaving the interface behind. Add an equal volume of Phenol/Chloroform/Isoamyl alcohol extract thoroughly and spin in a microcentrifuge at 10000 rpm for 5 min.
- Transfer the supernatant to a fresh tube (≈400 µl).
- Add $1/10^{th}$ volume of 3 M sodium acetate and mix.
- Add 0.6 volumes of isopropanol to precipitate the nucleic acids, keep on ice for 10 minutes.
- Centrifuge at 13500 rpm for 15 minutes.
- Discard the supernatant.
- Wash the obtained pellet with 1 ml of 95% ethanol for 5 minutes. Then centrifuge at 12000 rpm for 10 minutes.
- Discard the supernatant.
- Dry the pellets as there is no alcohol.
- Resuspend the pellet in 50 µl of TE and then 7.5 µl of RNase. Store DNA at 4^0C for short term and at -20^0C for long term.

3.2.9.2 PCR amplification and sequencing of 16S rRNA

Table 9: Condition of PCR.

Step	Temperature	Duration	Cycles
1. Initial denaturation	95ºC	5 min	01
2. Denaturation	95ºC	30 Sec	35
3. Annealing	56ºC	30 Sec	
4. Extension	72ºC	1.5 min	
5. Final extension	72ºC	10 min	01
6. Holding	4ºC	hold	-

3.2.9.3 Electrophoresis

Process of Electrophoresis:

- Preparation of gel.
- Sample application in the gel.
- Adjustment of voltage or current (gel-electrophoresis about 70-100 volts).
- Set up run time about 30-60 minute
- When DNA migrated sufficiently, as judged from the migration of bromphenicol blue of loading buffer, the power supply was switched off.
- The gel stained in ethidium bromide (0.5μg/ml) for 10 minutes, in a dark place.
- The gel was distained in distilled water for 10 minutes. The distained gel was placed on the imaging system in the dark chamber of the image documentation system.
- The UV light of the system was switched on; the image was viewed on the monitor, focused, acquired and saved in an USB flash drive.

3.2.9.4 Nucleotide sequence accession number and BLAST analysis

The nucleotide sequence 16S rRNA gene region data was submitted to NCBI nucleotide sequence database. Using BLAST tool, phylogenic tree, primer pairs were designed from NCBI database search tool.

3.2.9.4.1 Chain-termination methods (Sanger sequencing)

Steps of Sanger sequencing using ABI 3130 Genetic analyzer

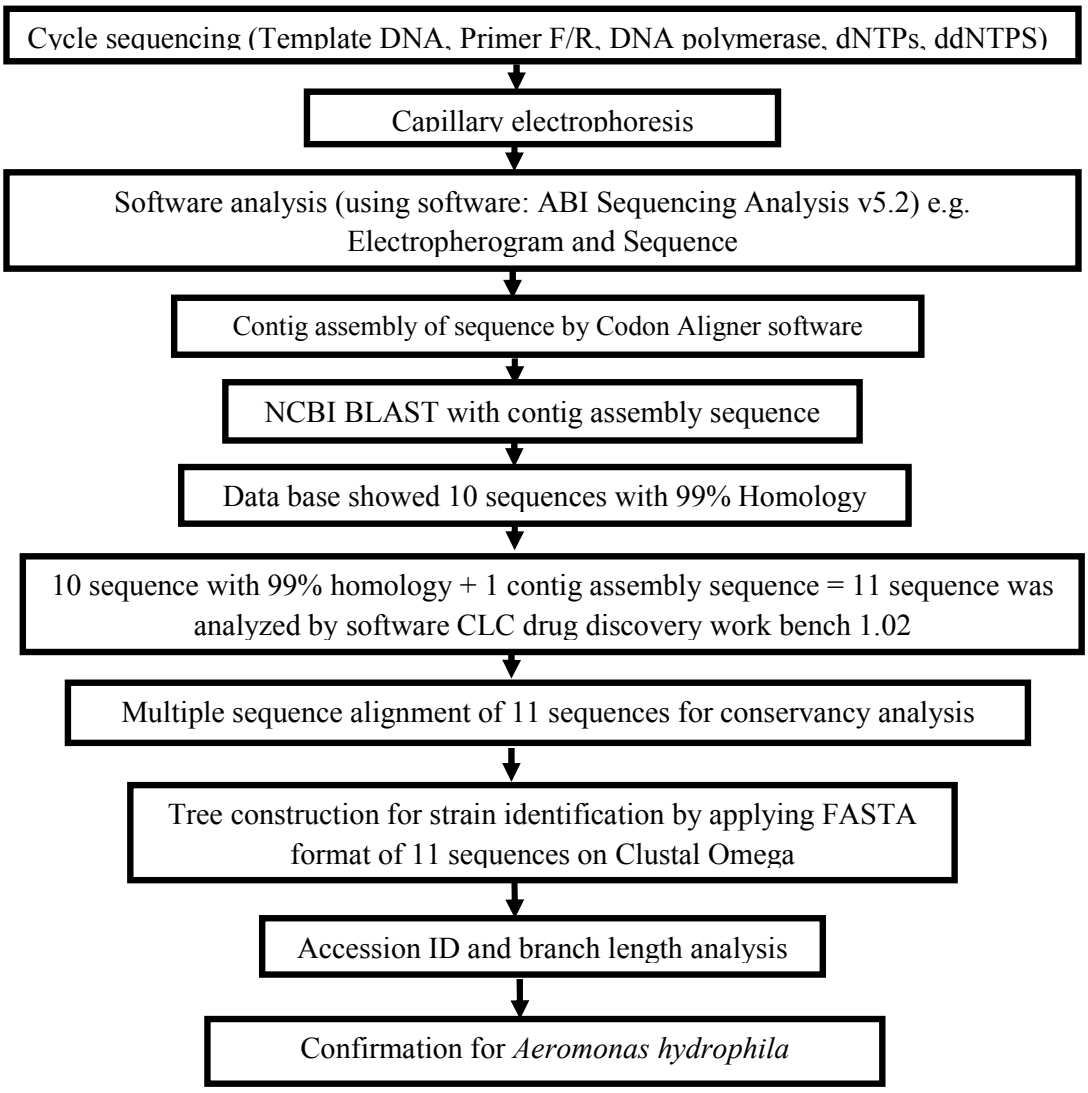

Figure 6: Steps of Sanger sequencing using ABI 3130 Genetic analyzer.

3.2.9.5 Maintenance of stock culture

The stock culture was maintained following the procedure of Choudhury *et al.* (1987). Nutrient agar slants were used for the maintenance stock culture for each of the bacterial isolates. One slant was used for an individual isolate. After growth of the organism in the slant, the sterile mineral oil was overlaid and the culture was kept at room temperature for further used as seed.

CHAPTER 4

RESULTS

The present study was conducted for isolation, characterization of bacteria from dead-in-shell chicks which collected from different hatcheries of Bogra District of Bangladesh with their antibiogram study. A total 60 dead-in-shell chicks egg samples (yolk, lungs and intestine) collected were subjected to various bacteriological, biochemical examination in the laboratory of the Department of Microbiology, HSTU, Dinajpur.

4.1 Results of Isolation of organism

Five different genera of bacteria such as: *E. coli, Salmonella* spp.*, Staphylococcus* spp. and *Pseudomonas* spp. and *Aeromonas hydrophila* were isolated from different samples of dead-in shell chicks such as yolk, lungs and intestine. During the study period a total 60 dead- in shell chicks egg (20 yolk, 20 lungs and 20 intestine) samples were collected from different hatcheries. In case of yolk samples 7 were positive for *Escherichia coli*, 8 for *Salmonella* spp., 5 for *Staphylococcus* spp., 3 for *Pseudomonas* spp. 2 for *Aeromonas hydrophila* respectively. In case of lungs samples 16 were positive for *Escherichia coli*, 14 were positive for *Salmonella* spp., 8 were positive for *Staphylococcus* spp. and 6 were positive for *Pseudomonas* spp. And lastly in case of intestinal samples 6 were positive for *Escherichia coli*, 9 were positive for *Salmonella* spp., 4 were positive for *Staphylococcus* spp. and 2 were positive for *Pseudomonas* spp. Among 90 isolates, 29 (32.2%) *Escherichia coli*, 31 (34.5%) *Salmonella* spp., 17 (18.9%) *Staphylococcus* spp.*,*11 (12.2%) *Pseudomonas* spp. and 2 (2.22%*) Aeromonas hydrophila* were identified which are shown in table no 10.

Table 10: Summary of isolation of bacteria from different samples of dead-in-shell chicks

Samples	No of isolated bacteria (n=90)				
	E. coli	***Salmonella* spp.**	***Staphylococcus* spp.**	***Pseudomonas* spp.**	***Aeromonas hydrophila***
Yolk (n=20)	7	8	5	3	2
Lungs (n=20)	16	14	8	6	0
Intestine (n=20)	6	9	4	2	0
Total (percentage)	29 (32.2)	31 (34.5)	17 (18.9)	11(12.2)	2 (2.2)

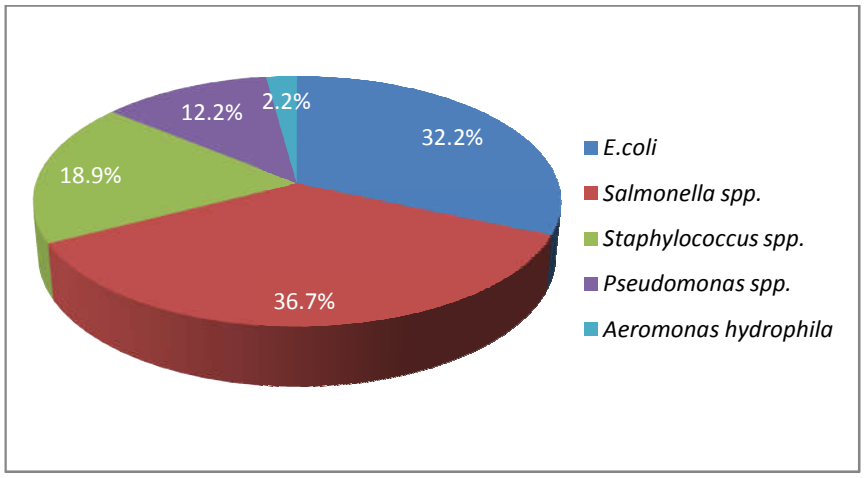

Fig 7: Percentage of bacterial species isolated from yolk, lungs and intestine of dead-in-shell chicks.

4.2 Isolation and identification of bacteria by different bacteriological methods

4.2.1 Results of Cultural Examination

The cultural characteristics of the isolated *E. coli*, *Salmonella* spp. *Staphylococcus* spp., *Pseudomonas* spp. and *Aeromonas hydrophila* on different media are presented in Table no 11.

Table 11: The results of cultural characteristics of the isolated organisms

Sl. No.	Name of bacteria	Name of media	Colony characteristics
01	*E. coli*	Nutrient agar	Circular, smooth, white to grayish or white colony.
		MacConkey agar	Bright pink colored colony.
		EMB agar	Metallic sheen (greenish black) colony.
02	*Salmonella* spp.	Nutrient agar	Circular, smooth, white to grayish or white colony with feted odor.
		MacConkey agar	Pale colored colony.
		SS agar	Opaque, smooth, round with black centered.
03	*Staphylococcus* spp.	Nutrient agar	Gray white or yellowish colony
		MSA	Golden yellowish colony
04	*Pseudomonas* spp.	Nutrient agar	Colonies are surrounded by bluish green coloration.
		MacConkey agar	Pale colored colony.
		Cetrimide agar	Yellow-green to blue colony.
05	*Aeromonas hydrophila*	Nutrient agar	Circular, smooth, white to grayish or white colony with feted odor.
		Mac Conkey agar	Pale colored colony.
		SS agar	Colourless colony

SS = Slamonella-Shigella agar; EMB = Eosin Methylene Blue Agar; MSA = Mannitol Salt Agar.

4.2.1.1 Nutrient Agar (NA)

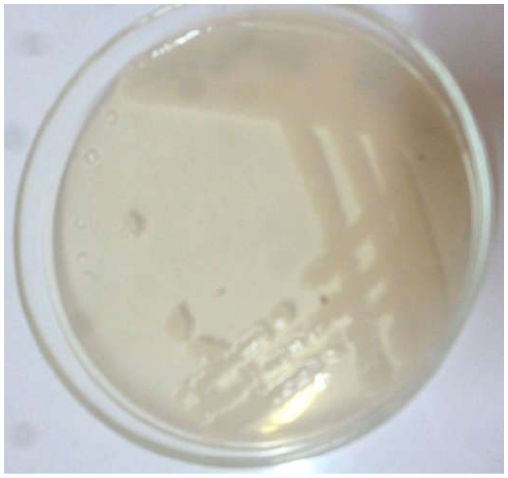

Plate. 1 (a) Nutrient agar (inoculated)

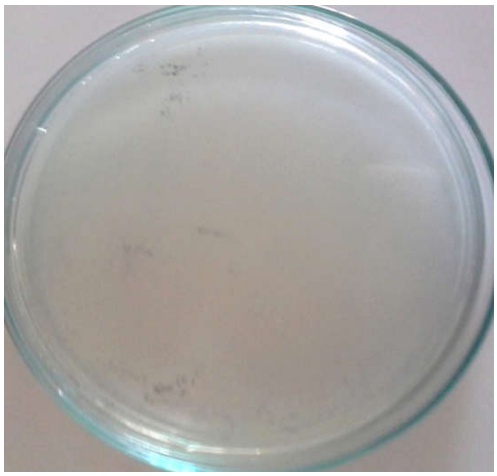

Plate. 1 (b) Nutrient agar (control)

Plate 1: Growth on Nutrient agar (left) and control (right)

4.2.1.2 MacConkey Agar

Plate. 2 (a). Lactose fermenting organisms produce bright pink coloured colony

Plate. 2 (b). MacConkey agar (control)

Plate 2: Growth on MacConkey agar (left) and control (right)

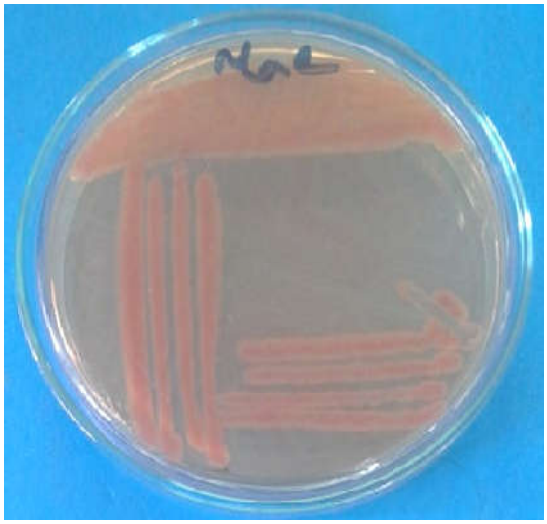

Plate. 3 (a). Non lactose fermenting organisms produce pale colored colony

Plate. 3 (b). MacConkey agar (control)

Plate 3: Growth on MacConkey agar (left) and control (right)

4.2.1.3 Eosin Methylene Blue Agar (EMB)

Plate. 4 (a). Metallic sheen (greenish black) colony on EMB agar

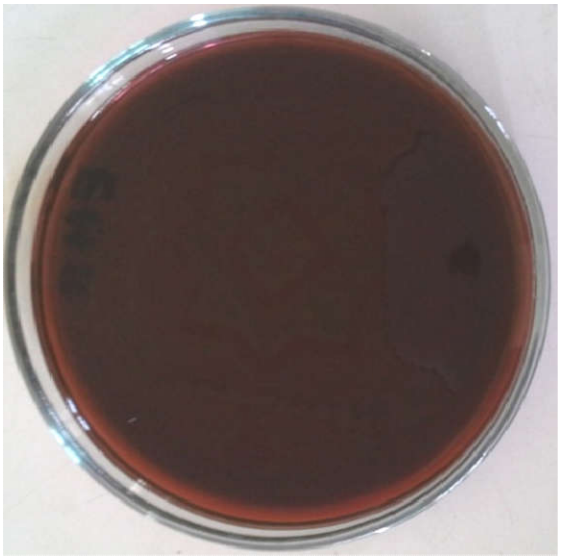

Plate. 4 (b). EMB agar (control)

Plate 4: Growth of *E. coli* on EMB agar (left) and control (right)

4.2.1.4 Salmonella-Shigella Agar (SS)

Plate. 5 (a). Black colour colonies on SS agar

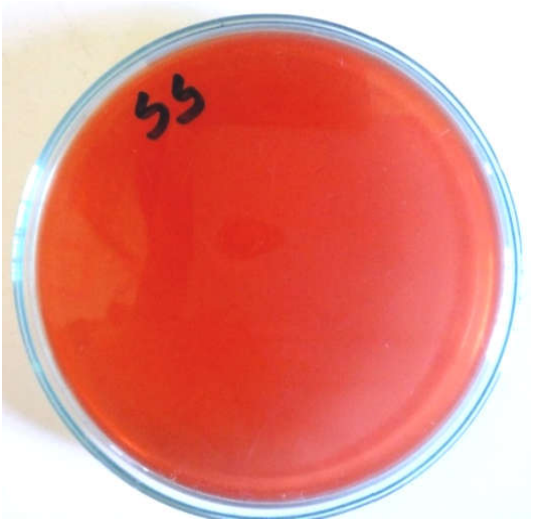

Plate. 5 (b). SS agar (control)

Plate 5: Growth of *Salmonella* spp. on SS agar (left) and control (right)

Plate. 6 (a). Colourless colonies on SS agar

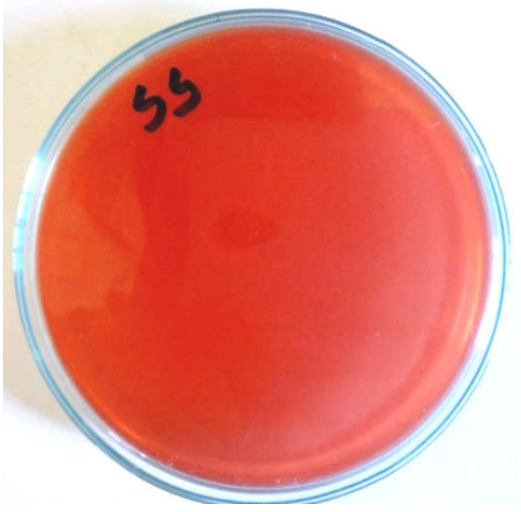

Plate. 6 (b). SS agar (control)

Plate 6: Growth of *Aeromonas hydrophila*. on SS agar (left) and control (right)

4.2.1.5 Manitol Salt Agar (MSA)

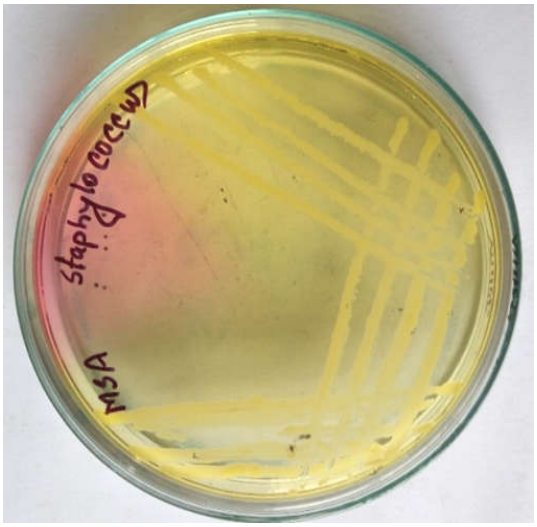

Plate. 7 (a). Golden yellowish colony of *Staphylococcus* spp. on MSA.

Plate. 7 (b). MSA (control)

Plate 7: Growth of *Staphylococcus* spp. on MSA (left) and control (right)

4.2.1.6 Staphylococcus Agar No. 110

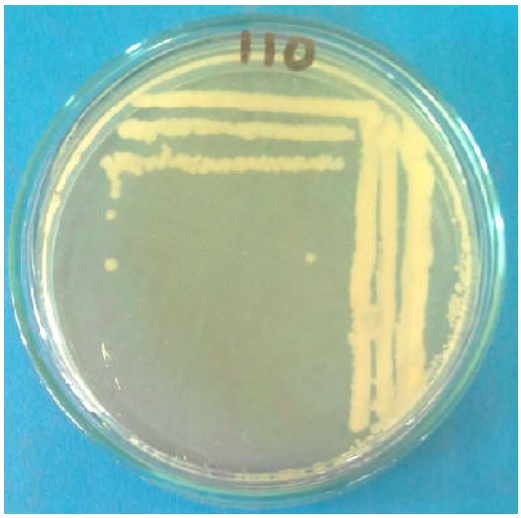

Plate. 8 (a). Yellowish colony of *Staphylocooccus* spp. on Staphylococcus agar 110

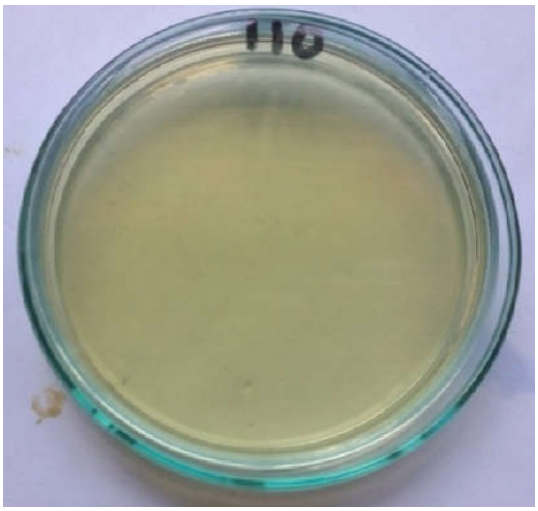

Plate. 8 (b). Staphylococcus agar 110 (control)

Plate 8: Growth of *Staphylococcus* spp. on Staphylococcus agar 110 (left) and control (right)

4.2.1.7 Cetrimide Agar

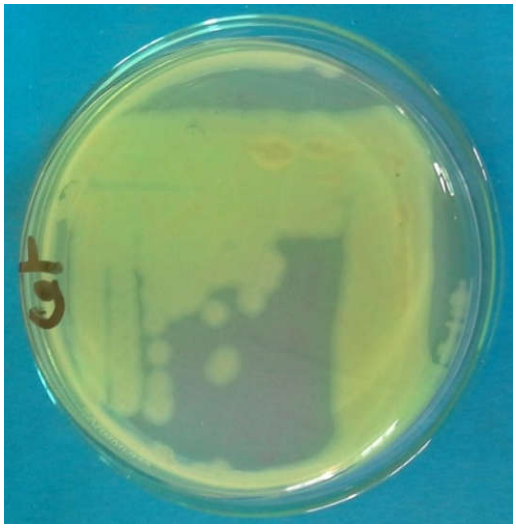

Plate. 9 (a). Bluish green colony of *Pseudomonas* spp. on cetrimide agar

Plate. 9 (b). Cetrimide agar (control).

Plate 9: Growth of *Pseudomonas* spp. on Cetrimide agar (left) and control (right)

4.2.2 Results of Gram's staining

The microscopic examination of Gram's stained smears from EMB agar revealed Gram-negative, pink colored,short rod shaped *E. coli* arranged in single, pairs or short chain. (Plate-10. a)

The microscopic examination of Gram's stained smears from SS agar revealed Gram-negative, pink colored,very short rod shaped *Salmonella* spp. arranged in single, pairs or short chain. (Plate-10. b)

The microscopic examination of Gram's stained smears from Mannitol salt agar revealed Gram-positive cocci arranged in grape like clusters of *Staphylococcus* spp. (Plate-10. c).

The microscopic examination of Gram's stained smears from Cetrimide agar revealed Gram-negative, pink colored, rod shaped of *Pseudomonas* spp. (Plate-10. d)

The microscopic examination of Gram's stained smears from SS agar revealed Gram-negative, pink colored, rod shaped of *Aeromonas hydrophila* (Plate-10. e)

4.2.2.1 Microscopic examination

Microscopic observation was performed to observe shape and gram reaction of the isolates. Both of the isolates were found to be gram positive and gram negative, curved, comma and rod shape.

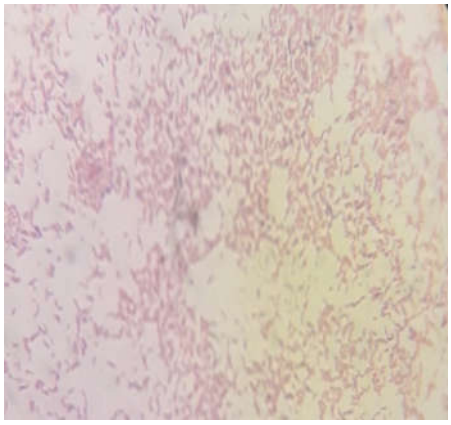

Plate. 10 (a). Gram-negative single or paired short rods of *E. coli*

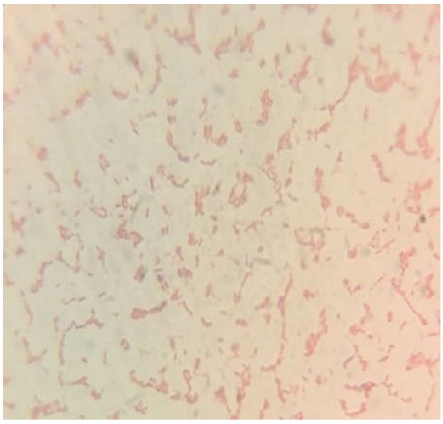

Plate. 10 (b). Gram-negative single very short rods of *Salmonella* spp.

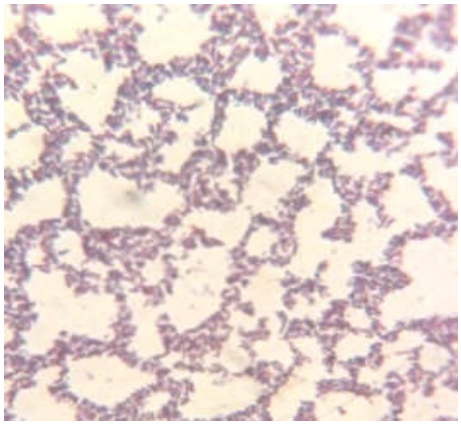

Plate. 10 (c). Gram-positive small coccus of *Staphylococcus* spp. arranged in cluster

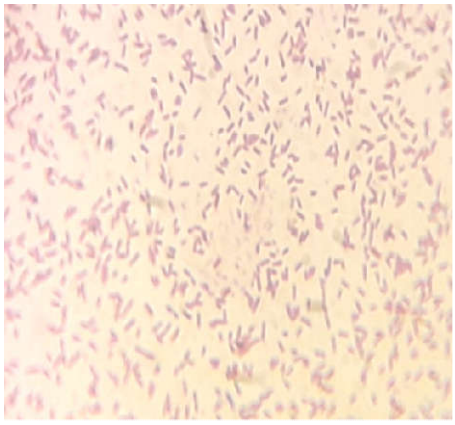

Plate. 10 (d). Gram-negative rods of *Pseudomonas* spp.

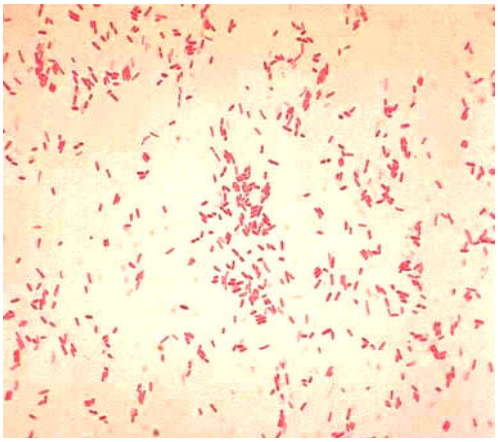

Plate. 10 (e). Gram-negative rods of *Aeromonas hydrophila*

Plate 10: Microscopic studies and staining properties of the bacterial isolates under alight

4.2.3 Results of Biochemical Test

Isolated organisms were confirmed by different biochemical tests.

Table 12: Result of biochemical test for the representative isolates

Name of isolates	In	MR	VP	SC	TSI		MIU	SB
					slant	butt		
E. coli	+	+	-	-	A(yellow)	A(yellow)	+	+
Salmonella spp.	-	+	-	-	Al(red)	A(yellow)	+	+
Staphylococcus spp.	-	+	+	+	A(yellow)	A(yellow)	-	-
Pseudomonas spp.	-	-	-	+	Al(red)	Al(red)	+	-
Aeromonas hydrophila	+	-	+	ND	Al(red)	A(yellow)	ND	ND

Legends: (-) = Negative), (+) = Positive, A=acid, Al= Alkaline, In = Indole, MR= Methyl-red, VP= Voges-proskauer, SC= Simmon's citrate, TSI= Triple sugar iron, MIU= Motility indole urease, SB= Selenite broth, ND=Not done.

4.2.3.1 Indole Test

The *E. coli and Aeromonas hydrophila were* positive and *Salmonella, Staphylococcus, Pseudomonas* spp. were negative for indole test.

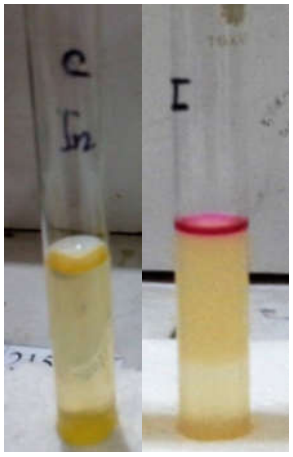

Plate 11: Indole test for ***E. coli*** showing positive result by red coloration of the medium (right) and control (left)

Plate 12: Indole test for ***Salmonella* spp.** showing negative result by no colour change of the medium (right) and control (left).

Plate 13: Indole test for ***Staphylococcus* spp.** showing negative result by no colour change of the medium (right) and control (left).

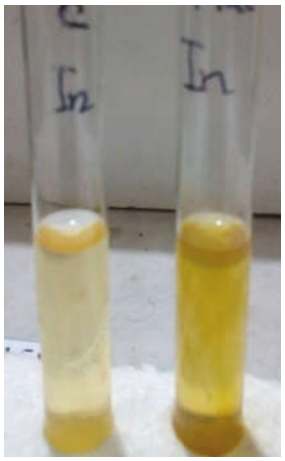

Plate 14: Indole test for ***Pseudomonas* spp.** showing negative result by no colour change of the medium (right) and control (left).

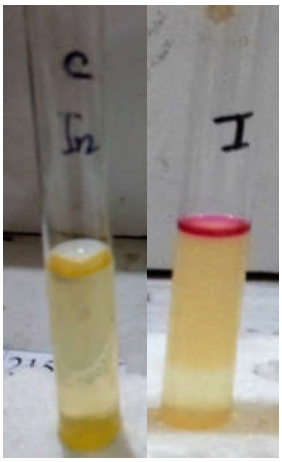

Plate 15: Indole test for ***Aeromonas hydrophila*** showing positive result by red coloration of the medium (right) and control (left).

4.2.3.2 Methyl Red test

The *E. coli, Salmonella, Staphylococcus* were positive and *Pseudomonas* spp. and *Aeromonas hydrophila* were negative for methyl red test.

Plate 16: Methyl-Red test for ***E. coli*** indicated positive by changing the medium into bright red colour (right) and control (left).

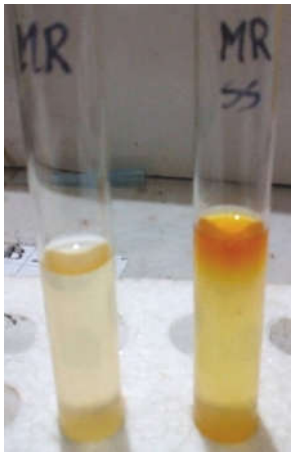

Plate 17: Methyl-Red test for ***Salmonella spp.*** indicated positive by changing the medium into bright red colour (right) and control (left).

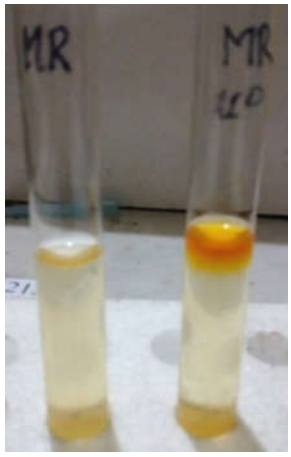

Plate 18: Methyl-Red test for ***Staphylococcus*** **spp.** indicated positive by changing the medium into bright red colour (right) and control (left).

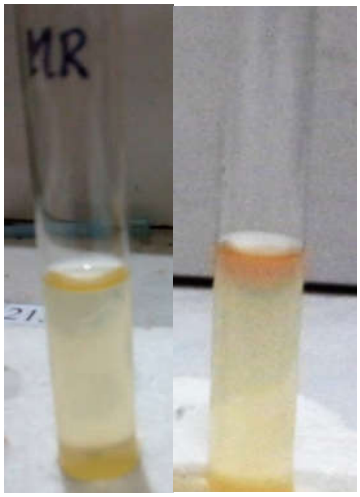

Plate 19: Methyl-Red test for ***Pseudomonas*** **spp.** indicated negative by no colour change of the medium (right) and control (left).

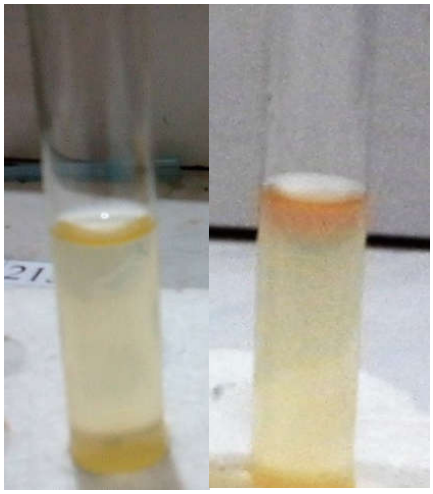

Plate 20: Methyl-Red test for ***Aeromonas hydrophila*** indicated negative by no colour change of the medium (right) and control (left).

4.2.3.3 Voges-Proskauer Test

The *E. coli, Salmonella* spp., *Pseudomonas* spp. were negative and *Staphylococcus* spp. and *Aeromonas hydrophila* were positive for Voges-Proskauer test.

Plate 21: Voges-Proskauer test for ***E. coli*** showing negative result by no change of the medium (right) and control (left)

Plate 22: Voges-Proskauer test for ***Salmonella* spp.** showing negative result by no change of the medium (right) and control (left). ((left).

Plate 23: Voges-Proskauer test for ***Staphylococcus* spp.** showing positive result by changing the medium into rose red colour (right) and control (left).

Plate 24: Voges-Proskauer test for ***Pseudomonas* spp.** showing negative result by no change of the medium (right) and control (left).

Plate 25: Voges-Proskauer test for ***Aeromonas hydrophila*** showing positive result by changing the medium into rose red colour (left) and control (right)

4.2.3.4 MIU Test

The *E. coli*, *Salmonella* spp. and *Pseudomonas* spp., were positive but *Staphylococcus* spp. was negative for MIU test.

Plate 26: MIU test for ***E. coli*** showing positive result by the diffuse, hazzy growth and slightly opaque media.

Plate 27: MIU test for ***Salmonella* spp.** showing positive result by the diffuse, hazzy growth and slightly opaque media.

Plate 28: MIU test for ***Staphylococcus* spp.** showing negative result by no colour change of the media.

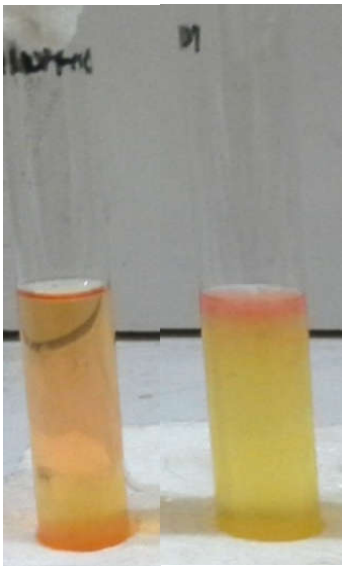

Plate 29: MIU test for ***Pseudomonas* spp.** showing positive result by the diffuse, hazzy growth and slightly opaque media.

4.2.3.5 Triple Sugar Iron (TSI) Test

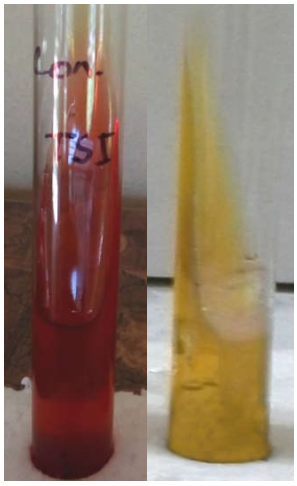

Plate 30: Triple Sugar Iron test for ***E. coli*** showing yellow colour butt & yellow colour slant inoculated (right) and control (left).

Plate 31: Triple Sugar Iron test for ***Salmonella* spp.** showing yellow colour butt & red colour slant with gas and H_2S production inoculated (right) and control (left).

Plate 32: Triple Sugar Iron test for ***Staphylococcus* spp.** showing yellow colour butt & yellow colour slant inoculated (right) and control (left)

Plate 33: Triple Sugar Iron test for ***Pseudomonas* spp.** showing red colour butt & red colour slant inoculated (right) and control (left)

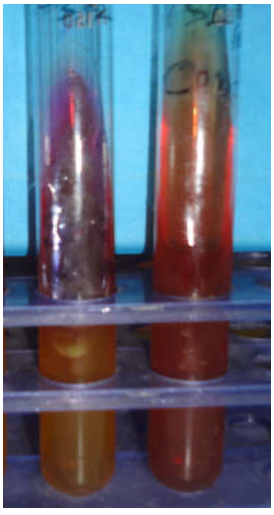

Plate 34: Triple Sugar Iron test for ***Aeromonas hydrophila*** showing yellow colour butt & red colour slant inoculated (left) and control (right)

4.2.3.6 Simmons Citrate test

The *E. coli* and *Salmonella* spp. were negative and *Staphylococcus* spp., *Pseudomonas* spp. were positive for Simmons Citrate test.

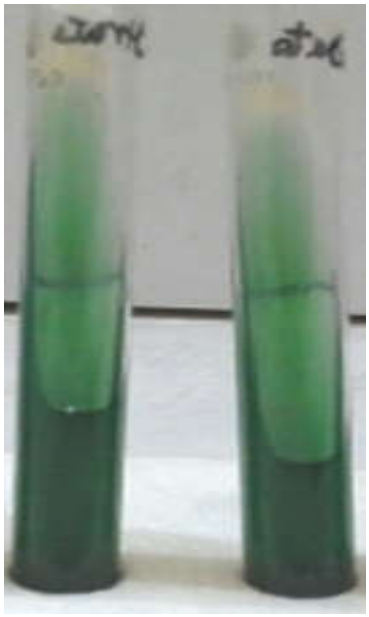

Plate 35: Simmons Citrate test for ***E. coli*** showing negative result by no change of the medium (right) and control (left).

Plate 36: Simmons Citrate test for ***Salmonella* spp.** showing negative result by no change of the medium (right) and control (left).

Plate 37: Simmons Citrate test for ***Staphylococcus* spp.** showing positive result by colour change of the medium into blue colour (right) and control (left).

Plate 38: Simmons Citrate test for ***Pseudomonas* spp.** showing positive result by colour change of the medium into blue colour (left) and control (right).

4.2.3.7 Selenite Broth

The *E. coli, Salmonella spp.* were positive and *Staphylococcus spp., Pseudomonas spp.* were negative for selenite broth test.

Plate 39: Selenite Broth test for ***E. coli*** showing positive result by the change of the medium to brick red colour (right) and control (left).

Plate 40: Selenite Broth test for ***Salmonella*** **spp.** showing positive result by the change of the medium to brick red colour (right) and control (left).

Plate 41: Selenite Broth test for ***Staphylococcus*** **spp.** showing negative result by no change of the medium (right)

Plate 42: Selenite Broth test for ***Pseudomonas*** **spp.** showing negative result by no change of the medium (right)

4.2.4 Results of Antibiotic Sensitivity Test

A total four isolates such as *E. coli, Salmonella* spp., *Staphylococcus* spp. *Pseudomonas* spp. and *Aeromonas hydrophila* obtained from yolk, lungs and intestine samples of dead-in-shell chicks were subjected to Antibiotic Sensitivity assay shown in Table no 13, 14, 15, 16, 17 and plate no, 43, 44, 45, 46 and 47.

Table 13: Antimicrobial profile of *Escherichia coli*

Antimicrobial agents	Diameter of zone of inhibition(mm)	Interpretation
Levofloxacin	22	S
Amoxicillin	-	R
Neomycin	15	I
Cephradine	10	R
Chloramphenicol	15	I
Penicillin G	-	R
Cefalexin	10	R
Azithromycin	15	S
Erythromycin	10	R
Cloxacillin	-	R

(Source: CLSI, 2013)

Table 14: Antimicrobial profile of *Salmonella* spp.

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Ciprofloxacin	23	S
Amoxicillin	-	R
Ampicillin	-	R
Amikacin	10	R
Chloramphenicol	10	R
Gentamicin	20	S
Vancomycin	-	R
Kanamycin	15	I
Erythromycin	-	R
Cephradine	-	R
Penicillin G	-	R
Piperacillin-Tazobactam	-	R

(Source: CLSI, 2013)

Table 15: Antimicrobial profile of *Staphylococcus* spp.

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Levofloxacin	22	S
Kanamycin	16	I
Ciprofloxacin	25	S
Cefixime	10	R
Gentamicin	22	S
Chloramphenicol	20	S
Penicillin G	-	R
Amoxicillin	10	R
Azithromycin	16	I
Cloxacillin	17	I

(Source: CLSI, 2013)

Table 16: Antimicrobial profile of *Pseudomonas* spp.

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Levofloxacin	13	R
Gentamicin	17	**S**
Ciprofloxacin	18	I
Cefixime	-	R
Cefalexin	-	R
Cephradine	-	R
Chloramphenicol	-	R
Amoxicillin	-	R
Erythromycin	-	R
Tetracycline	-	R

(Source: CLSI, 2013)

Table 17: Zone diameter interpretative standards for *Aeromonas hydrophila*

Antimicrobial agents	Diameter of zone of inhibition (mm)	Interpretation
Levofloxacin	22	S
Gentamicin	-	R
Ciprofloxacin	23	S
Azithromycin	15	I
Ampicillin	-	R
Penicillin	-	R
Kanamycin	13	R
Erythromycin	-	R
Chloramphenicol	-	R
Tetracycline	-	R

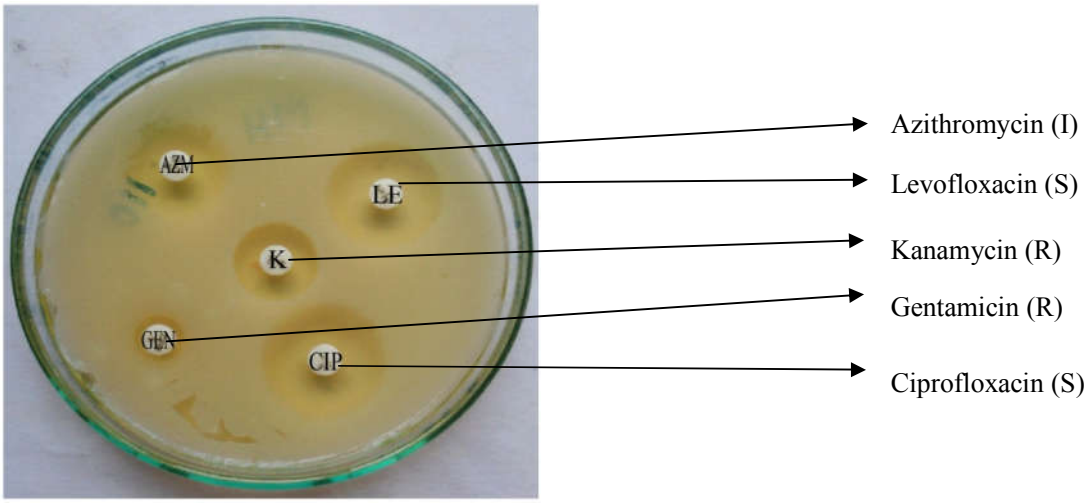

Plate 43 (A): Antibiogram profile of *Aeromonas hydrophila*

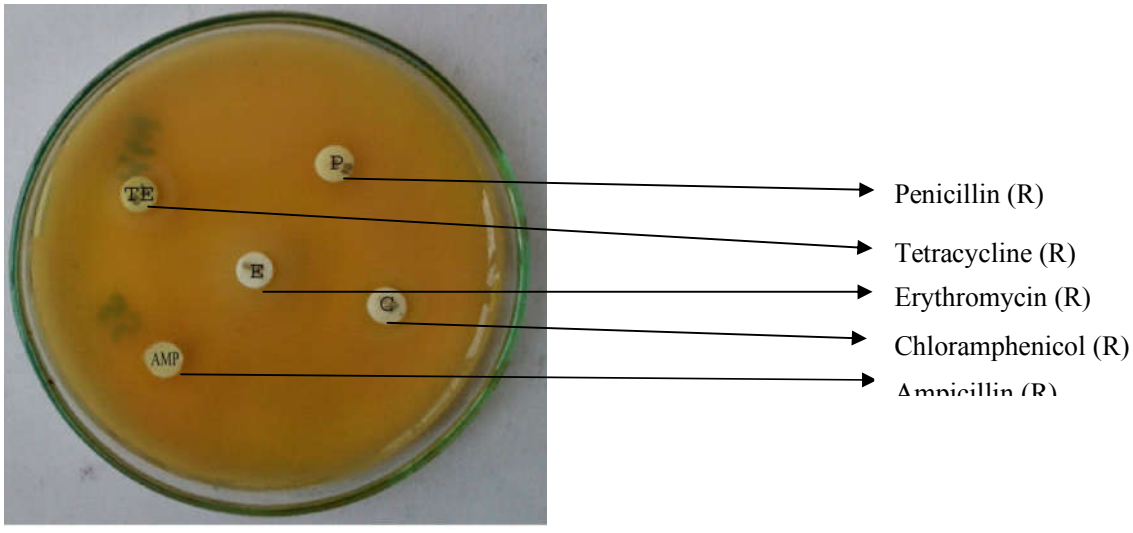

Plate 43 (B): Antibiogram profile of *Aeromonas hydrophila*

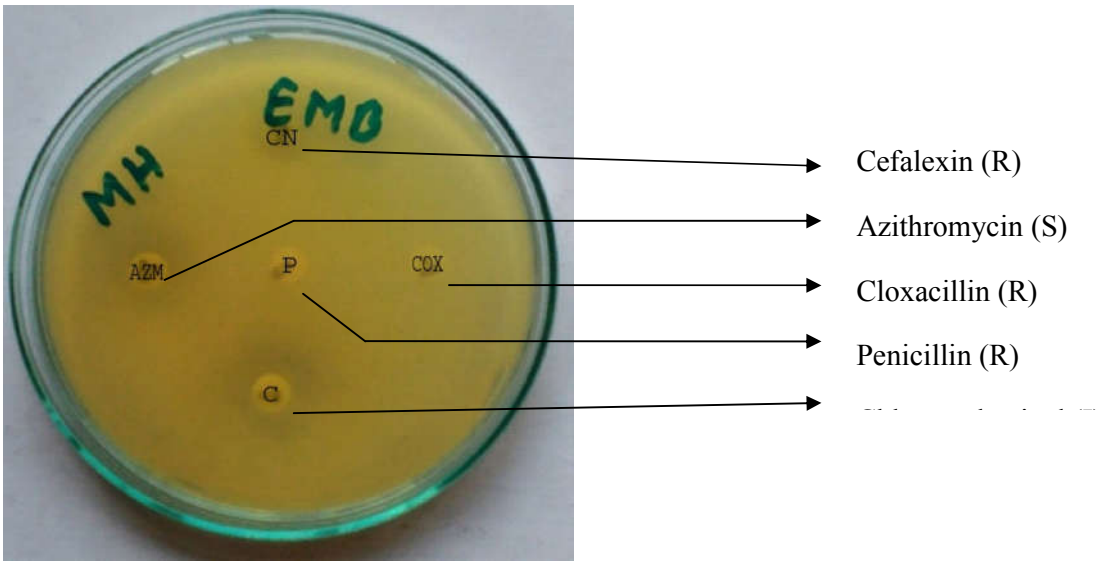

Plate 44 (A): Antibiogram profile of *E. coli*

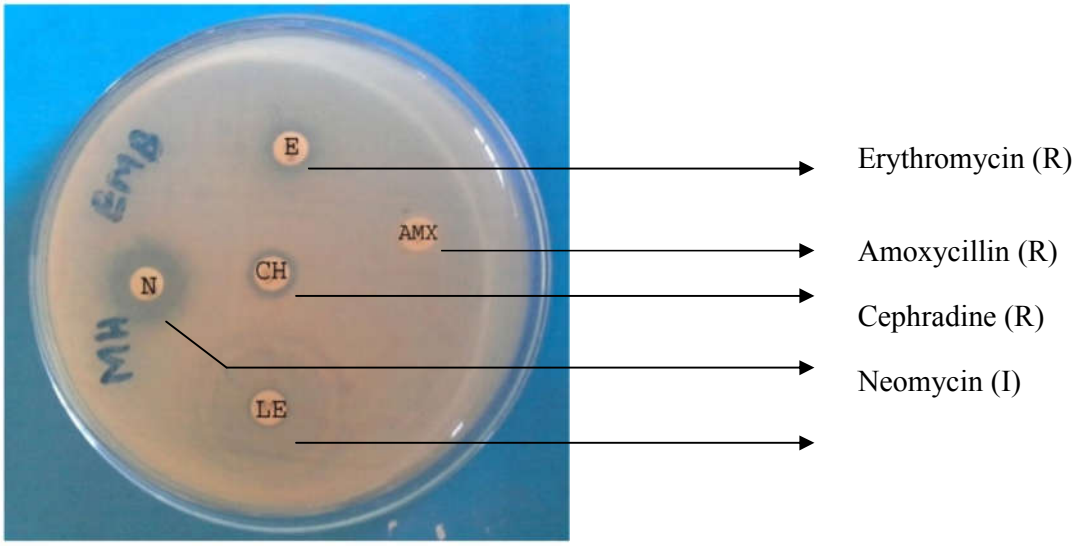

Plate 44 (B): Antibiogram profile of *E. coli*

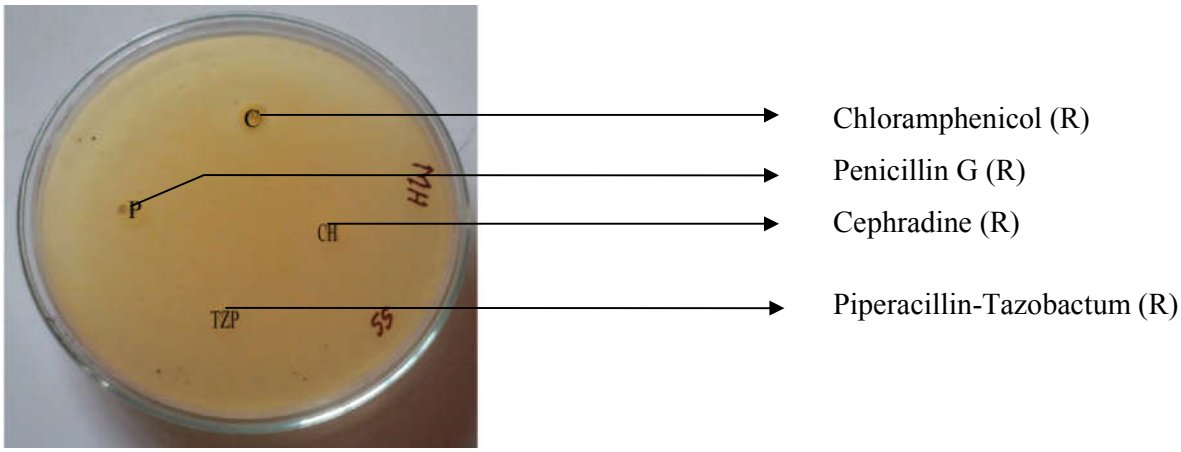

Plate 45 (A): Antibiogram profile of *Salmonella* spp.

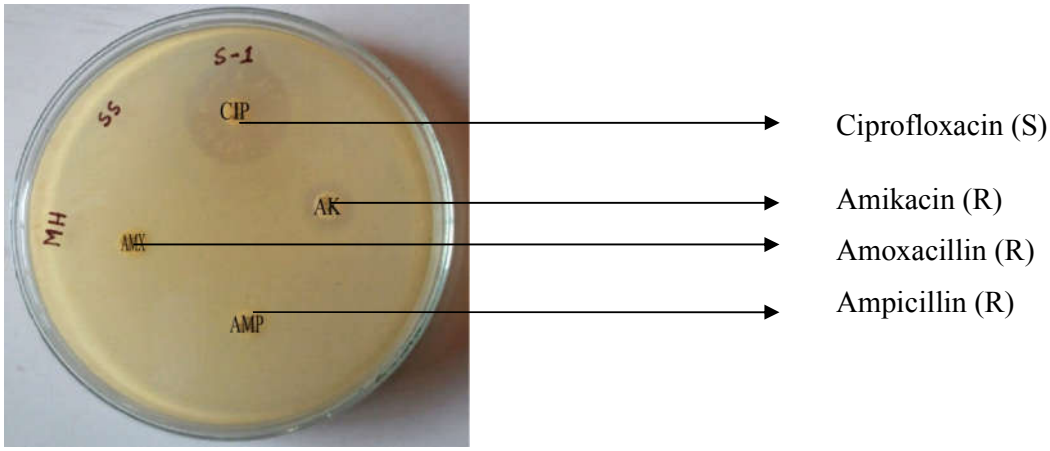

Plate 45 (B): Antibiogram profile of *Salmonella* spp.

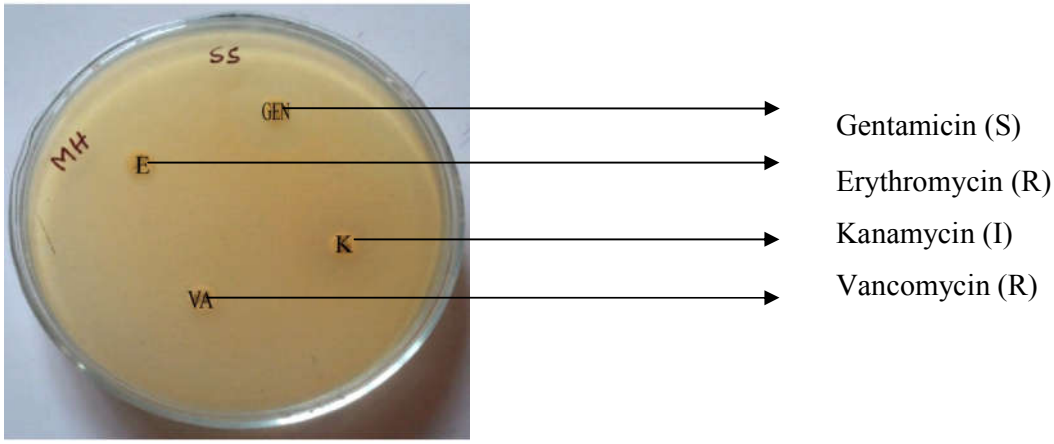

Plate 45 (C): Antibiogram profile of *Salmonella* spp.

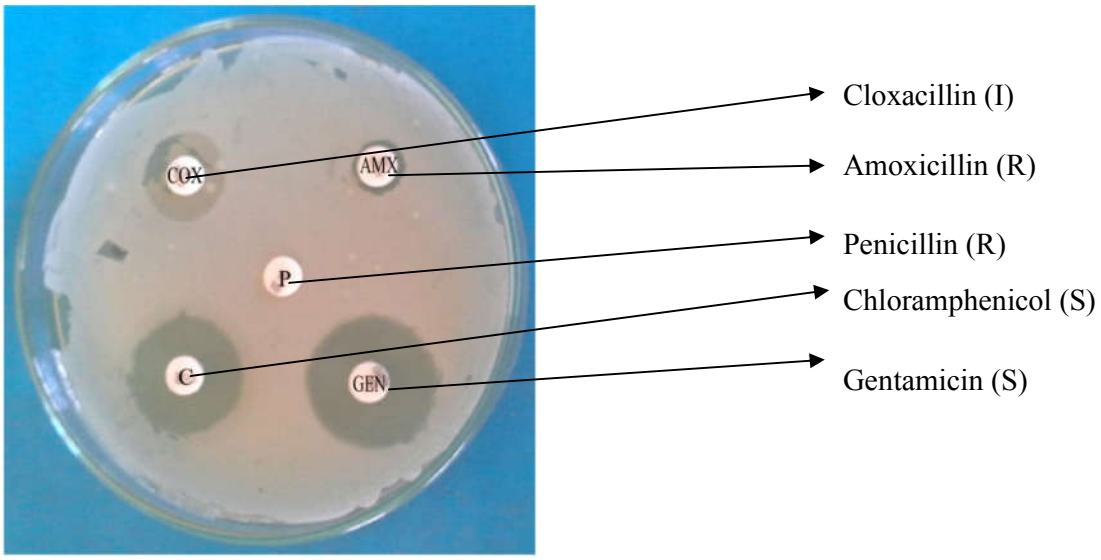

Plate 46 (A): Antibiogram profile of *Staphylococcus* spp.

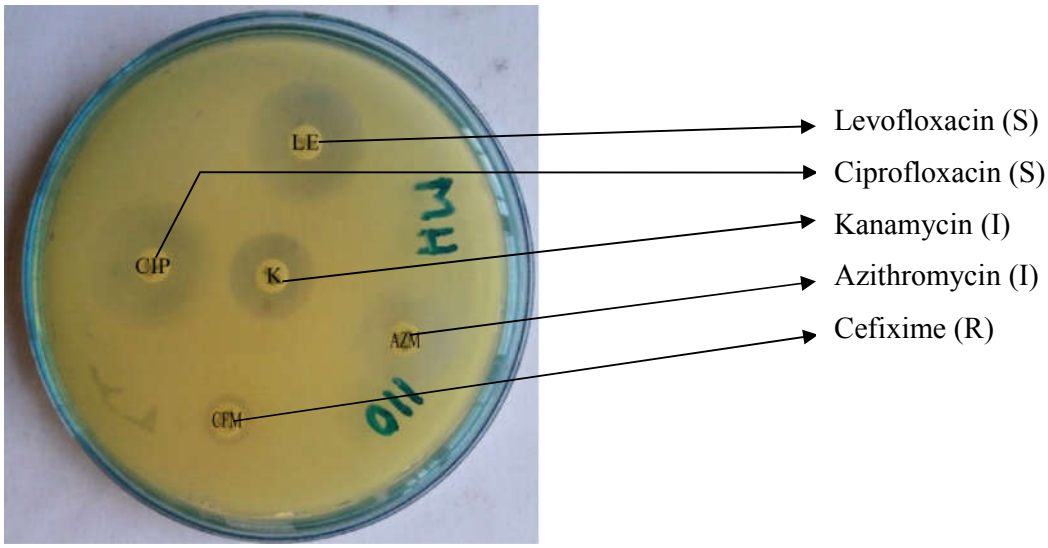

Plate 46 (B): Antibiogram profile of *Staphylococcus* spp.

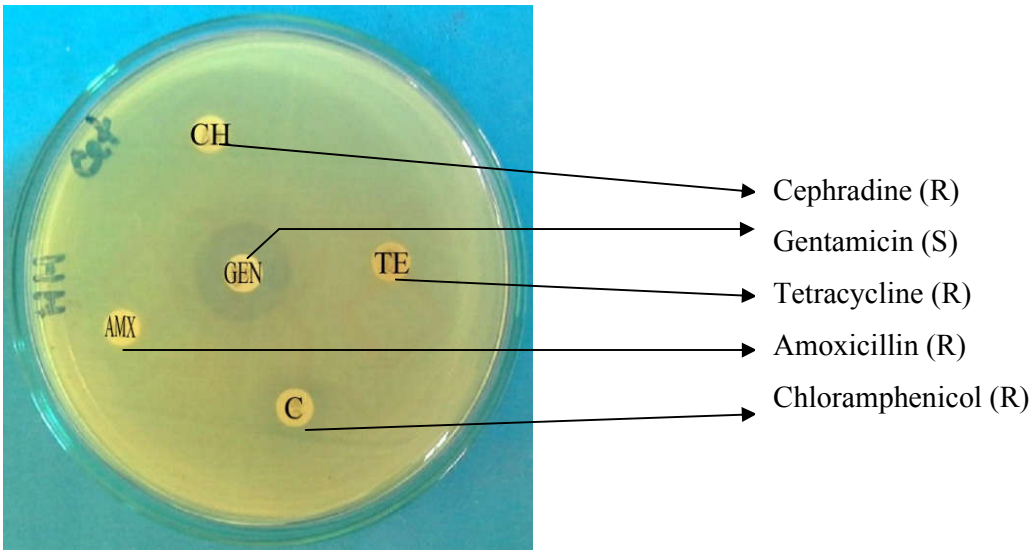

Plate 47 (A): Antibiogram profile of *Pseudomonas* spp.

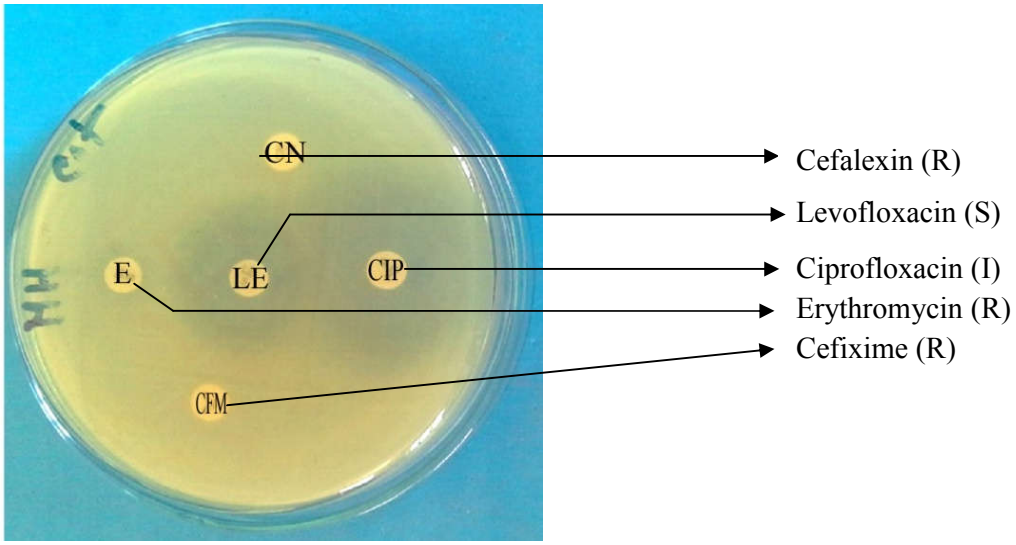

Plate 47 (B): Antibiogram profile of *Pseudomonas* spp.

4.2.5 Result of PCR Amplification, Sequencing of 16S rRNA Genes with Universal Primers and Phylogenetic Analysis of *Aeromonas hydrophila*

Aeromonas hydrophila was present in dead-in-shell chicks.16S rRNA gene region was amplified with the universal primers, Forward primer-27F (5'AGAGTTTGATCCTGGCT CAG3') Reverse primer- 1492R (5' TACCTTGTTACGACTT 3'). PCR Amplification band was found at 1414bp.

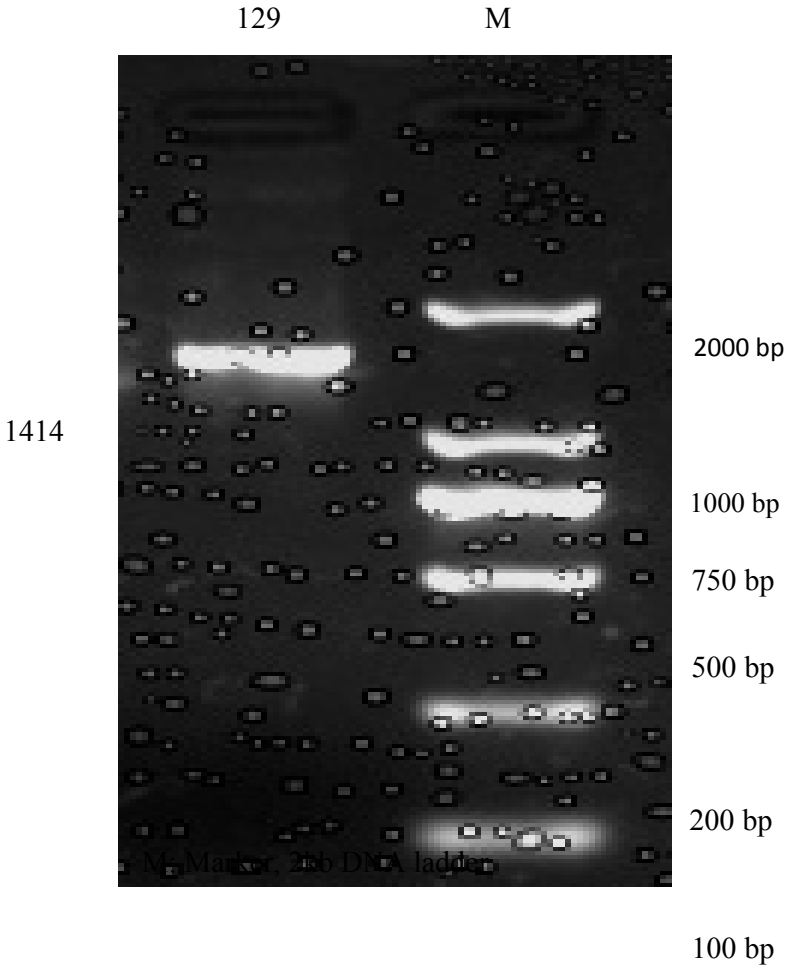

Fig 8: Result of amplification of 16S rRNA gene region of *Aeromonas hydrophila* by PCR.

Note: PCR= Polymerase Chain Reaction, kb= kilo base.

4.2.5.1 Electropherogram

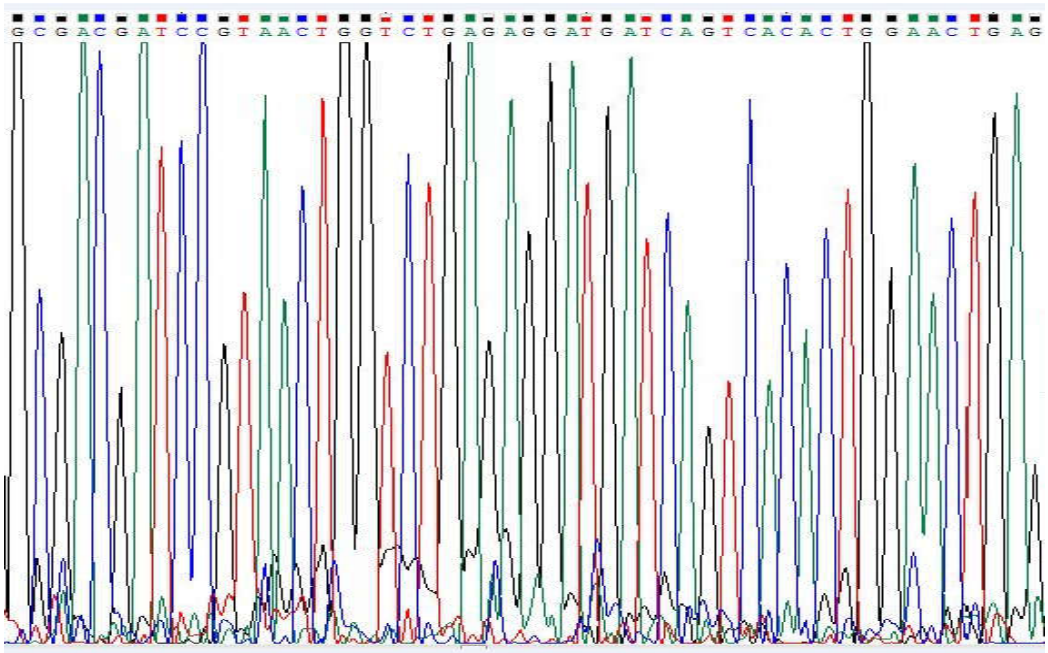

Fig 9: Electropherogram of 16s rRNA Gene Sequence of *Aeromonas hydrophila*

4.2.5.2 Phylogenic tree analysis of *Aeromonas hydrophila*

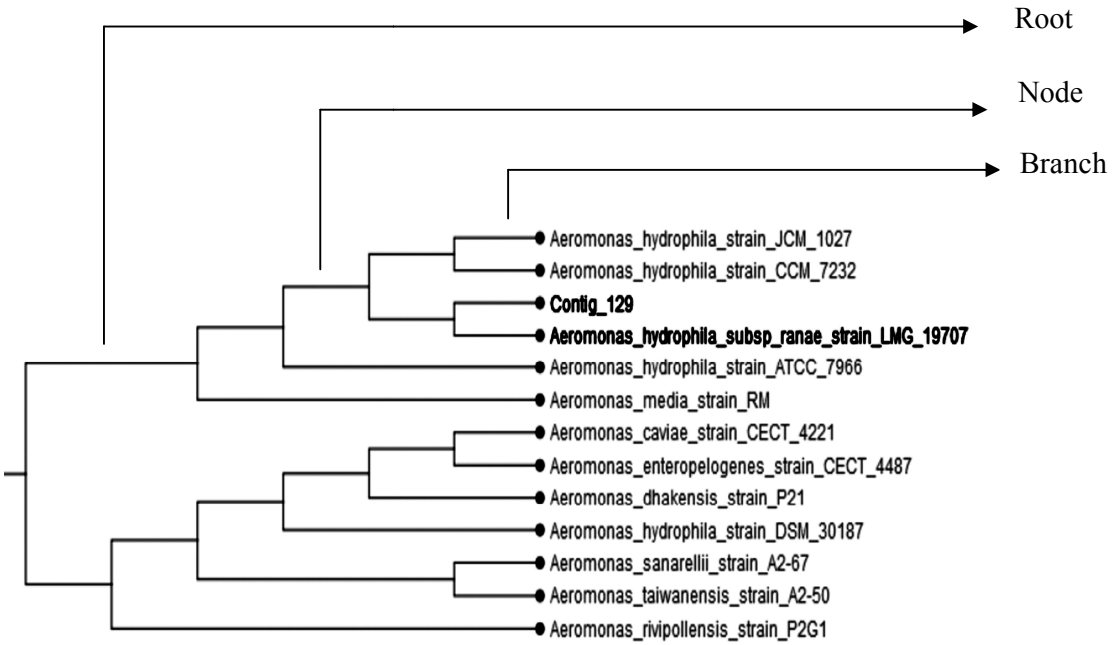

Fig 10: Phylogenic tree analysis of *Aeromonas hydrophila*

4.2.5.3 Contig Sequence of *Aeromonas hydrophila*

Contig129: 1414 bp, Blast: 98% similar

Identified strain: *Aeromonas hydrophila subsp ranae* strain LMG 19707

GCGGCTACTGTAATTAGCTTGCTCCTTTTGCCGGTTATCGGCGGACGGGTGAGTA
ATGCCTGGAAAATTGCCCAGTCGAGGGGTATAACCTGTGGAAACGACTGCTAAT
ACCGCATACGCCCTACGGGGGAAAGCGGGGGACCTTCGGGCCTTGCGCGATTGG
ATATGCCCAGGGGGGGATTATCTAGTTGGGGAGGTAATGGCTCACCAGGGCGACT
ATCCCTATGTGGTCTGAGAGGAAGATCACCCACTCTGGAACTGACACACGGCCC
ACACTCCTACGGGAGGCAGCAGGGGGAAATATTGCACAGTGGGGGAAACCCTGA
TGCACCCGTGCCGCGTGTGAGAAAAAGGCCTTCGGGTTGAAAAGCTTTTTCAGCG
AGGAGGAAAGGTTGATGCCTAATACTTATCAACTGTGACTTTCCTCGCAAAAAAA
GCCCCGGATAACTCCGTGCCAGCCCCCGCGGTAATACAGAGGGTGCAAGCGTTA
ATCGAAATTACTGGGCGTAAAGCGCACGCGGGCGGTTGGATAAGTTAGATGTGA
AACCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGT
AGAGGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGA
ATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTGACGCTCAGGTGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTC
GATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGA
CCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCT
GGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGGAGTGCCTTCGGGAATCAG
AACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAATGTTGGGTTAAGTC
CCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTCA
AGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCA
TGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGCGCGTACAGAGGGAAG
CAACCTAGCGATAGCAAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGATCGGAG
TCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGTT
GCGGTGAATACGTTCCCGGGCCTTGTACACACCAACCGTCACACCATGGGAGTG
GGTGCACCAGAAGTAGATAGCTTAACCTTCGGGAGGGCGTTACCCGTAGAGATG
CC

CHAPTER 5

DISCUSSION

Hatchery industry is considered as one of the major steps in poultry production cycles. Good sanitation and low bacterial contamination play an important role in lowering hatchability and decreasing the performance of hatched chicks. The present research work was conducted for the isolation and molecular identification of the bacterial flora causing dead-in-shell chicks. Routine bacteriological methods were performed for the isolation and identification of bacteria. Antibiotic sensitivity of the isolated bacteria against commonly used antibiotics was also performed.

In these study five (5) different genera of bacteria isolated and identified were *E. coli, Salmonella* spp.*, Staphylococcus* spp., *Pseudomonas* spp. and *Aeromonas hydrophila.* The results of isolation are in agreement with the findings of Al-Aboudi *et al.,* (1992), Nazer *et al*., (1994), Sharada *et al.,* (1999), Al-Sadi *et al.,* (2000), Ciocîrlan (2008) and Azmy (2010), Lin *et al*., (1996).

The result of bacterial isolation from dead-in-shell chicks revealed that the recovered *E. coli* was percentage of 32.2% which agreed with Lin *et al*., (1996) who recorded its percentage 29.8% but different from Raji *et al*., (2007) who reported it at 4.67% and 7.5% from Simtu farm and NAPRI respectively and it was also different from Rezk (2010) who reported it at 10.8%.On the other hand, the recovered *E. coli* percentage were lower than the result obtained by Sharada *et al.,* (1999) who recorded its percentage 52.54%. The variation in the percentage of *E. coli* isolates may be partly related to the prophylactic and therapeutic usage of certain antibiotic, vaccination against respiratory viruses and improved hatchery sanitation. In this study, different selective and differential media were used for isolation of *E. coli* from dead-in-shell chicks egg samples. Colony characteristics of *E. coli* observed in NA, EMB, and SS agar were similar to the findings of Nazir *et al.*, (2005) and Buxton and Fraser (1977). Morphologically *E. coli* were Gram negative short rod arranged in single or paired and motile. This characteristics of *E. coli* is also similar to that described by Jones *et al.,* (2005), Freeman (1985), Buxton and Fraser (1977). The identified bacteria were re-confirmed through the use of different sugar fermentation and other biochemical tests which were found similar with the findings of Sandhu *et al.,* (1989).

With regard to isolation of *Salmonella* spp. its percentage was 34.5% which was nearly similar with the result reported by Sharada *et al.,* (1999), who recorded a percentage of 30.5% but higher than the result obtained from Abd El-Galil *et al.,* (1994) who recorded it in 6%. However the result of the present study were in complete agreement with Calnek *et al*., (1997) who described coliforms and *Salmonella* spp. to be the major contaminating bacteria of hatching eggs.

According to bacterial isolation, Enterobacteriaceae represent 67 % which is nearly agreed with Kabilika *et al*., (1999), who recorded that Enterobacteriaceae represented for over 50% from samples. This result is higher than those reported by Nashed (1992) who recorded it in a percentage 38.2%. Cultural media and biochemical tests used in this experiment for the characterization of *Salmonella* spp. were also employed by a number of workers such as Khan *et al.,* (2005). In this study, the colonies of *Salmonella* spp. on SS agar plate were opaque, translucent with black centers, which were similar to the findings of Hossain (2002), Cheesbrough (1984). In Gram's staining bacteria exhibited short rods, Gram negative, single or paired in arrangement. Similar findings were also reported by Samad (2000) and Freeman (1985). In this study, *Salmonella* spp. isolated from dead-in-shell chicks were motile in MIU medium. Li *et al.,* (2013), Freeman (1985), Buxton and Fraser, (1977).

The results revealed prevalence of *Staphylococcus* spp. in a percentage of 18.9% which is more or less similar with the result recorded by Kabilika *et al.,* (1999), who mentioned that its percentage was 14.5%.The *Staphylococcus* spp. were Gram-positive cocci arranged in grape like cluster was similar to Freeman (1985), Buxton and Fraser, (1977). *Staphylococcus* spp. reveled positive reaction in MR test negative reaction in Indole test and variable reaction in VP test which was supported by Buxton and Fraser, (1977).

The results revealed prevalence of *Pseudomonas* spp. in a percentage of 12.2% which is similar with the results obtained by Rezk (2010) and Azmy *et al.,* (2010) who recorded it as 15.9% and 20.0% respectively. Morphology and staining characteristics of *Pseudomonas* spp. recorded in this study are in agreement with the finding of Buxton and Fraser, (1977). The isolates also revealed positive reaction in VP test and MR test, negative reaction in Indole test which was supported by Buxton and Fraser, (1977).

The results revealed prevalence of *Aeromonas hydrophila* in a percentage of 2.2% which is similar with the results obtained by Lin *et al.,* (1996). Morphology and staining characteristics is more or less similar with the family of Enteriobacteriaceae.

In this study, it was found that *E. coli* isolated from dead-in-shell chicks were only sensitive to Levofloxacin and resistant to Azithromycin, Erythromycin, Clindamycin and Amoxicillin. This result is not agreed with the result of Azmy (2010) who recorded that *E. coli* isolates were highly sensitive to ciprofloxacin. The *Salmonella* spp. isolated in the present study was found highly sensitive to Ciprofloxacin and Gentamicin which agreed with the result of Khan *et al.,* (2005) who also found *Salmonella* spp. highly sensitive to Ciprofloxacin. In the present study *Staphylococcus* spp. isolates of dead-in-shell chicks showed sensitivity to Ciprofloxacin, Gentamicin, Chloramphenicol and Levofloxacin and resistant to Penicillin, Amoxicillin and Cefixime. This finding of antibiotic sensitivity assays are somewhat in agreement with the findings of Farzana *et al.,* (2004).In present study, antimicrobial sensitivity assay of *Pseudomonas* spp. was found sensitive to Gentamicin and intermediately sensitive to Ciprofloxacin. Almost similar antibiogram profiles were also recorded by Amer *et al.,* (2017). In the present study *Aeromonas hydrophila* was found sensitive to Ciprofloxacin and Levofloxacin, intermediately sensitive to Azithromycin and resistant to Penicillin, Erythromycin, Ampicillin, Gentamicin, Tetracycline and Kanamycin.

The variation in antibiogram profile might be due to indiscriminate use of antibiotic for treatment purposes in birds which results in enzymatic degradation, mutation at binding sites, down regulation of outer membrane proteins, efflux pumps and transduction of genes in bacterial isolates. Antibiotics are extensively used as growth promoters in poultry feed or to control infectious disease. Anti-microbial exercise and/or especially abuse are considered to be the most vital selecting force to antimicrobial resistance of bacteria (Moreno *et al.,* 2000). Due to enormous exploitation of antibiotics in the field of veterinary medicine, an increased number of resistant bacterial strains were developed in recent years. The bacterial resistance against antimicrobial agents is known to be driven by the interplay of several mechanistic and epidemiologic factors including the chromosomal defects, random mutation, plasmid exchange, and by the transfer of drug resistance genes by integron or transposon (Canton 2008). Occupational exposure to antimicrobial resistant *E. coli* from live bird contact in the broiler chicken industry could be another important route of entry for antimicrobial resistant *E. coli* into the human population (Ojeniyi 1989, Levy *et al.,* 1976). There was evidence of human exposure to 3rd generation Cephalosporin resistant *E. coli* through consumption of broiler meat in Belgium (Depoorter *et al.,* 2012). To reduce the emergence and spread of antibiotic resistance in Bangladesh, over-the counter sales of antibiotics should be controlled

and veterinarians should authorize all antibiotics for animal and aquaculture use. Illegal and non-prescription based selling of antibiotics should be stopped in human medicine.

The recent study was conducted for molecular characterization of isolated *Aeromonas hydrophila* by Polymerase chain reaction using 16S rRNA. The identified organism is *Aeromonas hydrophila* subsp ranae strain LMG 19707.

In relation to the present study, further investigation might be performed on the following aspects;

- Genome analysis to have an idea about the genes responsible for pathogenicity and multidrug resistancy of bacterial isolates of dead-in-shell chicks.
- Further detailed study on various extrinsic and intrinsic factors, which might have direct or indirect influence on the development of dead-in-shell chicks in association with microbes that are present.
- Comparison of pathogenic characteristics and antigenic properties of the isolated organisms.

CHAPTER 6
CONCLUSION

Bacterial agents play an important role in the hatcheries by decreasing the rate of hatchability and affecting the health of newly hatched chicks and their future performance. This is due to the ignorance with hygienic and therapeutic measures. Egg shell contamination leads to bacterial penetration through the shell and shell membrane and subsequently infects the developing embryo. The results collected during this study designated that dead-in-shell-chicks are susceptible to a number of bacterial agents which are common to other poultry species. The number of bacteria isolated from dead-in-shell chicks were *E. coli* 29 (32.2%), *Salmonella* spp. 31 (34.5%), *Staphylococcus* spp. 17 (18.9%), *Pseudomonas* spp. 11 (12.2%) and *Aeromonas hydrophila* 2 (2.2%). The highest number of bacteria were recovered from lungs (44) followed by yolk (25) and intestine (21). It is important to carry out bacteriological examination in breeder flocks to investigate the bacterial affections with estimation of the changes in their similar to the used antibiotics. Antibiotic resistance of isolated organisms are considered as an important problem. Multidrug resistant bacteria isolated from samples of dead-in-shell chicks might have resulted from indiscriminate use of antibiotics for treatment.

Concluding remarks;

Based on the results of the study it may be concluded that

- *E. coli* and *Salmonella* spp. infection play an important role in producing dead-in-shell chicks.
- Other microorganisms like *Staphylococcus* spp., *Pseudomonas* spp. and *Aeromonas hydrophila* also increased the severity of the cases.
- Multidrug resistant bacteria isolated from samples of dead-in-shell chicks might have resulted from indiscriminate use of antibiotics for treatment.

CHAPTER 7

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APPENDICES

APPENDIX 1

Composition of Media

1. Nutrient broth

Ingredients per litter of deionized water	**g/L**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at25°C)	7.4±0.2

2. Nutrient agar

Ingredients per litter of deionized water	**g/L**
Beef extract	3.0
Peptone	5.0
Sodium chloride	5.0
Agar	20.0
Final pH	7.1±0.1

3. Salmonella Shigella agar

Ingredients per litter of deionized water	
Peptic digest of animal tissue	5.00 gm
Beef extract	5.00 gm
lactose	10.00 gm
Bile salts mixture	8.50 gm
Sodium citrate	10.00 gm
Sodium thiosulphate	8.50 gm
Ferric citrate	1.00 gm
Brilliant green	0.00033 gm
Neutral red	0.025 gm
Agar	15.00 gm
Distilled water	1000 ml
Final pH(at25°C)	7.0±0.2 gm

4. MacConkey Agar

Ingredients	**g/L**
peptone	17.0
Protease peptone	3.0
Lactose	10
Bile salt	1.5
Sodium cholride	5.0
Agar	13.5
Neutral Red	0.03
Crystal violet	0.001
Final pH	7.1±0.2

5. Eosine methylene blue agar

Ingredients	**g/L**
Peptone	100
Lactose	10.0
$K_2 HP0_4$	2.0
Eosin	0.4
Methylene blue	0.065
Agar	20.0
Final pH	6.8±0.2

6. MR VP medium (Himedium, India)

Composition	
Buffered peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Final pH(at 25°CO	6.9±0.2

7. Simmon's citrare agar

Ingredients	**g/L**
Magnessium sulphate	0.20
Ammunium dihydrogen phosphate	1.0

	Dipotassium phosphate	1.0
	Sodium citrate	2.0
	Sodium chloride	5.0
	Bromothvmol blue	0.08
	Agar	15.0
8.	**TSI Agar slant**	
	Ingredients Lab Lamco Powder	3.00 gm
	Yeast extract	3.00 gm
	Peptone	20.00 gm
	Sodium chloride	5.00 gm
	Lactose	10.00 gm
	Sucrose	10.00 gm
	Glucose	1.00 gm
	Ferric citrate	0.3 gm
	Sodium thiosulphate	0.3 gm
	Phenol red	0.3 gm
	Agar	12.00 gm
	Distilled water	1000 ml
9.	**Mueller Hinton Agar**	**Gram/Li**
	Beef infusion	2.0
	Bactocasamino acid (techinal)	17.5
	Starch	1.5
	Bacto agar	17.5
	Distilled water	1000 ml
	P^H	7.3
	Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.	
10.	**Mannitol Salt Agar**	**Gram/Li**
	Protease peptone	10.0
	Beef extract	1.0
	D-Mannitol	10.0

NaCl	75.0
Phenol red	0.025
Agar	20
Distilled water	1000ml

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes.

11.	**Normal Saline**	**Gram/Li**
	NaCl	0.85
	Distilled water	1000ml

Autoclaved at 121^0C for 15 minutes.

APPENDIX 2

Preparation of reagents

1. Peptone water

peptone	1 gm
Distilled water	1000 ml

2. Kovacs reagent for indole preparation

P- dimethyl aminobenzal dehyde	5 gm
Amyl alcohol	75 gm
Conc. HCL	25 ml

3. V-P reagent-1

5% alpha- naphthanol in absolute ethyl alcohol

4. V-P reagent-2

40% potassium hydroxide containing 0.3% creatine. The ingredient was dissolved by heating gently over a steam bath. When in solution, added 0.052 gm of cotton blue dye.

5. Methyl red Solution

Methyl red	0.05 gm
Ethanol(absolute)	28 ml
Distilled water	22 ml

6. Phenol red solution

0.2^0o aqueous solution of phenol red

7. Gram stain solutions

a. **Stock crystal violet**

crystal violet	10 gm
Ethy1 alcohol	1000m1

b. Stock oxalate

Ammonium oxalate	1 gm
Distilled water	1000 ml

Crystal violet working solution: 20 ml of solution no. I mixed with 80 ml of solution no. 2. Additional dilution was made when desired.

c. Lugol's Iodine solution

Iodine crystal	1 gm
Potassium iodide	2gm

Dissolved completely in 10 ml of distilled water, then added to distilled water to make 300 ml. stored in ambar bottle.

d.	**Ethyl alcohol**	250 ml
e.	**Acetone**	250 ml
f.	**Counterstain**	2.5 ml
	Safranine	
	Ethyl alcohol (95%)	100 ml

Safranine working solution:

The stock safranine is usually diluted as 1:4 with distilled water.