Prevalence of *Salmonella* spp. and Shiga-Toxin Producing *Escherichia coli* (STEC) among Living Poultry in Tulkarm by Culture and PCR.

By

Hind Jamal Abdo

Supervisor

Dr. Ahmad Saleh

This Thesis was Submitted in Partial Fulfillment of the Requirements for the Master's Degree of Science in:

Agricultural Biotechnology

Deanship of Graduate Studies

Palestinian Technical University- Kadoorie

January, 2019

انتشار بكتيريا السالمونيلا و بكتيريا القولون المنتجة لسموم الشيجا في الدجاج اللاحم الحي في محافظة طولكرم عن طريق استخدام تقنيات الزراعة التقليدية و تفاعل البلمرة المتسلسل

الطالبة:

هند جمال عبدو

المشرف:

الدكتور أحمد صالح

قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في:

التكنولوجيا الحيوية الزراعية

عمادة الدراسات العليا

جامعة فلسطين التقنية–خضوري

كانون الثاني، ٢٠١٩

Committee Decision

This thesis / dissertation entitled: (Prevalence of *Salmonella* spp. and Shiga-Toxin Producing *E. coli* (STEC) among Living Poultry in Tulkarm by Culture and PCR) was successfully defended and approved on 06/Jan./2019.

Examination Committee **Signature**

- Dr. Ahmad Saleh, (Supervisor) _____

Assistant Professor / Faculty of Applied Sciences, Palestine Technical University-Kadoorie (PTUK).

- Dr. Ghaleb Adwan, (External Examiner) _____

Professor / Faculty of Science, An-Najah National University.

- Dr. Eyad Badran, (Internal Examiner) _____

Assistant Professor / Faculty of Agricultural Sciences and Technology, Palestine Technical University-Kadoorie (PTUK).

خضوري -جامعة فلسطين التقنية

نموذج التفويض

- أنا هند جمال محمد عبدو ، أفوض جامعة فلسطين التقنية خضوري بتزويد نسخ من - رسالتي / أطروحتي للمكتبات والمؤسسات أو الهيئات أو الأشخاص عند طلبهم حسب التعليمات النافذة في الجامعة.

التوقيع:

التاريخ: 6/Jan./2019

Palestine Technical University-Kadoorie

(PTUK)

Authorization form

I, Hind Jamal Mohammad Abdo, authorize the PTUK to supply copies of my thesis / dissertation to libraries or establishments or individuals on request, according to the university of PTUK regulations.

Signature: _____

Date: 6/Jan./2019

Prevalence of *Salmonella* spp. and Shiga-Toxin Producing *Escherichia coli* (STEC) among Living Poultry in Tulkarm by Culture and PCR.

By:

Hind Jamal Abdo

Supervised by:

Dr. Ahmad Saleh

قدمت هذه الرسالة استكمالا لمتطلبات الحصول على درجة الماجستير في: التكنولوجيا الحيوية الزراعية.

This thesis was submitted in partial fulfillment of the requirements for the master degree in: Agricultural Biotechnology.

Deanship of Graduate Studies عمادة الدراسات العليا

PTUK جامعة فلسطين التقنية –خضوري

January/ 2019 كانون الثاني/ ٢٠١٩

Prevalence of *Salmonella* spp. and Shiga-Toxin Producing *Escherichia coli* (STEC) among Living Poultry in Tulkarm by Culture and PCR.

Dedication

I would like to dedicate this thesis to my parents, sisters, brothers, husband and friends.

Acknowledgment

I would like to sincerely thank my supervisor Dr. Ahmad Saleh for his continuous support, constructive comments, indispensable guidance throughout this work, guidance that he has given me throughout the research and writing this thesis and specially for his confidence in me.

I would like to thank my parents for their love and support throughout my life. I thank you both for giving me strength to reach my dreams.

I would like to thank my sisters, my brothers and my husband for their support and standing by me.

I would like to express my appreciation to the team of Biotechnology Research Center at Palestine Technical University and to Dr. Abdul Rahman Issa for helping in samples collection.

Finally, I would like to express my sincere thanks to all the people who directly or indirectly have contributed to this work.

List of Contents

List of Tables

List of Figures

List of Diagrams

List of Abbreviations

S	Second
Min	Minute
0 C	Degree Celsius
EMB	Eosin methyline blue
%	Percentage
EDTA	Ethylene diamine tetra acetic acid
BPW	Buffered peptone water
Rpm	Revolution per minute
PCR	Polymerase chain reaction
SBG	Selenite brilliant green
NaCl	Sodium chloride
Ml	Millilitre
XLD	Xylose lysine deoxychockolate
G	Gram
NB	Nutrient broth
DNA	Deoxyribonucleic acid
E.coli	*Escherichia coli*
Stx	Shiga toxin
Bp	Base pair
STEC	Shiga toxin producing *Escherichia coli*
mM	Millimolar
GE	Gel electrophoresis
TAE	Tris acetate EDTA
UV	Ultra violet
w/v	Weight per volume
V	Voltage
x *g*	Times gravity
Aw	Water activity
PH	Potenz hydrogen
/	Per
µl	Microlitre
NaOCl	Sodium hypochloride
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
EHEC	Enterohemorrhagic *E. coli*
VTEC	Verocytotoxigenic *E. coli*
EIEC	Enteroinvasive *E. coli*
EAggEC	Enteroaggregative *E. coli*
DAEC	Diffuse-adherent *E. coli*
FAO	Food and Agriculture Organization

Prevalence of *Salmonella* spp. and Shiga-Toxin Producing *Escherichia coli* (STEC) among Living Poultry in Tulkarm by Culture and PCR.

By: Hind Abdo

Supervised by: Dr. Ahmad Saleh

Abstract

Salmonella and Shiga-toxin producing *Escherichia coli* (STEC) causing avian salmonellosis and colibacillosis are considered to be the major bacterial diseases in the poultry industry world-wide, causing heavy economic losses through mortality and reduced production. Colibacillosis and salmonellosis are the most common avian diseases that are communicable to humans. The morbidity and mortality associated with several recent outbreaks due to *Salmonella* and STEC have highlighted the threat that these organism poses to public health. This study aimed to investigate the prevalence of *Salmonella* and STEC in living broiler chicken by using cultural and molecular techniques. Two hundred cloacal samples were collected during the period of April 2017 to November 2017 from four different broiler chicken farms in Tulkarm district, Palestine. *Salmonella* and STEC isolates were identified by chromogenic culture media and PCR targeting pathogen-specific genes (*invA, stx1 and stx2*). Results of this study showed that the overall prevalence of *Salmonella* and STEC by chormogenic

culture media was 14% (28/200) and 47% (94/200) using HiCrome *Salmonella* improved agar and HiCrome EC O157:H7 agar, respectively; while it was found to be 12.5% (25/200) and 6.5% (13/200) by using *invA*-PCR and *stx1*-PCR, respectively. However, infection levels were found to be different among the four investigated farms. Moreover, concurrent infections with both pathogens was found be very low. Thus we conclude that infection with *Salmonella* and STEC among broiler chicken in Palestine is high by using either cultural or PCR techniques, however, it is important for accurate detection of both *Salmonella* and STEC to combine both of them. Such high prevalence of *Salmonella* and STEC poses high risk to the public health as well as for the poultry farming sector.

Key words: *Salmonella*, Shiga toxin producing *E. coli* (STEC), PCR, *InvA*, *Stx1*, *Stx2*, Poultry, Chromogenic media.

Chapter One

Introduction

1.1 General backgroun

Poultry farming is the raising of domesticated birds such as chickens, ducks, turkeys and geese for the purpose of farming meat or eggs for food. Poultry are farmed in great numbers with chickens being the most numerous. More than 50 billion chickens are raised annually in the world as a source of food, for both; their meat and their eggs (Compassion in World Farming – Poultry, n.d). Chickens raised for eggs are usually called layers while chickens raised for meat are often called broilers (Compassion in World Farming – Poultry, n.d). Globally, poultry meat output is expected to rise by more than 1 percent to a record high of 116.2 million tons. Asia is easily the leading producing region with North America and South America competing closely for second place (Global Poultry Trends: Poultry to Boost Global Meat Market Share Above 36 Per Cent, 2016). By 2024 an OECD/FAO report (OECD-FAO Agricultural Outlook 2015-2024) forecasts poultry meat consumption to almost 133 million tons, compared with 111 million tons in 2015. Uptake in developed countries in 2015 is put at 43.6 million tons with 67.5 million tons being consumed in developing nations (Global Poultry

Trends - Developing Countries Main Drivers in Chicken Consumption, 2016).

There are many problems facing the poultry farming sector worldwide. Locally in Palestine, the main problems that face the poultry are; lack of marketing channels, the poor planning, the control of occupation on feed, species, chicken breeds, meat prices, massive production of broiler chicks and eggs in the settlements, lack of experience which should be available in the farm, the scarcity of possibilities, and infectious diseases resulting from bacterial, viral and parasitic pathogens which causes high morbidity, mortality, and production losses.

Different bacterial pathogens causing serious diseases to poultry and humans are identified; the most significant of them, among others, are *Salmonella* spp. and pathogenic *Escherichia coli*.

Poultry and poultry products have been implicated as a major source of *Salmonella* infection in human (Amavisit, Browning, Lightfood, & Anderson, 2001). *Salmonella* spp. are the most important bacterial pathogens of poultry in the world causing an important economic loss in poultry rearing and food industries. It has been reported that one of the most frequent cause of human infections by *Salmonella* species is due to uncooked poultry

meat, in addition to mishandling of poultry products and raw poultry carcasses, (Panisello, Rooney, quantick, & Stanwell-Smith, 2000).

Salmonella is a genus of gram-negative bacteria, rod-shaped (bacillus) that belongs to the Enterobacteriaceae family (Bennasar, Luna, Cabrer, & Lalucat, 2000; Grimont, P., Grimont, F & Bouvet, 2000). *Salmonella* is characterized as ubiquitous, intracellular, straight rod shaped, nonencapsulated, facultative, non-spore forming, and generally motile with peritrichous flagella (Gray & Fedorka-Cray, 2002; Kwang, Littledike, & Keen, 1996). *Salmonella* has a width of 0.7 to 1.5 μm and a length of 2.0 to 5.0 μm (Holt, Krieg, Sneath, Stanley, & William, 1994). *Salmonella spp*. are typically found in different environments like soil, water, food, and the gastrointestinal tract of humans and other animals mainly poultry (Anderson & Ziprin, 2001). Most *Salmonella* are motile, with the exception of the poultry-specific serotypes of *S. gallinarium* and *S.pullorum* (Grimont *et al.*, 2000). *Salmonella* temperature growth range lies between 8 to 45 ºC (Hanes, 2003), but the optimum temperature is 37 ºC. Typically, *Salmonella* can grow within a range of pH from 4.5 to 9.0 (D'Aoust, 1989); however, the most favorable pH for growth is between 6.5 to 7.5 (Garcia-Del Portillo, 1999), *Salmonella* is tolerant to high moisture and grows best in conditions with a water activity (*a*w) of 0.93 (Gray & Fedorka-Cray, 2002;).

Salmonella grows optimally when sodium chloride (NaCl) range is between 3 to 4% and 350 mg/L of sodium nitrite (NaNO2) (Portillo, 2000).

Salmonella is classified into two species: *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* is further divided into six subspecies that include over 2500 serovars (Su & Chiu, 2007). *Salmonella* spp. are important zoonotic pathogens which cause significant morbidity, mortality, and economic losses (Chiu *et al.,* 2010, Sanchez, Hofacre, Lee, Maurer, & Doyle, 2002). Consumption of contaminated poultry products such as eggs and meats cause human infections (Foley *et al.,* 2011). It is estimated that about 94 million cases of gastroenteritis due to *Salmonella* species occur annually worldwide, leading to 155,000 deaths every year (Majowicz *et al.*, 2010). Strains of *Salmonella* cause health problems such as food poisoning (salmonellosis), typhoid fever, and paratyphoid fever (Ryan & Ray, 2004). Contaminated food products derived from beef, pork, poultry and eggs are the main reasons for approximately 75% of human *Salmonella* infection cases (Hald, Vose, Wegener, & Koupeev, 2004). Poultry often become infected through the consumption of contaminated feed, cross-contamination in breeding houses, or during slaughter and processing (Doyle & Erickson, 2006; Fratamico, 2003). With increasing regulatory pressure placed on poultry and livestock processors to

reduce pathogen contamination in processed meats, more emphasis is likely to be focused on reducing pathogen contamination on farms (Rasschaert *et al.*, 2008). Therefore, development of a rapid and sensitive method to detect *Salmonella* spp. and their serovars is important. Several techniques for improving the detection of *Salmonella* serovars in fecal material such as the use of a selective culture medium and enzyme-linked immunosorbent assay (ELISA) have been developed (Araj & Chugh, 1987; Aspinall, Hindle, & Hutchinson, 1992). However, problems remain with sensitivity and specificity that have limited routine use of these procedures. In general, these methods are laborious and time-consuming, in contrast with molecular methods that reduce the time of diagnosis with the same and even higher efficiency (Aabo, Rasmussen, Rossen, Sørensen, & Olsen, 1993; Malorny, Huehn, Dieckmann, Kramer, & Helmuth, 2009).

PCR methods are a powerful tool in microbiological diagnostics for *in vitro* amplification of desired DNA sequence (Malorny, Hoorfar, Bunge, & Helmuth, 2003). Several genes have been used to detect *Salmonella* in natural environmental samples as well as food and faecal samples. *InvA*, *invE*, *himA*, *phoP* are virulence chromosomal genes which are target genes for PCR amplification of *Salmonella* species (Jamshidi, Bassami, & Afshari-Nic, 2009). The *invA* gene of *Salmonella* contains sequences

unique to this genus and has been proved as a suitable PCR target, with potential diagnostic applications (Rahn, De Grandis, Clarke, Curtiss, & Gyles, 1992).

E. coli is a bacterium that is classified as a gram-negative, rod-shaped, flagellated, non sporulating, and facultative anaerobic bacterium that belongs to Enterobacteriaceae family (Holko, Bisova, Holkova, & Kmet, 2006). Over the last half-century it has become increasingly obvious that there are a number of different enteropathogenic groups of *E. coli.* At least six known pathotypes associated with gastrointestinal infections have been recognized, apart from those opportunistic "nonpathogenic strains" causing urinary tract infections, septicemia, and meningitis in humans and a number of similar diseases in animals. The pathotypes associated with gastrointestinal infections currently recognized are: Enteropathogenic *E. coli* (EPEC), Enterotoxigenic *E. coli* (ETEC), Enterohemorrhagic *E. coli* (EHEC) which are a subgroup of Verocytotoxigenic *E. coli* (VTEC) or Shiga toxin-producing *E. coli* (STEC), Enteroinvasive *E. coli* (EIEC), Enteroaggregative *E. coli* (EAggEC), and Diffuse-adherent *E. coli* (DAEC) **(**Nataro & Kaper., 1998).

Each of these pathotypes has unique features in their interaction with eukaryotic cells (Bélanger, Garenaux, & Harel, 2011). Among these avian

pathogenic *Escherichia coli* (APEC), some include shiga toxin-producing *E. coli* (STEC) which is also known as verotoxin producing E. coli (VTEC) causing disease in humans and animals.

Production of shiga toxins (Stx) is the common features of STEC that are considered to be the major virulence factors. The pathogenicity of these bacteria is mainly mediated by shiga toxins (Stx1, Stx2 and their variants) encoded by *stx1* and *stx2* genes and the products of the locus of enterocyte effacement (*LEE*), the pathogenicity island, with the *eaeA* gene that encodes for the intimin protein involved in the intimate adhesion of bacteria to enterocytes and production of attaching and effacing (AE) lesion (Paton & Paton 1998). The bacteria *Shigella dysenteriae* and the STEC are the most common sources for shiga toxins (Beutin, 2006). STEC are becoming an ever increasing problem as an etiological agent of food-borne gastrointestinal disease. The stx is characterized by the cytotoxicity due to disruption of protein synthesis within host cells. Shiga toxin-producing *E. coli,* is the most important foodborne pathogen which is the causal agent of mild diarrhea, bloody diarrhea, hemorrhagic colitis, thrombotic thrombocytic purpura, hemolytic-uremic syndrome (HUS) in human (Karmali, Petric, Lim, Cheung, & Arbus, 1985; O'Brien & Holmes, 1987;

Griffin *et al*,.1988; Smith & Scotland, 1988; Karmali, 1989; Kovacs *et al*,. 1990).

Transmission of STEC occurres through different routes, including faecal material contaminated water or food, person-to-person contact, and animal-to-person contact (Caprioli, Morabito, Brugere, & Oswald, 2005; Paton & Paton, 2002). Most human infections are caused by consumption of contaminated foods including poultry products (Erickson & Doyle. 2007).

Although most sporadic cases and outbreaks have been reported from developed countries, human infections also have been described in Latin America, India and other developing countries (Kaddu-Mulindw, Aisu, Gleier, Zimmermann, & Beutin, 2001; Leelaporn *et al*., 2003). Several works have been done regarding the isolation and molecular characterization of shiga toxin producing *E. coli* from the intestinal contents and meat of cattle, diarrheic human patients, the environment by several researchers (Hossain, Sultana, Deb, & Ahmed 2011; Islam *et al*., 2008; Alam *et al*., 2006).

To the best of our knowledge, no work has yet been conducted for the isolation and molecular characterization of *Salmonella* spp. and STEC from broiler birds in Palestine.

Significance of this study emerged from the limited studies conducted in Palestine to determine the burden of these bacterial pathogens on poultry farming sector. Available statistics and reports are basically based on arbitrary veterinarian and farmers observations which hardly includes dissection of infected/dead birds or via the use of very basic standard microbiological identification techniques. Based on scarcity of data and lack of application of modern techniques for estimating the prevalence of different poultry-pathogens in Palestine, this study came to unravel the prevalence of selected bacterial pathogens in poultry, among them are possibly human pathogens, using PCR technique. Two bacterial pathogens: *Salmonella* and STEC were selected for investigation. Based on huge economic losses in poultry farming and production due to infection with both of these pathogens, which could also have a significance to the human health, this study was designed to achieve the following aims:

- To estimate the prevalence of *Salmonella* spp. in broiler chickens in Tulkarm district by using microbiological culture methods as well as PCR.
- To estimate the prevalence of STEC in broiler chickens in Tulkarm district using microbiological culture methods as well as PCR.
- To investigate the possibility of concurrent infections.

Chapter Two

Literature Review

Several studies using different standard and molecular techniques were conducted to determine the prevalence of poultry infecting microorganisms; *Salmonella* and STEC worldwide. Despite the fact that some of these studies were conducted in the nearby countries such as Jordan, Egypt, Saudi Arabia, Iraq, and Iran, however and to the best of our knowledge, no studies were conducted in Palestine using PCR for detection of our selected pathogens in living broiler chicken.

2.1 *Salmonella*

Internationally, several studies were conducted to determine the prevalence of *Salmonella* spp in poultry. In China; Zhao *et al* (2016) reported that 38 (12.66%) *Salmonella* isolates were recovered from 300 samples from three free-range chicken farms verified by PCR amplification of inherent gene *invA*. The 38 *Salmonella* isolates were classified into three serotypes. The most common serotype was *S. enterica* serovar Enteritidis (81.6%), followed by *S. enteric* serovar Indiana (13.2%) and *S. enterica* serovar Typhimurium (5.3%). Whereas Gong *et al* (2014) reported that the prevalence of *Salmonella sp*. in rectal swabs samples was 9.8% (167/1,706)

in chickens using PCR amplification technique. *S. Pullorum* was most commonly isolated from chickens.

In Europe, several studies were conducted such as in Austria where Lassniq *et al* (2012) conducted a study on the prevalence of *Salmonella spp.* in broilers, 363 flocks were tested. The results showed that 28 flocks (7.7%) were infected with *Salmonella spp.*, (2.2%) had either *S. enteritidis* or *S. typhimurium*, more precisely the percentage has been found to be: *S. enteritidis* (1.7%), *S. typhimurium* (0.6%), *S. montevideo* (4.1%), *S. infantis* 0.6%, *S. senftenberg*, *S. tennessee* and *S. virchow* (0.3% each).

Other studies conducted in Brazil where Tejada *et al* (2016) reported that (4/200; 2%) from chicken fecal samples were positive for *salmonella* by using *invA* primers for PCR. While in another study conducted by (Paião *et al*., 2013), 90 fresh culture cloacal swab samples from poultry chicken were examined for the detection of *Salmonella* spp, *Salmonella* Enteritidis and Typhimurium by multiplex PCR using *Inv-A*, *IE1* and *Flic-C* genes. The final results showed the presence of *Salmonella spp*. in 25% of samples, *S.* Enteritidis was present in 12% of the *Salmonella*-positive samples and *S. Typhimurium* in 3% of the samples.

In a study conducted by (Paul, Kennedy, & Shoyinka, 2016) in Nigeria using universal primer set specific for genus *Salmonella 16S rRNA* for PCR

technique to detect the presence of *Salmonella* isolates from a total of 1420 samples (800 eggs, 420 clocal swabs and 200 poultry litter), the results showed that a total of 28 *Salmonella* isolates were recovered from egg, cloacal swab and litter presenting 18, 7 and 3 *Salmonella*, respectively and an isolation rate of 1.97%.

A study conducted in India by (Jinu *et al*., 2014) revealed that the prevalence rate of *Salmonella* in 510 poultry blood samples and 255 faeces samples was found to be 5.09% using conventional culture methods and 5.88% by PCR assay targeting the *invA* gene. Serotyping of 26 *Salmonella* isolates revealed 57.69% *Salmonella Typhimurium*, 19.23% rough type, 15.38% *Salmonella* Enteritidis and 7.69% untypable. Among *Salmonella Typhimurium* isolates, 73.33% were from poultry blood and 26.66% from faeces samples. All isolates belonging to *Typhimurium* and Enteritidis serotypes were confirmed by PCR targeting of *Salmonella Typhimurium* (*typh*) and *Salmonella* Enteritidis (*ent*) specific genes. However, 4 isolates found to be rough type also turned out to be positive for *ent* gene.

In a study conducted by Henry *et al.* (2012) investigated *Salmonella* contamination of 71 chicken broiler flocks at the slaughterhouses in the Reunion Island in the Indian ocean by using biochemical assays. Droppings ,

intestines, neck skins and carcass rinses samples were taken from live broiler chicken and chicken carcasses as well as the slaughterhouse environment. *Salmonella* spp. was isolated from 40 of 71 (56%) of broiler chicken flocks at slaughter. At the farm, 27% of the broiler chicken flocks tested positive. *Salmonella* spp. were isolated from 127 of 497 environmental samples (25%).

Regionally, several studies were conducted. A recent study conducted in Iraq by Abed and Ali (2018) where a total of 69 samples were isolated from chicken ceca. 21 (30.43%) *Salmonella* positive samples were found, 12 of which were *S. pullorum*, which were confirmed by differential biochemical tests and additionally confirmed by PCR targeting the *invA* gene. Another study conducted in the same country by (Al-Khayat & Khammas, 2016) using PCR technique for the detection of *salmonella* species in broiler and layer chicken, the PCR performed targeting *invA* gene, the study revealed that 60 out of 577 (10.4%) samples were positive for *Salmonella* sp. *Salmonella* isolates from poultry samples (liver, bile, spleen, heart, yolk sac, ceca, joint, ovary and oviduct) obtained from layer and broiler chicken. Another study in the previous country by (Al-Abadi & Mayah, 2011) found that 34 of 370 samples (50 samples for each of liver, yolk sac and cecal content, 100 cloacal swabs, 30 samples of poultry litter, 30 samples of

poultry ration and 60 samples of embryonated eggs) were *salmonella* positive by biochemical and serological tests; the overall prevalence of *salmonella* in broiler and layer chicken was 9.2%. The results obtained showed that no significant differences among the broiler and layer cloacal swabs. Three hundred and thirty broiler samples were collected, 30 out of these samples were positive for *Salmonella* (9.09%). While 40 layer cloacal swab samples were collected, 4 samples were positive for *Salmonella* (10%). PCR technique with specific primers for *fimC* and *sefA* genes respectively were used to detect *S. typhimurium* and *S. enteritidis*, 14 isolates were detected as *S. typhimurium* and 2 isolates were detected as *S. enteritidis* at 289 bp and 330 bp.

Several studies were also conducted in Egypt, El-Sharkawy *et al* (2017) performed PCR technique using primers *inv*A to detect *salmonella* sp. In a total of 615 samples collected from broiler flocks from different organs (liver, intestinal content and gall bladder), *Salmonella* infection was identified in 17 (41%) broiler chicken flocks and 67 *Salmonella* isolates were collected. Recovered isolates were serotyped as 58 (86.6%) *S. enterica* serovar *Typhimurium*, 6 (9%) *S. enterica* serovar Enteritidis and 3 (4.5%) were non-typable. *sop*E gene was detected in 92.5% of the isolates indicating their ability to infect humans. Ammar, Mohamed, Abd El-Hamid,

and El-Azzouny, (2016) used PCR technique for the detection of *invA* gene in broiler chicken. A total of 300 samples of liver, heart, and spleen (100 each) were aseptically collected from 100 freshly dead and diseased broiler chickens from different localities in Sharkia Province. The study revealed that 17% were infected with *Salmonella*, seven different serovars with the main one being *Salmonella typhimurium* (52.94%). Further PCR investigations of 17 *Salmonella* strains revealed different distribution patterns of eight virulence determinants among the isolates. The *inv*A gene was the most prevalent one (100%), followed by *hil*A (88.24%), *stn* (58.82%), and *fli*C genes (52.94%), while each of *sop*B and *pef*A genes had a similar prevalence (41.18%), and *sef*C and *spv*C genes had the lowest prevalence (11.76 and 5.88%, respectively). PCR genotyping allowed grouping of *Salmonella* strains into ten genetic profiles. A recent study in Egypt by Ibrahim, Abd El-Ghany, Nasef , and Hatem (2014) reported that the incidence of *Salmonella* among imported chicks was 11.67% compared to 21.67% among local chicks using conventional cultural isolation methods for liver samples and intestinal contents samples. *Salmonella newport* (*S. newport*) showed the highest incidence rate in imported chicks, while *Salmonella enteritidis* and *Salmonella typhimurium* were frequently detected in local chicks. The RT-PCR results for detection of *invA* gene of

Salmonella spp. were 58.33% and 66.67% positive samples in imported and local chicks, respectively. Another study conducted by Ibrahim, Emeash, Ghoneim, and Abdel-Halim, (2013) in the same country, where cloacal swabs were collected from different live poultry species including 150 broilers, 50 breeders, 50 layers, 50 turkeys, and 50 ducks, beside 30 litter samples from various poultry farms. By using cultural and biochemical methods, the recovered *Salmonella* strains were found belonging to S. *Kentucky*, *S. Typhimurium* and *S. SaintPaul*. The obtained results demonstrated that the occurrence of *Salmonella* spp. accounted for 16.66, 10.0, 2.0, 6.0 and 2.0% in broilers, breeders, layers, ducks and turkeys respectively. Investigation of litter samples revealed that the occurrence of *S. Kentucky* was 53.33, 66.66 and 28.57% in broiler's, breeder's and duck's litters respectively.

In Turkey, a study conducted by Carli, Eyigor, and Caner (2001), 151 (18.6%) of 814 ceca 28 broiler and 5 layer flocks were found to be contaminated with four different *Salmonella* serovars. Using Biochemical identification, serogrouping, and serotyping only *Salmonella enterica* subsp. enterica Serovar Enteritidis (*Salmonella* Enteritidis) was recovered from layer birds, whereas *Salmonella* Enteritidis (81.5%), *Salmonella* Agona

(7.6%), *Salmonella* Thompson (10.1%), and *Salmonella* Sarajane (0.8%) were isolated from broiler birds.

2.2 Shiga toxin producing *E.coli* (STEC)

Regarding prevalence of STEC in broiler chicken, several studies were conducted. In Bangladesh, a recent study conducted by Runa, Lijon, and Rahman (2018) found that 5 out of 8 *E. coli* isolates were STEC by polymerase chain reaction (PCR) assay targeting the *stx2* gene. The prevalence of the STEC in broiler cloacal swabs was 62.5%. Mamun *et al* (2016) conducted a survey using PCR technique for the detection of STEC in 60 cloacal swabs in broiler chicken, PCR was performed for the detection of virulence genes *stx1* and *stx2*. The prevalence of *E. coli* was 81.67%. These 49 *E. coli* (one isolate per sample) were examined further for the presence of *stx*1 and *stx*2 genes by PCR to identify STEC. It was found that 10.20% of the isolates were found positive for *stx*1 alone, 53.06% for *stx*2 alone, and 12.24% for both *stx*1 and *stx*2. On the other hand, the remaining isolates (24.28%) were non-STEC, since they were found negative for both *stx1* and *stx*2 genes. Himi *et al* (2015) conducted another survey, the PCR was performed by targeting *16s rRNA* gene and shiga toxin producing genes (*stx1* and *stx2*) in *E. coli*. Out of 150 collected samples, E. *coli* was found in

81 (54%) samples. The *stx2* gene was detected in all isolates while all samples were negative for *stx1* gene.

In a study conducted by (Chandran & Mazumder, 2014) in British Columbia, Canada using standard biochemical tests and also by PCR amplification, out of the 412 *E. coli* isolates obtained from the feces of 15 avian host sources, 93 isolates obtained from 8 hosts. None of the isolates were found to be positive for stx_1, while 23% (n = 93) were positive for only stx_2, representing STEC, and 15% (n = 63) were positive for only *eae*, representing EPEC. In addition, five strains obtained from pheasant were positive for both stx_2 and *eae* and were confirmed as non-O157.

In a study conducted in Argentina by (Alonso, Lucchesi, Rodríguez, Parma, & Padola, 2012), out of 859 cloacal samples, 102 (11.9%) were contaminated with EPEC and, in contrast, only one sample (0.1%) was contaminated with STEC by using multiplex PCR.

In a study conducted in Finland by (Kobayashi, Pohjanvirta, & Pelkonen 2002), fecal samples from live gulls (n=86), pigeons (n=33) and broiler chickens (n=199) from 23 flocks were analyzed for *stx* and *eae* by PCR. No *stx* positive samples were detected. In contrast, *eae E. coli* were highly prevalent among gulls (40%), and was also found in pigeons (7%) and

chickens (57% of the flocks contaminated). The *eae* positive isolates were analyzed genetically and O-serogrouped. One isolate from a pigeon was found to have *stx* $_{2f}$.

Regionally, several studies were conducted in Iran. A recent study conducted by Doregiraee *et al*. (2016) using PCR, found that out of the 500 collected cloacal swab samples, 444 *E. coli* strains were isolated. Three strains (0.67%) presented at least one of the studied virulence genes (*stx2*, *hly* and *eae*), two strains were identified as STEC (*stx2*$^{+}$, O157:nonH7) and one as an atypical EPEC strains (*eae*$^{+}$ *bfp*$^{-}$). Tabatabaei *et al* (2011) found that 4% STEC were isolated from a total of 350 fecal samples from 28 broiler farms by conventional culture methods and polymerase chain reaction (PCR). All STEC isolates were examined for *stx1* and *stx2* genes by multiplex PCR. About 2.8% STEC isolates were positive for both *stx1* and *stx2* genes. About 1.16% of the STEC isolates were positive for *stx1* gene only, and *stx2* alone gene was only detected in one isolate 0.28%.

Several studies conducted in Egypt, Byomi, Zidan, Diab, Reddy, and Abdela, (2017) conducted a study in El-Behera Province in 1278 samples (908 from broilers , 253 from ducks and 117 from backyard chicken). The prevalence of STEC in broilers chicken was 31.7% by using multiplex PCR. A study conducted by (Abd El Tawab, Amma, El-Hofy, Abdel Hakeem, &

Abdel Galil, 2016) in Sharkia and Dakahlia using PCR, multiplex PCR and uniplex PCR. The obtained results revealed that out of 451 samples from freshly slaughtered broilers, 236 *E. coli* isolates were successfully recovered with a total percentage 52.3% (representing 64, 51, 48 and 52 out of 100 examined lung, liver, heart blood and trachea respectively, and 21 out of 51examined spleen). Fifteen *E. coli* strains of different serogroups isolated from birds with colibacillosis were assigned to their phylogenetic groups and analyzed for the occurrence of 11 virulence associated genes. The virulence profiles showed that *ompA* was found in most isolates 14/15 (93.3%). The *iss* gene was found in13/15 (86.6%). Followed by *traT* and *iutA* genes which were found in12/15 (80%). *cvaC*, *stx2* and *tsh* genes were present in 9/15(60%), 7/15(46%) and of the isolates, respectively. Only one isolate gave positive amplification for *stx1* and *ibeA* genes each. Another study conducted by (Eid, Algammal, Nasef, Elfeil, & Mansour, 2016) at at Sharkia province, liver and heart blood samples were collected from 100 diseased broiler chickens. The prevalence of *E. coli* was 60% from the total collected samples. The PCR was used for detection of Shiga-like toxins genes *(stx*1 and *stx*2), attaching and effacing (*eae*A) gene and enterohaemolysin gene *(hly)* in the typable isolated *E. coli* strains. The detected virulence genes were *stx*1 in all *E. coli* strains

(100%), *stx*2 in 17 strains (47.2%), *eae*A in 12 strains (33.3%) and *hly* only in three strains (8.3%). In Ismailia city in Egypt (Selim *et al*., 2013) using multiplex PCR technique for detection of STEC in broiler chicken, the multiplex PCR was applied for the detection of virulence gene (*stx1, stx2, eaeA*). The overall prevalence of STEC was 17.3% (49/283) were from stools of sheep, cattle and chicken with diarrhea.

Locally in Palestine we couldn't find any published studies conducted using PCR for detection of both selected pathogens in living broiler chicken, whereas few studies were conducted to detect *Salmonella* spp in fresh and frozen chicken meat and in hen eggs and their environment in West bank and Gaza strip. However, another study focused on APEC in broiler chicken in the West Bank.

A study conducted by (Adwan, G., Alqarem, & Adwan, K., 2015) in Jenin district aimed to investigate the prevalence of enterotoxigenic *Staphylococcus aureus*, *Salmonella* and *Escherichia coli* pathotypes in different meat types. Forty meat samples fresh (n=35) and frozen (n=5) were purchased from local markets. Multiplex PCR was used to detect enterotoxigenic *S. aureus*, *Salmonella* and *E. coli* pathotypes. The prevalence of *S. aureus*, *Salmonella* and *E. coli* was 30%, 25% and 95%, respectively. The results also showed that 89.5% of meat samples

contaminated with *E. coli* that belonge to enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), diffuse adherent *E. coli* (DAEC) pathotypes. In Gaza strip Elmanama, El Kahlout, and Elnakhalaa (2013) conducted a study using biochemical identification of isolates and serological test, the study showed that 11 (7.3%) of samples were positive for *Salmonella* and the remaining 139 (93.7%) of samples were negative. The study demonstrated that 13.3% of fresh chicken, 10% of fresh minced meat, 6.7% of frozen minced meat, 3.3% of fresh meat and 3.3% of frozen meat were contaminated with *Salmonella*. In addition, it was found that samples collected from Khan-Youns had the highest *Salmonella* contamination (13.3%) and fresh chicken was of the most frequently contaminated with *Salmonella*. Another study conducted by (ElKichaoui, Elmanama, & Msallam, 2017) using biochemical identification and serological confirmation , a total of 596 samples (100 egg pools, 88 feed samples, 320 chicken excreta and cloacal swabs and 88 water samples) were collected from 12 poultry farms in Gaza strip. The study showed that egg pools, feed samples and water samples were negative for *Salmonella* spp., whereas one *Salmonella* spp. was isolated from chicken excreta pools from Khan-Younis poultry farm.

A study conducted in West Bank by (Qabajah, 2011), the multiplex PCR of the 83 tested liver samples of broiler chicken revealed 66 samples positive for *E. coli*, while the other 17 samples were negative based on morphological and biochemical characteristics, and a high prevalence of the following APEC genes: *iss* and *cvi* 100%, *astA* 98.48% and *iucD* 78.79%.

Chapter Three

Materials and Methods

The present research was carried out during the period of April 2017 to November 2017 in the research laboratory at Agricultural Biotechnology Research Center in Palestine Technical University (Khadoori).

3.1 Study area

Samples were collected from four different farms in Tulkarm district (A: Jbarah, South; B: Bala'a, East ; C: Shwaikah; North, and D: Nazlat Issa; Northwest) and directly transferred to the research laboratory in cold box for investigation. The samples were used to isolate and identify poultry *Salmonelae* and Shiga Toxin Producing *Escherichia coli* (STEC) by cultural and molecular methods.

3.2 Materials

3.2.1 Solid and liquid culture media

The following culture media purchased from Himedia, India were used for identification and isolation of both pathogens: Eosin methyline blue agar (EMB), HiCrome EC O157:H7 Selective Agar Base, HiCrome improved salmonella agar, Xylose lysine deoxychockolate agar (XLD), Nutrient broth

(NB), Buffered peptone water (BPW) and SBG Enrichment Broth (Twin Pack).

3.2.2 Polymerase Chain Reaction

Three primer sets were used in this study**.** *InvA*, a specific primer pair of the *invA* gene, selected to detect *Salmonella* at the genus level, *16S rRNA* is a specific primer pair to detect for *E.coli*, and *Stx1* and *Stx2* specific primer pairs to detect STEC (Table 1), Pwo master mix, DNA template and nuclease free water.

Table 1: Oligonucleotide primers used in the PCR

Target gene	**Sequence**	**Length (bp)**	**Anneal -ing Temp**	**References**
Inv-A	F-CGG TGG TTT TAA GCG TAC TCTT R-CGA ATA TGC TCC ACA AGG TTA	796	58 °C	Fratamico (2003)
16S r-RNA	F-GAC CTC GGT TTA GTT CAC AGA R-CAC ACG CTG ACG CTG ACC A	585	60 °C	Candrian et al., (1991); Wang, Cao, and Cerniglia (1996)
Stx1	F-CAC AAT CAG GCG TCG CCA GCG CAC TTG CT R-TGT TGC AGG GAT CAG TCG TAC GGG GAT GC	606	61 °C	Talukdar *et al*., (2013)
Stx2	F- CCA CAT CGG TGT CTG TTA TTA ACC ACA CC R- GCA GAA CTG CTC TGG ATG CAT CTC TGG TC	372	59 °C	Talukdar *et al*., (2013)

3.3 Methods

3.3.1 Experimental Design

The entire study was performed in to four phases. The first phase was collection of cloacal swabs of broiler chicken, followed by identification and isolation of *Salmonella* and STEC on the basis of cultural and biochemical characteristics (phase 2), followed by DNA extraction (phase 3) and the 4th phase was the molecular characterization of the isolated *Salmonella* spp and STEC by amplification of species specific genes by PCR.

3.3.2 Collection and transportation of samples

A total of 200 cloacal swab samples were collected randomly from adult chickens during the period from April 2017 to November 2017, from four different poultry farms in Tulkarm district: Farm A: Jbarah (South), farm B: Bala'a (East), farm C: Shwaikah (North), and farm D: Nazlat Issa (Northwest) (Figure 1), 50 cloacal samples from each farm. Samples were collected in transport media and all were directly transferred immediately in an icebox to the laboratory for investigation.

Figure 1 : Sample collection from the broiler chicken farms in Tulkarm district (Jbara farm: A).

3.3.3 Preparation of culture media and reagents:

3.3.3.1 Preparation of culture media

All media were prepared according to the manufacturer's instruction (Himedia, India) with taking care of not boiling chromogenic media during preparation to keep activity of dyes and other heat-sensitive ingredients. Solid media was poured aseptically in sterile Petri dishes while broth media was prepared in 10 ml volumes in sterile valcon tubes.

3.3.3.2 Preparation of 50X TAE Electrophoresis Buffer

TAE buffer is commonly prepared as a 50X stock solution for laboratory use. A 50X stock solution was prepared by dissolving 242g Tris base in water, adding 57.1mL glacial acetic acid, and 100mL of 500mM EDTA (pH 8.0) solution, and bringing the final volume up to 1 liter. This stock solution

was diluted fifty times with distilled water to make a 1X working solution. This 1X solution contains 40mM Tris, 20mM acetic acid, and 1mM EDTA.

3.3.4 Cultural identification and isolation of *Salmonella* and STEC

3.3.4.1 Cultural identification and isolation of *salmonella* spp.

Tips of the cotton swabs submerged in the transport media were cut and incubated in 5ml BPW at 37° C for overnight, then a loopful (10 µl) of BPW broth was inoculated to the 10ml enrichment broth (SBG) and were incubated at 37° C for overnight , a loopful (10 µl) of SBG broth was streaked on the surface of XLD agar plates and HiCrome improved *salmonella* agar plates followed by further incubation at 37° C for overnight. Pink with black center colonies were subjected to subculture on XLD agar and were incubated at 37° C for overnight to get pure colony culture. Single pure colony of suspected *Salmonella* was obtained for further subculture in nutrient broth media followed by further incubation at 37° C for overnight. These pure isolates were used for the further investigation.

3.3.4.2 Cultural identification and isolation of STEC.

For STEC isolates, a loopful (10 µl) of overnight incubated BPW was streaked onto EMB agar plates followed by further incubation at 37° C for overnight. Separated single greenish metallic sheen colonies were obtained

for further subculture on HiCrome EC O157:H7 Selective Agar and in nutrient broth media followed by further incubation at 37° C for overnight. These pure isolates were used for the further investigation.

3.3.5 DNA Extraction

DNA was extracted from all isolates by using a commercial DNA extraction kit (QIAamp® DNA Mini and Blood Mini Kit) according to the manufacturer's instructions. One ml of bacterial culture was pipeted into a 1.5 ml microcentrifuge tube, followed by centrifugation for 5 min at 5000 x *g* (7500 rpm). A 180 µl of ATL buffer, and 20 µl proteinase K were added to the pellet, vortexed, then incubated at 56ºC for 1 hour. 200 µl Buffer AL was added to the sample, mixed by pulse-vortexing for 15 s, and incubated at 70°C for 10 min. The tube was spinned to remove drops from inside the lid, 200 µl ethanol (96–100%) was added to the sample, and mixed by pulse-vortexing for 15 s. After mixing, the 1.5 ml microcentrifuge tube was spinned to remove drops from inside the lid. The mixture was carefully applied (including the precipitate) to the QIAamp Mini spin column (in a 2 ml collection tube) and centrifuged at 6000 x *g* (8000 rpm) for 1 min. The QIAamp Mini spin column was placed in a 2 ml collection tube and the tube containing the filtrate was discarded. 500 µl Buffer AW1 was added to QIAamp Mini spin column, and centrifuged at 6000 x *g* (8000 rpm) for 1

min. The QIAamp Mini spin column was placed in a 2 ml collection tube, and the collection tube containing the filtrate was discarded. Carefully the QIAamp Mini spin column was opened and 500 µl Buffer AW2 was added and centrifuged at full speed (20,000 x *g*; 14,000 rpm) for 3 min. The QIAamp Mini spin column was placed in a clean 1.5 ml microcentrifuge tube and the DNA was eluted by adding 200 µl Buffer AE, incubated at room temperature for 1 min, and then centrifugation at 6000 x *g* (8000 rpm) for 1 min. The eluted DNA was stored at -20°C for further analysis.

3.3.6 PCR Amplification

Amplification reactions were performed using the automated thermal cycler (Veriti 96 well thermal cycler). The PCR reaction mixture with final volume of 25 µl was performed with 12.5µl master mixture (Pwo Master), 2.5 µl of 5 µM working concentration of each primer, 5 µl nuclease free water and 2.5µl DNA template.

The *invA* gene was amplified according the following thermal conditions: initial denaturation (95ºC for 5min), followed by 30 cycles of denaturation (95ºC for 1 min), annealing (58ºC for 1 min), and extension (72ºC for 30s). Followed by final extension (72ºC for 7min), and final holding temperature was 4ºC.

The *16S r-RNA* gene was amplified according the following thermal conditions : initial denaturation (94ºC for 3min), followed by 30 cycles of denaturation (94ºC for 45s), annealing (58ºC for 45 sec.), and extension (72ºC for 1 min). Followed by final extension (72ºC for 3min), and final holding temperature was 4ºC.

The *stx1* gene was amplified according the following thermal conditions : initial denaturation (94ºC for 5min), followed by 30 cycles of denaturation (94ºC for 1 min), annealing (61ºC for 1 min), and extension (72ºC for 1 min). Followed by final extension (72ºC for 5min), and final holding temperature was 4ºC.

The *stx2* gene was amplified according the following thermal conditions: initial denaturation (94ºC for 5min), followed by 30 cycles of denaturation (94ºC for 1 min), annealing (59ºC for 1 min), and extension (72ºC for 1 min). Followed by final extension (72ºC for 5min), and final holding temperature was 4ºC.

3.3.7 Gel electrophoresis (GE)

The PCR products were detected by gel electrophoresis through a 1.5% agarose gel for *invA* and 2% for *16Sr-RNA, stx1* and *stx2* (w/v) in 1X TAE. Samples were loaded in wells mixed with Blue orange 6X dye. Gel red was used for DNA staining, using DNA marker 100bp and a current of 70 V was applied to each gel for 1 hour. PCR products were visualized with UV illumination and imaged by gel documentation system.

3.3.8 Maintenance of stock culture

For further investigation, it was necessary to preserve the isolated *Salmonella* and STEC organisms. Pure cultures of isolated *Salmonella* and STEC organisms were preserved in 50% sterile buffered glycerin and stored at -20º C. This method is more appropriate for preserving bacteria with no deviation of their original characters for several years.

3.4 Statistical analysis

For comparing the suitability of different media for identification of both pathogens, percentages for sensitivity, specificity and efficiency were calculated, where appropriate, as follows:

Sensitivity (%) = True Positives X 100 / (True Positives + False Negatives).
Specificity (%) = True Negative X 100 / (True Negative + False Positive).
Efficiency(%) = (True Positives + True Negative) X 100 / Total.

Chapter Four

Results

4.1 Identification and isolation of *Salmonella* spp. and STEC using culture methods.

4.1.1 Isolation of *Salmonella* spp. by cultural characteristics.

After 24 hours of culturing swab cotton-tips in BPW, a pre-enrichment medium used for increasing the recovery of injured *Salmonella* species from food prior to selective enrichment and isolation, the clear transparent broth was changed from clear to turbid indicating a bacterial growth in all inoculated tubes (Figure 2). After another 24 hours of sub-culturing in SBG, selective enrichment of *Salmonella* species, the light green color was changed to deep orange color which indicates possible growth of *Salmonella* spp. (Figure 3). Sub-culturing on XLD agar revealed that the percentage of the presence of *Salmonella* for the all set of samples was 42.5% (85/200), suspected isolates of *Salmonella* appeared as pink colonies with black centers (Figure 4), which are differently distributed among the farms as shown in the Table 2 . However, the percentage of *Salmonella* on HiCrome improved *Salmonella* agar was found to be only 14% (28/200) where suspected colonies appeared as pink to red (Figure 5), these samples are differently distributed among the farms as shown in Table 2 . Among the 85

suspected isolates of *Salmonella* detected on XLD, only 28 isolates (32.94%) were shown to have a positive chromogenic appearance on HiCrome improved *Salmonella* agar.

Figure 2: Bacterial growth in BPW after 24 hours incubation at 37C in both tubes.

Figure 3: Sub-cultured suspected *Salmonella* from BPW on SBG after 24 hours at 37C.

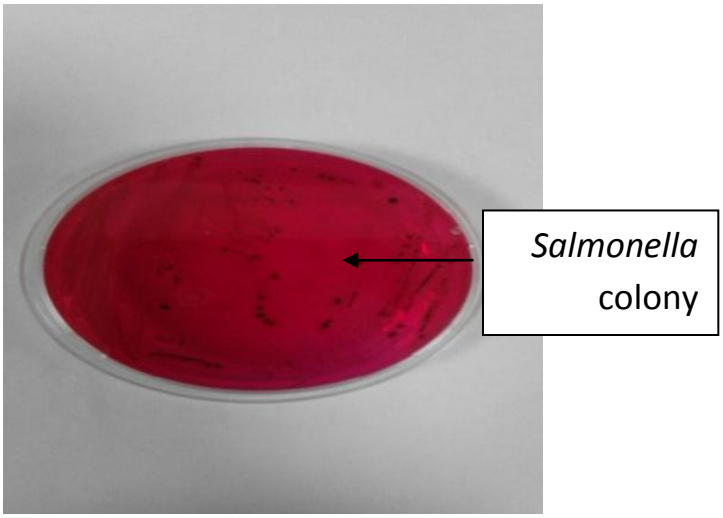

Figure 4: Suspected *Salmonella* colonies (pink with black centers) on XLD agar.

Figure 5: suspected *Salmonella* on Hicrome improved *Salmonella* agar.

Table 2: Distribution of suspected *Salmonella* spp. on XLD and Hicrome media in different farms.

Media	Farm A	Farm B	Farm C	Farm D
XLD	31	14	25	15
Hicrome improved *Salmonella* agar	20	7	1	0

Using the number of confirmed *Salmonella* spp. by PCR for calculations, XLD agar has shown a sensitivity and a specificity of 100% and 65.7%, while Hicrome agar has shown a 89% and 90%, respectively (Table 3). Low specificity (65.7%) of XLD indicates a high number of false positives showing shared colony characteristics with *Salmonella*, whereas low sensitivity (89%) of Hicrome indicates a relatively high number of false negatives that did not show a chromogenic features of *Salmonella* though they were confirmed to be *Salmonella* by PCR (Table 3).

Table 3: Sensitivity, specificity and efficiency of used culture media for the isolation of *Salmonella* from cloacal samples.

	Media	
	XLD	**Hicrome**
True positive	25	25
True negative	115	157
False Positive	60	18
False negative	0	3
Sensitivity %	100%	89%
Specificity %	65.7%	90%
Efficiency %	70%	91%

4.1.2 Isolation of STEC by cultural characteristics.

After 24 hours of sub-culturing swab cotton-tips in BPW as a pre-enrichment media, the clear transparent broth was changed to turbid indicating bacterial growth (Figure 2). EMB agar plates streaked with pre-enriched inoculums and incubated at 37°C for 24 hrs. The growth of *E. coli* was identified as smooth, circular, greenish color colonies with metallic

sheen (Figure 6). Results revealed the presence of *E.coli* in 98% (196/200) of samples on EMB agar. A 196 *E.coli* samples were examined further for the presence of STEC using HiCrome EC O157:H7 Selective Agar Base that appeared as dark purple magenta (Figure 7), this selective chromogenic media showed that the percentage of STEC was 47% (94/200) in all tested samples and 48% (94/196) among the *E. coli* isolates which are distributed differently among the farms as in the table (4).

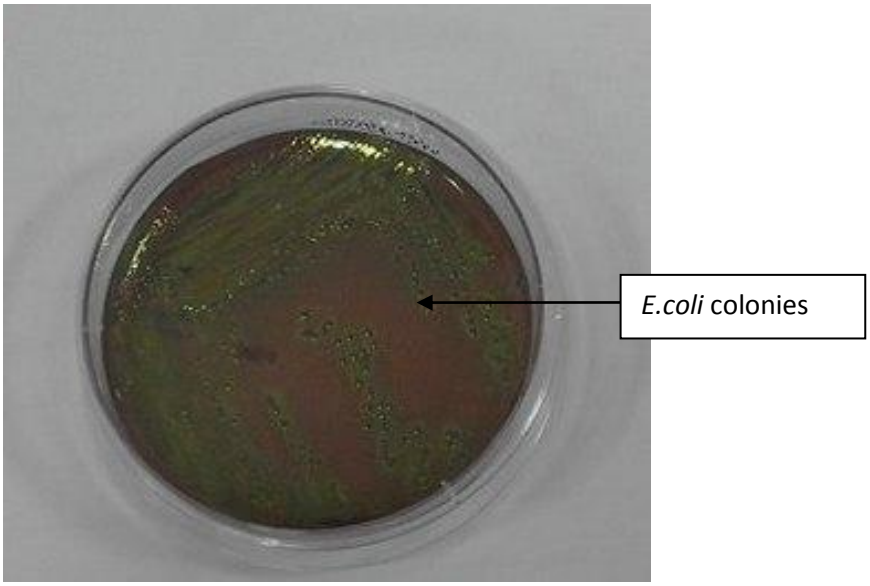

Figure 6: Growth of *E. coli* colonies (greenish colonies with metallic sheen) on EMB.

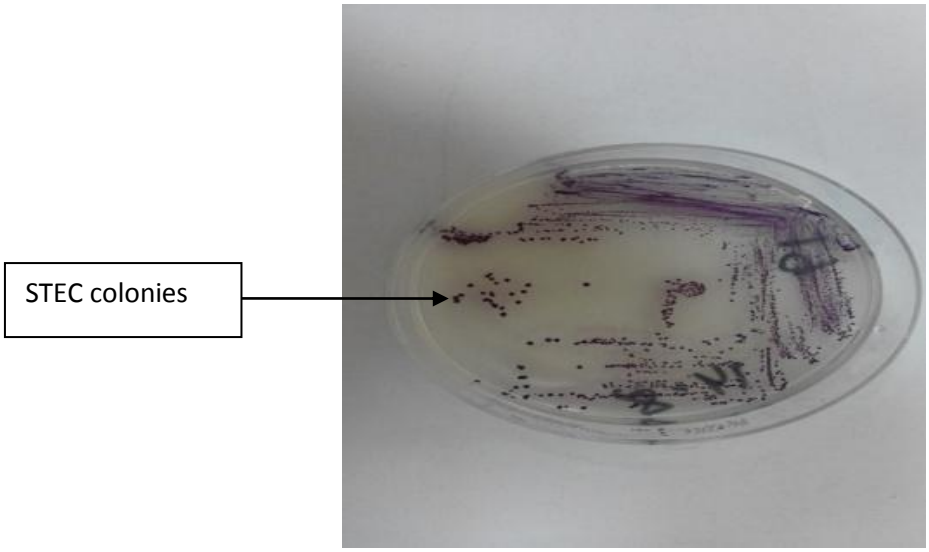

Figure 7: STEC colonies on Hicrome EC O157:H7 Selective Agar Base.

Table 4:Distribution of *E.coli* on EMB and STEC on Hicrome media in different farms.

Media	Farm A	Farm B	Farm C	Farm D
EMB	50	46	50	50
Hicrome ECO157:H7	19	17	32	26

Using the number of confirmed STEC by PCR for calculations, we found that EMB agar has shown a very high sensitivity (100%) and a very low specificity (2.13%), while Hicrome agar has shown a relatively high sensitivity (86.66%) and a moderate specificity (56.68%). Low to moderate specificity of both media led to a high number of false positives (Table 5).

Table 5: Sensitivity, specificity and efficiency of used culture media for isolation of STEC from cloacal samples.

	Media	
	EMB	**Hicrome**
True positive	13	13
True negative	4	106
False positive	183	81
False negative	0	2
Sensitivity %	100%	86.66%
Specificity %	2.13%	56.68%
Efficiency %	8.5%	59.5%

4.2 Identification of *Salmonella* and STEC by PCR.

Suspected isolates of both bacterial pathogens recovered by culture methods were subjected for further investigations using molecular characterization by PCR method to confirm their identity as *Salmonella* and STEC .

4.2.1 *InvA* PCR for identification of *Salmonella* spp.

Totally, 85 out of 200 (42.5%) samples were positive for *Salmonella spp.* on XLD. All suspected *Salmonella* colonies were purely cultured and then isolated DNA was subjected to PCR amplification for the detection of *invA* gene which is present in all *Salmonella* spp. The results showed that only 25 of 85 (29.41%) were positive an expected PCR product of 796 bp of *inv*A gene (Figure 8).

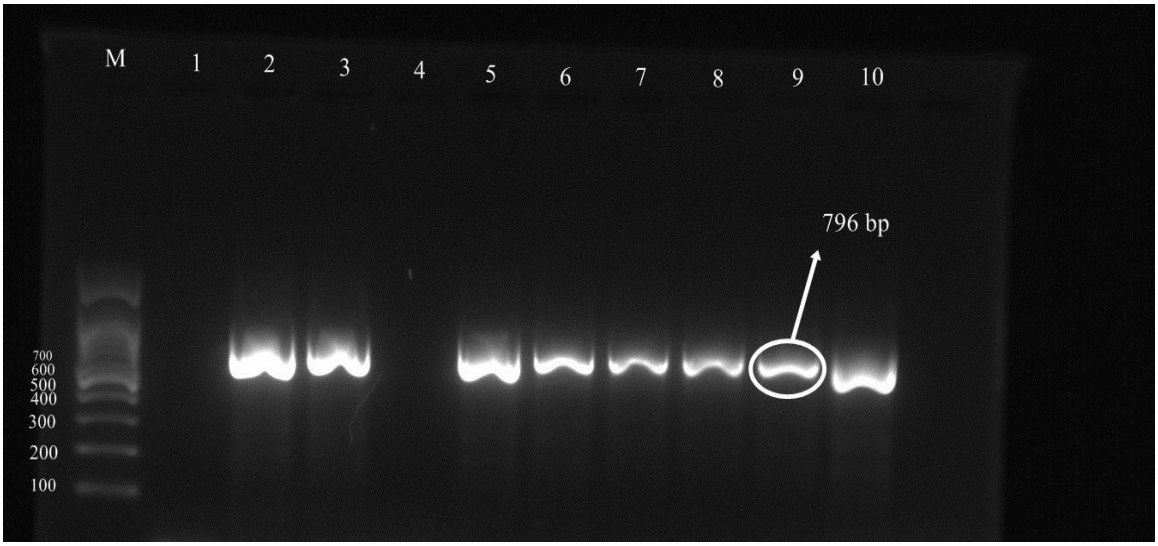

Figure 8: Representative image of agarose gel electrophoresis showing amplification of *inv*A gene. M: 100 bp ladder. Lanes 2,3,5,6,7,8,9 and 10 are positive *salmonella* exhibiting an amplified *inv*A gene (796 bp). Lanes 1and 4 are negative *Salmonella*.

4.2.2 16S *r-RNA* PCR for the identification of *E. coli*

Totally, 196 out of 200 (98%) of all tested samples were found positive for *E.coli* on EMB. All suspected *E.coli* isolates were subjected to PCR amplification targeting the 16S *r-RNA* gene which is specific for *E. coli* species, the results has shown an expected PCR product of 585 bp fragment in all suspected isolates (100%) confirming their *E. coli* identities as obtained by EMB (Figure 9).

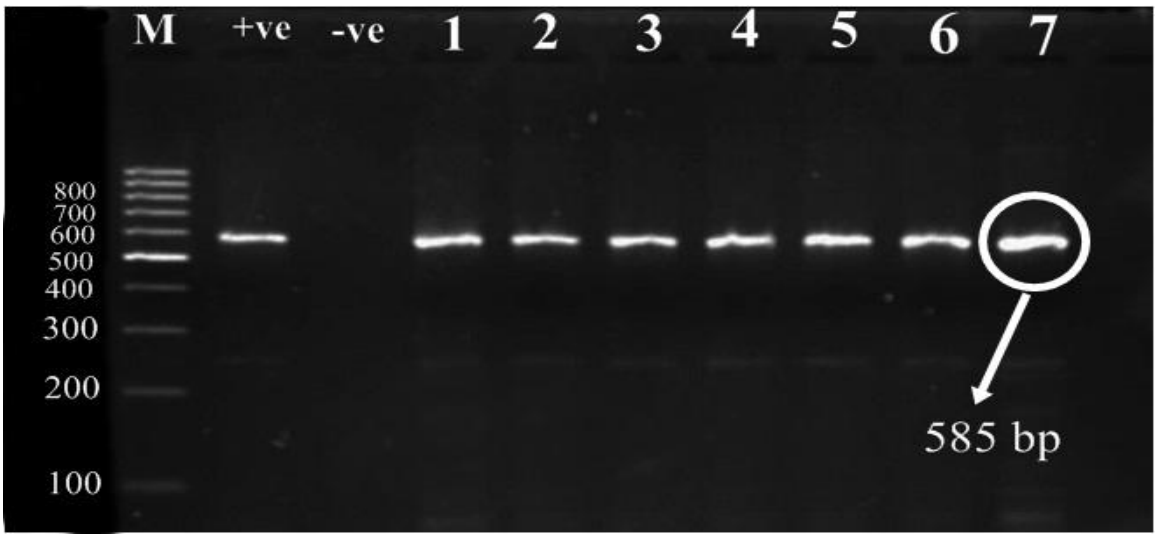

Figure 9: Representative image of agarose gel electrophoresis showing amplification of *16S r-RNA* gene. M: 100 bp ladder. Lanes 1:positive control. Lane 2: negative control. Lanes 3,4,5,6,8 and 9 are positive *E.coli* isolates.

4.2.3 Detection of STEC by PCR targeting *stx1* and *stx2*

All suspected *E. coli* isolates (196 out of 200) recovered on EMB, were subjected to PCR amplification targeting the STEC specific genes, *stx1* and *stx2*. The results has shown that only 13 out of 196 (6.63%) isolates were positive for *stx1* showing a a PCR product with an expected size of 606 bp of *the stx1* gene (Figure 10). However, PCR failed to detect any positive isolate for *stx2* with a an expected PCR product of 372 bp of the *stx2* gene (Figure 11).

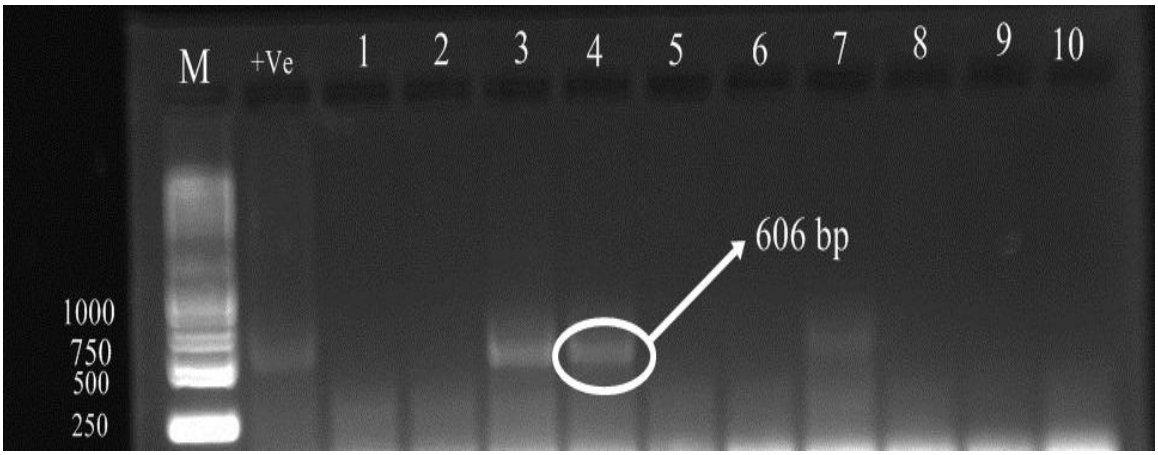

Figure 10: Representative image of agarose gel electrophoresis showing amplification of *stx1* gene. M: 1Kb ladder. Lane 1: positive control. Lanes 4,5, and 8 are positive STEC exhibits the amplified *stx1*gene (606bp). Lanes 2,3,6,7,9,10and 11 are negative STEC.

Figure 11: Agarose gel electrophoresis image showing no amplification of *stx2* gene. Lane 1: 1Kb ladder. Lanes 1,2,3,4,5,6,7,8,9,10,11,12,13,14,15and 16 are negative STEC by amplification of *stx2* gene with a PCR product of 372 bp.

4.3 Overall prevalence of *Salmonella*

Out of 200 samples, 85 (42.5%) were suspected to be contaminated with *Salmonella* on XLD. Among them 28 (32.94%) isolates showed expected characteristics of *Salmonella* on Hicrome and 25 (29.41) were positive for

invA gene. However, among the 25 isolates confirmed by PCR, only 10 (40%) were suspected to be *Salmonella* on Hicrome. Based on the total number of samples, this study revealed the presence of *Salmonella* in 12.5%(25/200) cloacal swabs collected from healthy broiler chickens by PCR (Table 6) and (Diagram 1). On the other hand, the remaining 87.5% (175/200) isolates were found negative for *invA* gene by PCR (Diagram 1).

Table 6: *Salmonella* isolates recovered on XLD, HiCrome *Salmonella* agar and *invA* PCR.

SN	Sample Number	Farm	Sample Type	On XLD	On Hicrome	InvA PCR
1	13	A	Cloacal	+	+	+
2	14	A	Cloacal	+	+	+
3	22	A	Cloacal	+	-	+
4	29	A	Cloacal	+	-	+
5	30	A	Cloacal	+	+	+
6	39	A	Cloacal	+	+	+
7	40	A	Cloacal	+	+	+
8	45	A	Cloacal	+	+	+
9	49	A	Cloacal	+	+	+
10	50	A	Cloacal	+	+	+
11	83	B	Cloacal	+	-	+
12	85	B	Cloacal	+	-	+
13	91	B	Cloacal	+	-	+
14	93	B	Cloacal	+	+	+
15	101	C	Cloacal	+	-	+
16	102	C	Cloacal	+	-	+
17	122	C	Cloacal	+	-	+
18	123	C	Cloacal	+	-	+
19	135	C	Cloacal	+	-	+
20	142	C	Cloacal	+	+	+
21	143	C	Cloacal	+	-	+
22	145	C	Cloacal	+	-	+
23	147	C	Cloacal	+	-	+
24	149	C	Cloacal	+	-	+
25	194	D	Cloacal	+	-	+

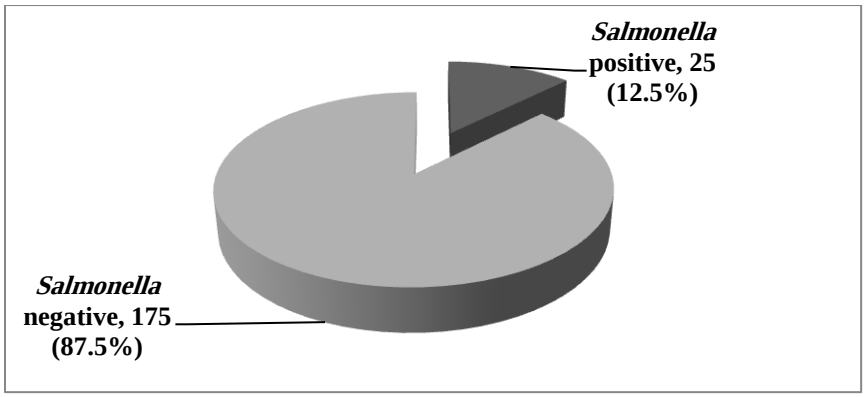

Diagram 1: The prevalence of *Salmonella* in cloacal samples collected from broiler chicken by PCR targeting *invA* gene .

4.4 Overall prevalence of STEC

Out of 200 samples, 196 (98%) were suspected to be contaminated by *E.coli* on EMB which were confirmed by PCR targeting the *E. coli* universal *16S r-RNA* gene (Table 7) and (Diagram 2). Among 196 isolates, 94 (47.95%) isolates has shown chromogenic characteristics of STEC on Hicrome and 13 (6.63%) were positive for *stx1* gene. Among the 13 isolates, only 11 (84.61%) were suspected to be STEC on Hicrome. Based on the total number of samples, this study revealed the presence of STEC in 6.5% (13 /200) cloacal swabs collected from healthy broiler chickens by PCR (Table 7) and (Diagram 3). On the other hand, the remaining 93.5% (187/200) isolates were found negative for *stx1* by PCR (Diagram 3). However, all tested isolates has shown negative results for *stx2* gene by PCR. Indeed, we cannot rule out the possibility of PCR failure for detecting *stx2* in all tested

isolates due to technical reasons specially with the lack of DNA from a positive control of *stx2*.

Table 7: Prevalence of STEC on EMB, HiCrome and by 16S r-RNA PCR, stx1 PCR and stx2 PCR.

SN	Sample number	Farm	Sample Type	On EMB	On Hicrome	16S r-RNA PCR	Stx1 PCR	Stx2 PCR
1	1	A	Cloacal	+	+	+	+	-
2	16	A	Cloacal	+	+	+	+	-
3	64	B	Cloacal	+	+	+	+	-
4	65	B	Cloacal	+	+	+	+	-
5	68	B	Cloacal	+	+	+	+	-
6	82	B	Cloacal	+	+	+	+	-
7	102	C	Cloacal	+	+	+	+	-
8	113	C	Cloacal	+	+	+	+	-
9	141	C	Cloacal	+	+	+	+	-
10	150	C	Cloacal	+	+	+	+	-
11	163	D	Cloacal	+	-	+	+	-
12	177	D	Cloacal	+	+	+	+	-
13	185	D	Cloacal	+	-	+	+	-

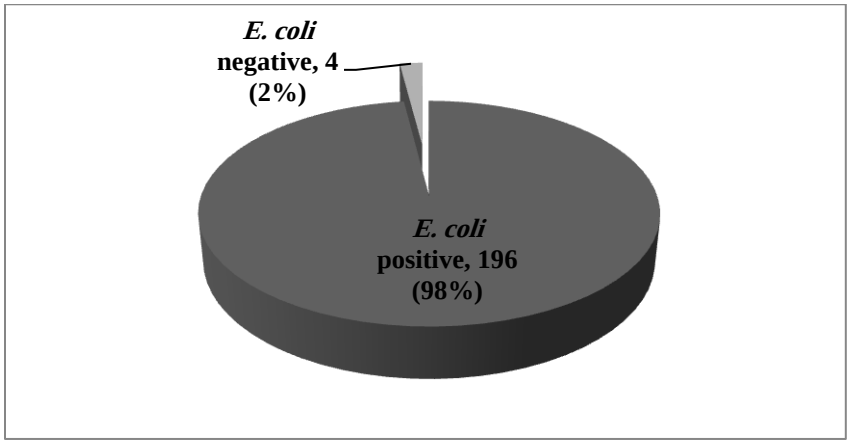

Diagram 2: *E.coli* prevalence in cloacal samples by EMB and PCR targeting 16S r-RNA gene.

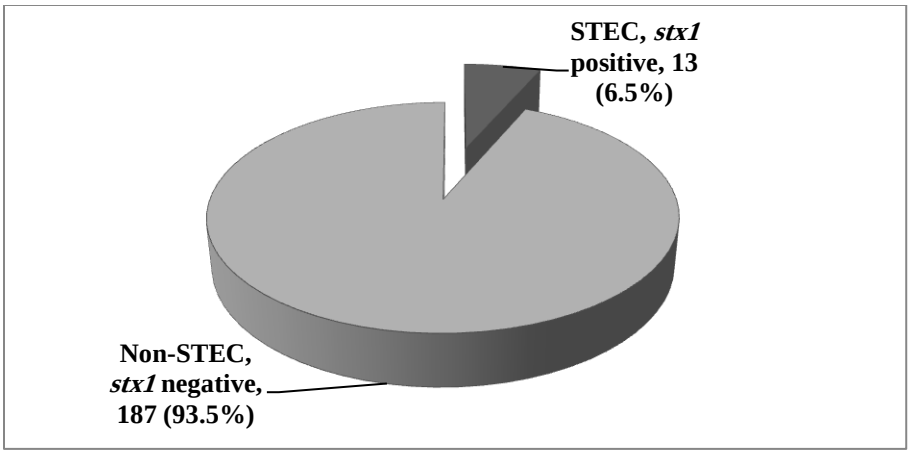

Diagram 3: Prevalence of STEC/*stx1* in cloacal samples by PCR targeting *stx1* gene.

Comparing between HiCrome chromogenic media and PCR for identification of *Salmonella* and STEC in suspected isolates recovered from selective differential media has shown that PCR was more sensitive for both pathogens, thus proving the reliability of PCR as an accurate molecular method of identification (Diagram 4).

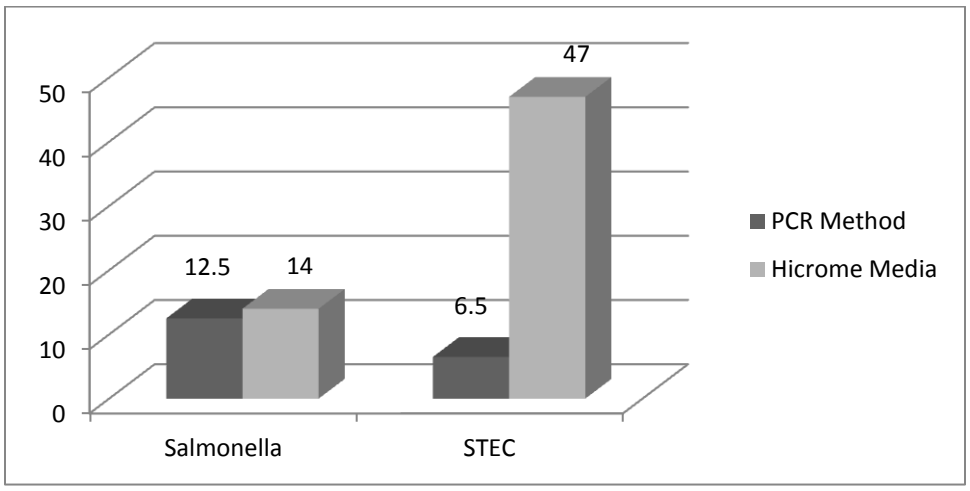

Diagram 4: Prevalence (%) of *Salmonella* and STEC using HiCrome media and PCR.

A difference in the prevalence of *Salmonella* and STEC in broiler chicken from the investigated farms was observed, where it was found to be (20%, 4%) in Jbarah, (8%, 8%) in Bala'a, (20%, 8%) in Shwaikah and (2%, 6%) in NazlatIssa for both *Salmonella* and STEC, respectively (Diagram 5).

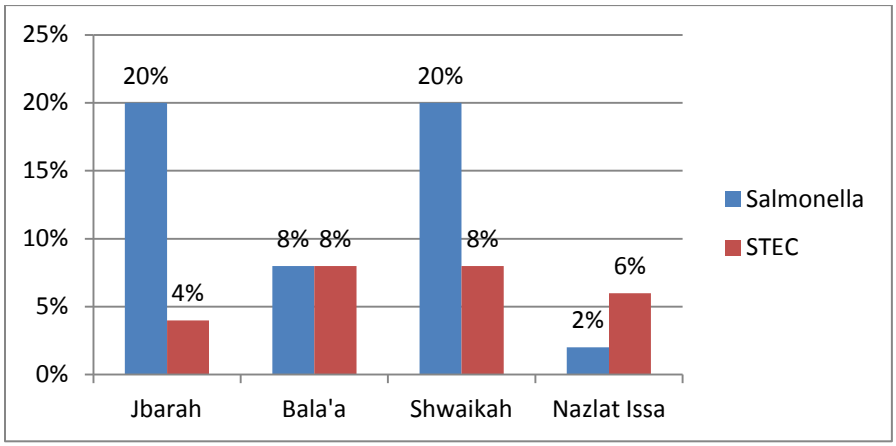

Diagram 5 : Prevalence of *Salmonella* and STEC in broiler chicken by farm.

Results has shown that only one sample was found to be contaminated with both *Salmonella* and STEC, therefore the possibility of concurrent infections has been found to be very low (0.5%).

Chapter Five

Discussion

To our knowledge, this was the first study on molecular determination of *Salmonella* and STEC in living broiler in Palestine. Poultry production plays an important role in providing valuable proteins, poverty alleviation and economic development. Despite great potential and opportunities, poultry production is threatened by many disease outbreaks, these diseases are the major constrains for developing the poultry industry (Ewers, Janssen, Kiessling, Philip, & Wieler, 2005). The present study was carried out to determine the prevalence of *Salmonella* and STEC in broiler chickens by culture and molecular method.

Salmonella and STEC are the among most virulent bacteria that cause life-threatening diseases for both animal and human. Every year, millions of salmonellosis cases are reported worldwide (Majowicz *et al*., 2010). STEC, is the most important foodborne pathogen which is the causal agent of mild diarrhea, bloody diarrhea, hemorrhagic colitis, thrombotic thrombocytic purpura, hemolytic-uremic syndrome (HUS) in human and animals (Karmali *et al*,.1985, O'Brien and Holmes, 1987, Griffin *et al*,.1988, Smith and Scotland, 1988, Karmali, 1989, Kovacs *et al*,. 1990). In the United

States alone, approximately 1.2 million illnesses, 43,000 hospitalizations, and 400 deaths occur each year as a result of STEC and nontyphoidal *Salmonella* infections (Scallan *et al.,* 2011). *Salmonella* and STEC not only poses serious threat to public health but also causes huge economic losses by generating mortality and morbidity to poultry industry. Monitoring and control are two important aspects to reduce the prevalence at farm level of this zoonotic disease.

Effective surveillance of foodborne pathogens and investigations of foodborne illness outbreaks rely on rapid, robust, and sensitive methods for pathogen detection and strain discrimination. Traditional culture techniques and molecular-based methods, such as polymerase chain reaction (PCR), are commonly used for detecting foodborne pathogens in clinical, environmental, and food samples. Due to their specificity and sensitivity, PCR-based methods provide distinct advantages in time sensitive outbreak investigations and are continually being improved to allow fewer target pathogens to be detected (Levin, 2009). Small modifications in PCR methods can sometimes lead to significant improvements in foodborne pathogen detection speed and sensitivity.

This study revealed that the prevalence of *Salmonella* using the culture method for all set of the samples was 42.5% (85/200) on XLD agar, with a

calculated sensitivity and a specificity of 100% and 65.7% with a total 60 false positive on XLD. Represents results is in agreement with a study done by (Park *et al* , 2012) who reported that the sensitivity and the specificity of XLD for the detection of *Salmonella* sp from food samples are 100% , 73.0% respectively, with a total of 47 false-positive results found on XLD. Very high sensitivity compared to PCR ensures the usefulness of this selective differential media as a primary identification choice where a few if any of *Salmonella* spp. will be dismissed. However, moderate specificity of XLD posing a challenge due to high number of false positives, such false positive colonies results from other bacterial species that grow on XLD with black colonies because of H2S production such as *Proteus* spp. (*P. mirabilis*), *Citrobacter* spp. (*C. freundii*) and *Pseudomonas* spp. Therefore, suspected colonies should be confirmed for *Salmonella* by a secondary procedure such as biochemical identification and/or PCR. On the other hand, some of *Salmonella* species like *S.* Paratyphi A and *S*. Berta are known as hydrogen sulfide negative; thus, their colonies do not appear as black on XLD media that detect hydrogen sulfide formation (Cox, 1993, Janda & Abbott, 2008). *S. Gallinarum* and *S. Pullorum* rarely produce hydrogen sulfide and thus appear without black colonies (Christensen, Olsen, Hansen, & Bisgaard, 1992). Also the hydrogen sulfide-generating

ability of *S. Typhi* is weak or negative (Janda & Abbott, 2008). This could be, however, a reason for the moderate prevalence (42.5%). of *Salmonella* among the investigated broiler chicken. In other words, some *Salmonella* spp. might have been dismissed due to unusual colony characteristics on XLD.

The prevalence of *Salmonella* on HiCrome improved *Salmonella* agar was determined to be 14% (28/200), we found that Hicrome agar had a high sensitivity and a specificity of 89% and 90% respectively. Despite the high sensitivity of Hicrome, a good number of false negatives where misidentified as there are some *Salmonella* species that do not appear as a red colonies like *Salmonella typhi* and *Salmonella paratyphi A*, which appear colorless on Rambach chromogenic media (Gruenewald, Henderson, & Yappow, 1991) which has the same formula as HiCrome *Salmonella* agar used in this study. According to the results that appeared with us when using XLD and Hicrome media, we found that the percentage of false positive colonies determined by PCR for both media was 70% (60/85) and 64% (18/28) respectively, high number of false positive colonies reduce the specificity of the media. Therefore, it is better to use these two types of media in addition to PCR technique to obtain accurate results for *Salmonella* detection.

It was also observed that there was a difference in the number of samples that were positive on XLD and Hicrome which is used to detect *Salmonella* in the farms studied. The highest number of *Salmonella* on XLD was recorded in farms A (31/50) followed by, Farm C (25/50), Farm D(15/50) and farm B (14/50). For Hicrome media, the highest number of *Salmonella* was recorded in farm A (20/50), followed by farm B (7/50), farm C (1/50) and farm D (0/50). These differences may be due to the environment of each farms.

Using PCR for the detection of *invA* gene that is shared among all *Salmonella* spp., 25 samples (12.5%) out of 200 samples were positive for *Salmonella,* this is in agreement with the findings of other studies conducted to determine the prevalence of *Salmonella* in broiler chicken. Zhao *et al*., (2016) reported that a total of 39 *Salmonella* isolates were identified from 300 samples (12.66%), Al-Khayat and Khammas (2016) identified the prevalence of *Salmonella* in 577 broiler samples to be 10.4%. In another study by Ammar *et al*., 17% of *salmonella* isolates were identified from 300 samples (Ammar *et al*., 2016). Moreover, Gong et al. (2014) reported that the prevalence of *Salmonella* sp. was 9.8% (167/1,706) in chickens. Al-Abadi and Mayah (2011) found that the overall prevalence of *salmonella* was 9.2% (34/370) samples among investigated chicken.

Many investigators (Guo, Killfer, Kenny, & Amick Morris, 1999; Ferretti, Mannazzu, Cocolin, Comi, & Clementi, 2001; Schneder *et al.*, 2002) tried to establish a method, which can reduce the time of *Salmonella* identification procedures. In an international research project for the validation and standardization of PCR for the detection of five major foodborne pathogens including *salmonella* , the most selective primer set was found to be 139-141, which targets the *invA* gene. This specific PCR assay, which was validated in that project, showed high selectivity on 242 *Salmonella* strains (sensitivity 99.6%) and 122 non-*Salmonella* strains (specificity 100%). Amplification of *invA* gene now has been recognized as an international standard for detection of *Salmonella* genus (Malorny *et al*., 2003). This gene encodes a protein in the inner membrane of bacteria which is responsible for invasion to the epithelial cells of the host (Darwin & Miller, 1999). *Salmonella* specific PCR with primers for *invA* is rapid, sensitive, and specific for detection of *Salmonella* in many clinical samples (Lampel, Orlandi, & Kornegay, 2000). The present study supports the ability of these specific primer sets to confirm the suspected isolates as *Salmonella*. In the present study we used *invA* primers for specific detection of *Salmonella* at the genus level. A total of 25 *Salmonella* isolates were found in 200 chicken samples (12.5%) by PCR. All strains were subjected to

Salmonella-specific gene (*invA*) and were confirmed as *Salmonella* positive by the predicted product a 796-bp DNA fragment. The results obtained in the present study were inconsistent with Paiao et al. (2013). The ability of *Salmonella* specific primers to detect *Salmonella* species rapidly and accurately in the present study is primarily due to the primer sequences that are selected from the gene *invA*. Culture techniques with biochemical testing are universally recognized as the standard methods for the detection of bacterial pathogens, such as *Salmonella* in food stuffs (White, Meglli, Collins, & Gormely, 2002). These techniques generally take longer time (Malorny *et al.*, 2003) and are less sensitive compared to PCR based methods (Oliveira *et al.*, 2002). Our results support the previous conclusion where only 25 out of the 85 suspected isolates based on XLD identification were confirmed by i*nvA*-PCR as *Salmonella*. Among them, only 10 were showing colonial characteristics of *Salmonella* on the chromogenic media (HiCrome *Salmonella* agar media). The use of *invA* gene specific PCR method in most diagnostic and research laboratories is possible, this method is the simplest and less expensive. Application of PCR technique as a diagnostic tool for *Salmonella* detection in poultry in Palestine is highly recommended to replace the time-consuming cultural technique.

This study found that *E.coli* was present in 98% (196/200) of tested samples by using culture on EMB agar, whereas the prevalence of STEC on Hicrome EC O157 agar was 47% (94/200). We have determined that EMB agar had a sensitivity and a specificity of 100% and 2.13%, the result of this study were fairly consistent with the result of other studies such as (Antony *et al*., 2016) who reported that the sensitivity and specificity of EMB media for E.coli detection in food and environmental samples was 68.5% and 20% respectively. Sensitivity and specificity of Hicrome agar were 86.66% and 56.68% respectively, (Al-Wasify, El-Taweel, Kamel, & El-Laithy, 2011) reported that the sensitivity and specificity of Hicrome agar for STEC detection in water samples were 93.7% and 100%. We can say that the EMB is very excellent for detecting *E.coli* but not enough to determine the different types of *E.coli* such as STEC so we should use selective media with also relying on the molecular technique as PCR. Low to moderate specificity of EMB and Hicrome led to a high number of false positives that were initially mis-identified as STEC based on colony characteristics to be later proved to be non-STEC by PCR.

It was also observed that there was a convergence in the number of samples that were positive for *E.coli* on EMB in the farms where the study is conducted (farm A, C and D was 50, farm B was 46). The reason for the

high convergence and spread of *E.coli* in the farms is due to that *E.coli* is considered as normal flora in the poultry. However, according to Hicrome media for STEC, there was a difference in the number of samples that were positive on Hicrome for STEC detection in the farms studied. The highest number of STEC on Hicrome was recorded in farms C (32/50), followed by farm D (26/50), farm A (19/50) and farm B (17/50). These differences may be due to the environment of each farms.

The prevalence of *E. coli* in present study using 16S r-RNA PCR was 98% (196/200), the same as found by culturing on EMB. Higher rates of *E. coli* also were recorded by Mamun *et al* (2016) and Doregiraee *et al* (2016) which was (49/60) 81.67%, (444/500) 88.8% respectively. High prevalence of *E.coli* can be explained as *E.coli* isolates are part of the normal enteric flora in the boiler chicken. Young aged broiler chickens up to 3 weeks have high affinity to the disease, but older chickens are more resistant. Different predisposing factors may increase the affinity of chickens to colibacillosis, such as viruses affecting the respiratory tract of chickens and bad hygienic mesearues. Traditional confirmation techniques for *E. coli* O157:H7 are complicated and time consuming. Polymerase chain reaction (PCR) technique is rapid, sensitive, specific and able to detect minute amounts of target genes in a sample (Toze, 1999), and have been adopted for routine

detection of food and water borne pathogens including *E. coli* O157: H7 (Deisingh & Thompson, 2004). PCR-based STEC detection is primarily focused on virulence associated genes (Nataro & Kaper, 1998). Specifically, Shiga toxin 1 (*stx1*), Shiga toxin 2 (*stx2*), and intimin (*eae*), an intestinal adherence factor, are the primary targets used for PCR detection of STEC (Nataro & Kaper, 1998). All three of these genes were recently incorporated into a single multiplex PCR assay developed by Fratamico *et al.* (2011) which is very specific and sensitive for STEC detection. However, some studies have described potential spontaneous loss of *stx1* and/or *stx2* naturally (Feng, Dey, Abe, & Takeda, 2001) and a loss of *stx* genes in *stx*-positive isolates can already occur after the first subculturing step of STEC isolated from naturally contaminated samples. Consequently, this may lead to an underestimation of STEC in animals, food and humans (Joris *et al*., 2013) leading to false negative results. As a result, this study adopted the use of both genes for confirmatory purposes. The results of the current study showed a low prevalence (6.63%) of STEC in broiler chicken by detecting of *Stx1* gene only by PCR. However, no *stx2* gene was detected in all tested samples, because some of *E. coli* O157: H7 might have lost the *stx* genes which was also reported by Feng *et al*. (Feng *et al*., 2001) or due to mutations in the primer binding sites of *stx2*. This finding is consistent with

the results of other studies conducted for prevalence of STEC in other geographical regions. In broilers, the *stx2* gene was detected in 4.5% of the isolates in Iran (Ghanbarpour, Sami, Salehi, & Ouromiei, 2011), Tabatabaei *et al* (2011) found that 4% STEC were isolated from chicken 350 fecal samples. Doregiraee *et al* (2016), reported that three strains (0.67%) were confirmed as STEC from 500 samples. On the other hand, some studies found no Shiga toxin genes in *E.coli* strains from poultry (Wani, Samanta, Bhat, & Nishikawa, 2004; Farooq *et al*., 2009). Similarly, *stx1* or *stx2* genes were not detected in *E. coli* from wild birds (Kobayashi, Kanazaki, Hata, & Kubo, 2009). As reported previously (Zeibell, Read, Johnson, & Gyles, 2002), the multiplex-PCR was not able to identify *stx2f* subtype in the mentioned study. This is in agreement with the findings Kobayashi *et al.* (2002) who also found no STEC in fecal samples from 199 broiler chickens.

The prevalence of *Salmonella* and STEC differs depending upon sample types, sample number, collection and handling methods, the sensitivity of detection techniques, detected genes, the poor hygiene, sample transportation, geographic regions and management systems. These differences may mask the impact of other factors such as raising practices,

seasonal patterns and processing procedures that are actually causing true changes in the distribution of the bacteria (Myint, 2004).

Prevalence of *Salmonella* as identified by invA PCR and STEC as identified by stx1 PCR was different among tested broiler chickens investigated from the four farms. The highest prevalence of *Salmonella* was recorded in farms A and C (20%, 10/50), followed by farm B (8%, 4/50) and farm C (2%, 1/50). However, the prevalence of STEC was highest in farms B and C (8%, 4/50), followed by farm D (6%, 3/50) and farm A (4%, 2/50). The highest infection with both pathogens was found in broiler chickens from farm C.

Conclusion

This study showed that the overall prevalence of *Salmonella* and STEC using PCR was 12.5% and 6.5% among the whole set of tested samples. This percentage obtained through our study could be considered relatively high when compared to other studies. This high prevalence could be harmful to the poultry sector and also to the human health, because *Salmonella* and STEC pathogens play an important role in causing diseases in poultry leading to devastating economic losses as well as to the human consumer due to presence of highly pathogenic *Salmonella* and shiga-toxins of STEC. Identification of both pathogens by conventional and chromogenic culture media as well as PCR methods has shown that using conventional culture media when combined with specific target gene-PCR but not the chromogenic media was more specific, sensitive, and cost effective.

Recommendations

1. Establish a permanent program is recommended for surveillance of *Salmonella*, STEC and other food-borne pathogens in poultry in Palestine.
2. Further studies are recommended at the national level to obtain accurate statistics for the prevalence rates of these pathogens.
3. Further studies are recommended to identify the *Salmonella* serotypes occurrence among poultry farms in Palestine.
4. For farmers, is recommended to apply a healthy poultry environment to limit such spread of poultry pathogens.
5. For consumers, is recommended to consume healthy food sources that are certified by the Ministries of Agriculture and Health, and poultry meat must be properly handled, refrigerated, and cooked.

انتشار بكتيريا السالمونيلا و بكتيريا القولون المنتجة لسموم الشيجا في الدجاج اللاحم الحي في محافظة طولكرم عن طريق استخدام تقنيات الزراعة التقليدية و تفاعل البلمرة المتسلسل

الطالبة: هند عبدو

المشرف: د. أحمد صالح

الملخص

تعتبر بكتيريا السالمونيلا وبكتيريا القولون المنتجة لسموم الشيجا (STEC) المسببات لداء السالمونيلوسز (Salmonellosis) وداء كوليباسيلوسز (Collibacilosis) من الأمراض البكتيرية الرئيسية التي تصيب قطاع تربية الدواجن في جميع أنحاء العالم، مسببة خسائر اقتصادية كبيرة من خلال الوفيات وانخفاض الإنتاج. تعد هذه الأمراض من أكثر أمراض الطيور انتقالاً للإنسان، حيث أن نسبة الإصابات والوفيات المرتفعة للبشر و المرتبطة بهذين النوعين من البكتيريا سلطت الضوء على التهديد الحقيقي الذي تمثله على الصحة العامة.

هدفت هذه الدراسة إلى التحقق من مدى انتشار بكتيريا السالمونيلا و بكتيريا القولون المنتجة لسموم الشيجا (STEC) في الدجاج اللاحم الحي في محافظة طولكرم عن طريق استخدام تقنيات الزراعة التقليدية و تفاعل البلمرة المتسلسل، ولهذا الغرض تم جمع مائتي عينة مسحة شرجية من دواجن حية

خلال الفترة من أبريل ٢٠١٧ إلى نوفمبر ٢٠١٧ من أربع مزارع دجاج في منطقة طولكرم ، فلسطين.

تم الكشف عن وجود السالمونيلا و STEC باستخدام وسائط غذائية ملونة (Chromogenic) بالإضافة إلى تقنية تفاعل البلمرة المتسلسل (PCR) باستهداف جينات خاصة لكلا النوعين من البكتيريا وهي (*invA*، *16S r-RNA*، *stx2* and *stx1*). أظهرت نتائج هذه الدراسة أن معدل انتشار السالمونيلا و STEC بشكل عام كان ١٤٪ (٢٨/٢٠٠) و ٤٧٪ (٩٤/٢٠٠) باستخدام أجار HiCrome Salmonella المحسّن وأجار HiCrome EC O157: H7 على التوالي. بينما وجد أن مدى االانتشار لكلا النوعين من البكتيريا هو ١٢,٥٪ (٢٥/٢٠٠) و ٦,٥٪ (١٣/٢٠٠) باستخدام invA-PCR و stx1-PCR ، على التوالي. وقد دلت النتائج على ان معدل انتشار كلا النوعين من البكتيريا كان مختلفاً بين المزارع الأربعة التي تمت دراستها. علاوة على ذلك ، وجد أن العدوى المتزامنة من كلا النوعين من البكتيريا كانت منخفضة للغاية.

يمكن الاستدلال من النتائج على ارتفاع نسبة انتشار كلا النوعين من البكتيريا الممرضة في الدواجن اللاحمة الحية في فلسطين باستخدام طرق الكشف التقليدية والجزيئية، وهنا يجب التأكيد على ضرورة استخدام هذه الطرق معاً للحصول على نتائج دقيقة. كما أن هذه النسبة المرتفعة لانتشار كلا

النوعين من البكتيريا تشكل تهديداً صحيا للمستهلك ولقطاع تربية الدواجن على حد سواء بما يستوجب الاهتمام بدراستها بشمولية اكبر ووضع الخطط المناسبة لمكافحتها.

References

1. Aabo, S., Rasmussen O.F., Rossen, L., Sørensen, P.D, & Olsen, J.E. (1993). *Salmonella* identification by the polymerase chain reaction. Mol Cell Probes. 7:171–178.

2. Abdeltawab, A., Amma, A. M., El-Hofy, F., Abdel Hakeem, M. & Abdel Galil, N. M. (2016). Molecular Screening Of Virulence Genes In Avian Pathogenic *Esherichia coli*. benha veterinary medical journal, 30: 137-149.

3. Abed, T. K. & Ali, B. H. (2018). Molecular Detection of *Salmonella pullorum* from Poultry Ceca and Vaccine Preparation. International Journal of Poultry Science, 17: 71-77.

4. Adwan, G. M., Alqarem, B. R. & Adwan, K. M. (2015). Prevalence of foodborne pathogens in meat samples in Palestine. International Food Research Journal 22(5): 1806-1812.

5. Al-Abadi, I. K. M. & AL- Mayah, A.A.S. (2011). Isolation and identification of *Salmonella spp.* from chicken and chicken environment in Basrah province. African J. Biol. Sci., 7 (1): 33-43.

6. Alam, M., Hassan, N.A., Ahsan, S., Pazhani, G.P., Tamura, K., Ramamurthy,T., Gomes,D.J.& Rahman,S.R. *et al.* (2006). Phenoty pic and molecular characteristics of *Escherichia coli* isolated from aquatic environment of Bangladesh. Microbiol Immunol 50, 359–370.

7. Al-Khayat, L. Dh ., Khammas, E. J., Ali, R. M., Al-Owaini, B. A. & Abdulrasool, L. M. (2016). Rapid detection and identification of poultry *Salmonella* serotypes using multiplex PCR assay. MRVSA. 5 (3), 31-40.

8. Al-Khayat, L. Dh. & Khammas, E. J. (2016). Detection of *Salmonella* isolated from layer and broiler chickens samples by using Polymerase Chain Reaction Technique. International Journal of Advanced Research in Biological Sciences. 3(8): 104-108.

9. Alonso, M. Z., Lucchesi, P. M. A., Rodríguez, E. M., Parma, A. E. & Padola, N. L. (2012). Enteropathogenic (EPEC) and Shigatoxigenic *Escherichia coli* (STEC) in broiler chickens and derived products at different retail stores. Food Control 23. 351e355.

10. Al-Wasify, R.S., El-Taweel, G.E., Kamel, M.M. & El-Laithy, M.A. (2011). comparative evaluation of different chromogenic media for detection of *E. coli* O157:H7, *Listeria monocytogenes* and *salmonella* spp. in water. international journal of academic research.Vol. 3. No. 3., I Part.

11. Amavisit, P., Browning, G. F., Lightfood, D. & Anderson, C. S. (2001) Rapid PCR detection of *Salmonella* in horse faecal samples. Vet. Microbiol.79: 63-74.

12. Ammar, A. M., Mohamed, A. A., Abd El-Hamid, M. I. & El-Azzouny, M. M. (2016). Virulence genotypes of clinical *Salmonella* Serovars from broilers in Egypt. The journal of infection in developing countries; 10(4):337-346.

13. Anderson, R. C. & Ziprin ,R. L. (2001). Bacteriology of *Salmonella*. Pages 247-263 in Foodborne Disease Handbook. Vol. 1. Y. H. Hui, M. D. Pierson, and J. R.Gorham, ed. Marcel Dekker, Inc., New York, NY.

14. Antony, A. C., Paul, M. K., Silvester, R., Aneesa, P.A., Suresh, K., Divya P. S., Paul, S., Fathima P.A. & Mohamed H.A. (2016). Comparative Evaluation of EMB Agar and Hicrome *E. coli* Agar for Differentiation of Green Metallic Sheen Producing Non *E. coli* and

Typical *E. coli* Colonies from Food and Environmental Samples. Journal of pure and microbiology. Vol. 10(4), p. proof.

15. Araj, GF. & Chugh, T.D. (1987). Detection of *Salmonella* spp. in clinical specimens by capture enzyme-linked immunosorbent assay. J Clin Microbiol. 25:2150–2153.

16. Aspinall, S.T., Hindle, M.A., & Hutchinson, D.N. (1992). Improved isolation of *Salmonellae* from feces using a semisolid Rappaport-Vassiliadis medium. Eur J Clin Microbiol Infect Dis. 11:936–939.

17. Bélanger, L., Garenaux, A. & Harel, J *et al*. (2011) *Escherichia coli* from animal reservoirs as a potential source of human extraintestinal pathogenic *E. coli*. FEMS Immunol Med Microbiol 62:1-10.

18. Bennasar, A., Luna, G., Cabrer, B. & Lalucat, J. 2000. Rapid identification of *Salmonella typhimurium, S.enteritidis and S. virchow* isolates by polymerase chain reaction based PCR. Appl. Env. Microbiol., 26: 2235-2237.

19. Beutin, L. (2006). "Emerging enterohaemorrhagic *Escherichia coli*, causes and effects of the rise of a human pathogen". Journal of veterinary medicine. B, Infectious diseases and veterinary public health. 53 (7): 299–305.

20. Buchanan, R.E. & Gibbon, N.E. (1994) Bergey's Manual of Determinative Bacteriology, 9th edn. Baltimore, MD: Williams& Wilkins, pp. 787.

21. Byomi, A., Zidan, S., Diab, M., Reddy, G. & Abdela, W, *et al*. (2017) Characterization of Diarrheagenic *Escherichia Coli* Serotypes Isolated from Poultry and Humans. SOJ Vet Sci 3(1): 1-8.

22. Candrian, U., Furrer, B., Hofelein, C., Meyer, R. , Jermini, M. & Luthy, J. (1991). Detection of *Escherichia coli* and identification of enterotoxigenic strains by primer-directed enzymatic amplification of specific DNA sequences. Int. J. Food Microbiol. 12:339–351.

23. Caprioli, A., Morabito, S., Brugere, H. & Oswald, E. (2005). Enterohaem- orrhagic *Escherichia coli*: emerging issues on virulence and modes of trans- mission. Vet. Res. 36:289–311.

24. Carli, K.T., Eyigor, A. & Caner, V. (2001). Prevalence of *Salmonella* serovars in chickens in Turkey. Journal of Food Protection 64(11): 1832-1835.

25. Chandran, A. & Mazumder, A. (2014). Occurrence of Diarrheagenic Virulence Genes and Genetic Diversity in *Escherichia coli* Isolates from Fecal Material of Various Avian Hosts in British Columbia, Canada. Appl Environ Microbiol. 80(6): 1933–1940.

26. Chiu, L.H., Chiu, C.H., Horn, Y.M., Chiou, C.S., Lee, C.Y., Yeh ,C.M., Yu, C.Y., Wu, C.P., Chang, C.C. & Chu, C. (2010). Characterization of 13 multi-drug resistant *Salmonella* serovars from different broiler chickens associated with those ofhumanisolates. BMCMicrobiol.10:86.http://dx.doi.org/10.1186/1471 -2180-10-86.

27. Christensen, J.P., Olsen, J.E., Hansen, H.C. & Bisgaard, M. (1992). Characterization *of Salmonella enterica* serovar gallinarum biovars gallinarum and pullorum by plasmid profiling and biochemical analysis. Avian Pathol. 21: 461–470.

28. Compassion in World Farming - Poultry". Ciwf.org.uk. Retrieved October 3, 2018, from https://www.ciwf.org.uk/farm-animals/chickens/.

29. Cox, JM. (1993). Lysine-mannitol-glycerol agar, a medium for the isolation of *Salmonella* spp., including *S. typhi* and atypical strains. Appl. Environ. Microbiol. 59:2602–2606.

30. D'Aoust, J. Y, 1989. *Salmonella*. Foodborne Bacterial Pathogens. M. P. Doyle, ed. Marcel Dekker Inc., New York, NY. pp 327-445.

31. Darwin, K. H. & Miller, V. L. (1999). Molecular Basis of the Interaction of *Salmonella* with the Intestinal Mucosa. *Clinical Microbiology Reviews*, *12*(3), 405–428.

32. Deisingh, A. K. & Thompson, M. (2004). Strategies for the detection of *Escherichia coli* O157:H7 in foods. J. Appl. Microbiol. 96, 419-429.

33. Doregiraee, F., Alebouyeh, M., Nayeri Fasaei, B., Charkhkar, S., Tajedin, E. & Zali, M.R. (2016). Isolation of atypical enteropathogenic and shiga toxin encoding *Escherichia coli* strains from poultry in Tehran, Iran. Gastroenterol Hepatol Bed Bench. 9(1): 53–57.

34. Doyle, M.P. & Erickson, M.C. (2006). Reducing the carriage of foodborne pathogens in livestock and poultry. Poultry Sci. 85:960–973.

35. Eid. H. I., Algammal, A .M., Nasef, S. A., Elfeil, W. K. & Mansour, G. H, 2016. Genetic Variation among Avian Pathogenic *E.coli* Strains Isolated from Broiler Chickens. Asian Journal of Animal and Veterinary Advances, 11: 350-356.

36. ElKichaoui, A.Y., Elmanama, A.A. & Msallam, A.K. (2017). Bacteriological Occurrence of *Salmonella* spp. in Hens Eggs and their Environment in Selected Farms in Gaza Strip. IUG Journal of Natural Studies. Vol 25, No 1, pp 13-20.

37. Elmanama, A.A., El Kahlout, K.A. & Elnakhalaa, S.A. (2013, December). Occurrence of *Salmonella* in fresh and frozen meat in the Gaza strip. Journal , Utility Reaction and Environment Vol.2 No.6:142-152.

38. El-Sharkawy, H., Tahoun, A., El-Gohary, A.E.A., El-Abasy, M., El-Khayat, F., Gillespie, T., Kitade, Y., Hafez, H. M., Neubauer, H. & El-Adawy, H. (2017, Feb) Epidemiological, molecular characterization and antibiotic resistance of *Salmonella enterica* serovars isolated from chicken farms in Egypt. Gut Pathog. 9: 8. 10. doi: 10.1186/s13099-017-0157-1.

39. Erickson, M. C. & Doyle, M. P. (2007). Food as a vehicle for transmission of Shiga toxin-producing *Escherichia coli*. J. Food Prot. 70:2426–2449.

40. Ewers,C., Janssen, T., Kiessling, S., Philip, H. C. & Wieler, L. H. (2005). Rapid detection of virulence associated genes in avian pathogenic *Escherichia coli* by multiplex Polymerase chain reaction. Avian Dis., 49:269-273.

41. Farooq, S., Hussain, I., Mir, M. A., Bhat, M. A. & Wani, S. A. (2009). Isolation of atypical enteropathogenic Escherichia coli and Shiga toxin 1 and 2f-producing *Escherichia* coli from avian species in India. Lett Appl Microbiol. 48:692–97.

42. Feng, P., Dey, M., Abe, A. & Takeda, T. (2001). Isogenic strain of *Escherichia coli* O157: H7 that has lost both Shiga toxin 1 and 2 genes. Clin. Diagn. Lab. Immunol. 8, 711–717.

43. Ferretti, R., Mannazzu , L., Cocolin, L., Comi, G. & Clementi, F. (2001). Twelve-hours PCR-based method for detection of *Salmonella* spp. In food. Appl. Environ. Microbiol., 74: 977-978.

44. Foley, S.L., Nayak, R., Hanning, I.B., Johnson, T.J., Han, J. & Ricke, S.C. (2011). Population dynamics of *Salmonella enterica* serotypes in commercial egg and poultry production. Appl. Environ. Microbial. 77:4273–4279.http: //dx.doi.org/10.1128/AEM.00598-11.

45. Fratamico, P.M., Bagi L.K., Cray W.C., Narang N., Yan X., Medina M. & Liu Y. (2011). Detection by multiplex real-time polymerase chain reaction assays and isolation of Shiga toxin–producing *Escherichia coli* serogroups O26, O45, O103, O111, O121, and O145 in Ground Beef. Foodborne Pathog. Dis. 8:601-607.

46. Fratamico, PM. (2003). Comparison of culture, polymerase chain reaction (PCR), TaqMan *Salmonella*, and Transia Card *Salmonella* assays for detection *of Salmonella* spp. in naturally-contaminated ground chicken, ground turkey, and ground beef. Mol Cell Probes. 17:215–221.

47. Garcia-del Portillo, F., Pucciarelli, M. G. & Casadesus, J. (1999). DNA adenine methylase mutants of Salmonella typhimurium show defects in protein secretion, cell invasion, and M cell cytotoxicity. Proc Natl Acad Sci U S A 96, 11578–11583.

48. Ghanbarpour, R., Sami, M., Salehi, M. & Ouromiei, M. (2011). Phylogenetic background and virulence genes of Escherichia coli isolates from colisepticemic and healthy broiler chickens in Iran. Trop Anim Health Prod. 43:153–7.

49. Global Poultry Trends - Developing Countries Main Drivers in Chicken Consumption. (2016, February 2). Retrieved October 3, 2018, from www.thepoultrysite.com/articles/3588/global-poultry-trends-developing-countries-main-drivers-in-chicken-consumption.

50. Global Poultry Trends: Poultry to Boost Global Meat Market Share Above 36 Per Cent**. (**2016, July 19). Retrieved October 3, 2018, from

http://www.thepoultrysite.com/articles/3645/global-poultry-trends-poultry-to-boost-global-meat-market-share-above-36-per-cent/.

51. Gong J., Zhang J., Xu M., Zhu C., Yu Y., Liu X., Kelly P, Xu B. & Wang C. (2014 Jan). Prevalence and Fimbrial Genotype Distribution of Poultry *Salmonella*Isolates in China (2006 to 2012). Appl Environ Microbiol. 80(2): 687–693.

52. Gray, J. T. & Fedorka-Cray, P. J. (2002). *Salmonella*. In Cliver, D. O. and Riemann, H. P. (Eds.). Foodborne diseases, p. 55-68. San Diego: Academic Press.

53. Griffin, P. M., Ostroff, S. M., Tauxe, R. V., Greene, K. D., Wells, J. H., Lewis, J. G. & Blake P. A. (1988). Illness associated with *Escherichia coli* 0157:H7 infections: a broad clinical spectrum. Ann. Intern. Med. 109:705-712.

54. Grimont, P.A.D., Grimont, F. & Bouvet, P. (2000). Taxonomy of the genus *Salmonella*. In Salmonella in domestic animals., Page: 1-17.

55. Gruenewald, R., Henderson, R. w. & Yappow, S. (1991). Use of Rambach propylene glycol containing agar for identification of *Salmonella* spp. J. Clin. Microbiol. 29, 2354-2356.

56. Guo, L., Killfer, Kenny, P.B. & Amick Morris, J.D. (1999). Use of arbitrarily primed Polymerase chain reaction to study *Salmonella* ecology in a turkey production. Environment. Poult.Sci.78:24-31.

57. Hald, T., Vose, D., Wegener, H.C. & Koupeev, T. A. (2004). Bayesian approach to quantify the contribution of animal-food sources to human salmonellosis. Risk Anal. 24:255–269.

58. Hanes, D. (2003) Nontyphoid *Salmonella*. Ch 9 In: Miliotis MD, Bier JW (eds) International handbook of foodborne pathogens. Marcel Dekker, New York, p. 137–149.

59. Henry, I., Sophie, G., Celine C., Francoise, L., Marianne, C., Gilles, S. & Cardinale, E. (2012). *Salmonella* enterica subsp. enterica isolated from chicken carcasses at slaughter in Reunion Island: prevalence, genetic characterization and antibiotic susceptibility. Trop. Anim. Health Prod. 10: 1250-012-0221-2.

60. Himi H. A., Parvej, Md. S ., Rahman, M. B ., Nasiruddin K. M ., Ansari, W. K. & Ahamed, Md .M. (2015). PCR Based Detection of Shiga Toxin Producing *E. coli* in Commercial Poultry and Related Environments. Turkish Journal of Agriculture - Food Science and Technology, 3(6): 361-364.

61. Holko, I., Bisova, T., Holkova, Z. & Kmet, V. (2006). Virulence markers of *Escherichia coli* strains isolated from traditional cheeses made from unpasteurise sheep milk in Slovakia. Food Contr.17:393–396.

62. Holt, J.G., Krieg, N.R., Sneath, P.H.A., Stanley, J.T. & William, S.T. (1994) Bergey's Manual of Determinative Bacteriology. Williams and Wilikins, Baltimore, 786-788.

63. Hossain, Z., Sultana, P., Deb, S. & Ahmed, M.M. (2011). Multidrug resistance in large-plasmid-associated presumptive enterohaemorrhagic *Escherichia coli* isolated from contaminated lake water. Bangladesh Journal of Microbiology 28: 33-40.

64. Ibrahim, M.A., Emeash, H.H., Ghoneim, N. H. & Abdel-Halim, M.A. (2013). Seroepidemiological Studies on Poultry Salmonellosis and its Public Health Importance. J. World's Poult. Res. 3(1): 18-23.

65. Ibrahim, W. A., Abd El-Ghany, W. A., Nasef , S. A. & Hatem, M. E. (2014). A comparative study on the use of real time polymerase chain

reaction (RT-PCR) and standard isolation techniques for the detection of *Salmonellae* in broiler chicks. International Journal of Veterinary Science and Medicine. http://dx.doi.org/10.1016/j.ijvsm.2013.11.001.

66. Islam, M.A., Mondol, A.S., de Boer, E., Beumer, R.R., Zwietering, M.H., Talukder, K.A. & Heuvelink, A.E. (2008). Prevalence and genetic characterization of shiga toxin-producing *Escherichia coli* isolates from slaughtered animals in Bangladesh. Appl Environ Microbiol 74, 5414–5421.

67. Jamshidi, A., Bassami, M.R. & Afshari-Nic, S. (2009). Identification of *Salmonella* spp. and *Salmonella typhimurium* by a multiplex PCR-based assay from poultry carcasses in Mashhad- Iran Int.J.Vet.Res. 3,1:43-48.

68. Janda, J.M. & Abbott, S.L. (2008). The enterobacteria, 2nd ed. ASM Press, Washington, DC.

69. Jinu, M., Agarwal, R.K., Sailo, B., Wani, M.A., Kumar, A., Dhama, K. & Singh, M.K. (2014). Comparison of PCR and Conventional Cultural Method for Detection of *Salmonella* from Poultry Blood and Faeces. Asian Journal of Animal and Veterinary Advances, 9: 690-701.

70. Joris, M.-A., Vanrompay, D., Verstraete, K., De Reu, K., De Zutter, L. & Cox, E. (2013). Use of Antibody Responses against Locus of Enterocyte Effacement (LEE)-Encoded Antigens To Monitor Enterohemorrhagic *Escherichia coli* Infections on Cattle Farms. *Applied and Environmental Microbiology*, *79*(12), 3677–3683.

71. Kaddu-Mulindw, D. H., Aisu, T., Gleier, K., Zimmermann, S. & Beutin, L. (2001). Occurrence of Shiga toxin-producing *Escherichia coli* in fecal samples from children with diarrhoea and from healthy zebu cattle in Uganda. Int J Food Microbiol 66, 95–101.

72. Karmali, M. A. (1989). Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbial. Rev. 2:15-38.

73. Karmali, M. A., Petric, M., Lim, C., Cheung, R. & Arbus, G. S. (1985). Sensitive method for detecting low numbers of Verotoxin-producing *Escherichia coli* in mixed cultures by use of colony sweeps and polymixin extraction of Verotoxins . J. Clin. Micro- biol. 22:614-619.

74. Kobayashi H., Pohjanvirta T. & Pelkonen S. (2002). Prevalence and Characteristics of Intimin- and Shiga Toxin-Producing Escherichia coli from Gulls, Pigeons and Broilers in Finland. Journal of Veterinary Medical science. Volume 64 Issue 11 Pages 1071-1073.

75. Kobayashi, H., Kanazaki, M., Hata, E. & Kubo, M. (2009). Prevalence and characteristics of *eae*- and *stx*-positive strains of *Escherichia coli* from wild birds in the immediate environment of Tokyo Bay. Applied and Environmental Microbiology. 75:292-295.

76. Kovacs, M. J., Roddy, J., Gregoire, S., Cameron, W., Eidus, L. & Drouin J. (1990). Thrombotic thrombocytic purpura following hemorrhagic colitis due to *Escherichia coli* 0157:H7. Am. J. Med. 88:177-179.

77. Kwang, J., Littledike, E.T. & Keen, J.E. (1996) Use of the polymerase chain reaction for *Salmonella* detection. Letters in Applied Microbiology 22, 46–51.

78. Lampel, K.A., Orlandi, P.A. & Kornegay, L. (2000). Improved template preparation for PCR-based assay for detection of food-borne bacterial pathogens. Appl. Environ. Microbiol., 66: 4539- 4542.

79. Lassniq, H., Much, P., Schliessnig, H., Osterreicher, E., Kostenzer, K., Korns chober, C. & Kofer, J.(2012). Prevalence of Salmonella

spp. in Austrian broiler flocks in the context of the EU-wide baseline survey 2005-2006. Berl Munch TierarztI Wochen schr, 125: 129-137.

80. Leelaporn, A., Phengmak, M., Eampoklap, B., Manatsathit, S., Tritilanunt, S., Siritantikorn, S., Nagayama, K., Iida, T., Niyasom, C. & Komolpit, P. (2003). Shiga toxin- and enterotoxin-producing *Escherichia coli* isolated from subjects with bloody and nonbloody diarrhoea in Bangkok, Thailand. Diagn Microbiol Infect Dis 46, 173–180.

81. Levin, R. (2009). The use of molecular methods for detecting and discriminating *Salmonella* associated with foods. A review. Food Biotech. 23:313-367.

82. Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'Brien, S.J., Jones, T.F., Fazil, A. 7 Hoekstra, R.M. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritisClin Infect Dis, 50. pp. 882-889.

83. Malorny, B., Hoorfar, J., Bunge, C. & Helmuth, R. (2003). Multicenter validation of the analytical accuracy of *Salmonella* PCR: towards an international standard. Appl. Environ. Microbiol., 69: 290-296.

84. Malorny, B., Huehn, S., Dieckmann, R., Kramer, N. & Helmuth R. (2009). Polymerase chain reaction for the rapid detection and serovar identification of *Salmonella* in food and feeding stuff. Food Anal Meth. 2:81–95.

85. Mamun, M. M., Parvej, M. S., Ahamed, S., Hassan, J., Nazir, K. H. M. N. H., Nishikawa Y. & Rahman M. T. (2016). Prevelance and characterization of shiga toxigenic *Escherichia coli* in broiler birds in Mymensingh, Bangl. J. Vet. Med. 14 (1): 5-8.

86. Myint, M.S. (2004). Epidemiology of Salmonella contamination of poultry products; Knowledge gaps in the farm to store products. Faculty of the graduate school of the University of Maryland, dissertation.

87. Nataro, J. P. & Kaper J. B. (1998). Diarrheagenic *Escherichia coli*. Clin. Microbiol. Rev. 11**:**142-201.

88. O'Brien, A. D. & Holmes R. K. (1987). Shiga and Shiga-like toxins. Microbial. Rev. 51:206-220.

89. OECD/Food and Agriculture Organization of the United Nations (2015), OECD-FAO Agricultural Outlook 2015, OECD Publishing, Paris. http://dx.doi.org/10.1787/agr_outlook-2015-en.

90. Oliveira, S.D., RSantos, L., Schuch, D.M.T., Silva, A.B., Salle, C.T.B. and Canal, C.W. (2002). Detection and identification of salmonellas from poultry-related samples by PCR. Veterinary Microbiology Volume 87, Issue 1, 5, Pages 25-35.

91. Paião F.G., Arisitides L.G.A., Murate L.S., Vilas-Bôas G.T., Vilas-Boas L.A. & Shimokomaki M. (2013). Detection of *Salmonella* spp, *Salmonella* Enteritidis and Typhimurium in naturally infected broiler chickens by a multiplex PCR-based assay. Braz J Microbiol. 44(1): 37–41.

92. Panisello, P. J., Rooney, R., quantick, P. C. & Stanwell-Smith, R. (2000). Application of foodborne disease outbreak data in the development and maintenance of HACCP system. Int. J. Food Microbiol. 59: 221-234.

93. Park, S-H., Ryu, S. & Kang D-H. (2012). Development of an Improved Selective and Differential Medium for Isolation of *Salmonella* spp. Journal of Clinical Microbiology. p. 3222–3226.

94. Paton, A.W. & Paton, J.C. (1998) Detection and characterization of shiga toxigenic *Escherichia coli* by using multiplex PCR assays for stx1, stx2, eaeA, enterohemorrhagic E. coli ehxA, rfbO111 and rfbO157. J Clin Microbiol 36, 598–602.

95. Paton, A.W. & Paton, J.C. (2002) Direct detection and characterization of Shiga toxigenic *Escherichia coli* by multiplex PCR for stx1, stx2, eae, ehxA, and saa. J Clin Microbiol 40, 271–274.

96. Paul, N., Kennedy, F. C. & Shoyinka, S.V.O. (2016). Molecular Detection of *Salmonella* Isolated from Poultry Farms in Abia State Southeast Nigeria. Int.J.Curr.Microbiol.App.Sci. 5(7): 961-968.

97. Portillo, F. G. (2000). Molecular and cellular biology of *Salmonella* pathogenesis. In Cary, J. W. and Bhatnagar, D. (Eds.), Microbial foodborne diseases: Mechanisms of pathogenesis and toxin synthesis, p. 3-34). United States of America: Technomic Publishing Company, Inc.

98. Qabajah M. H. (2011). Avian Pathogenic *Escherichia coli* (APEC) in Palestine: Characterization of Virulence Factors and Antibiotic Resistance Profile. Puplished. Polytechnic University and the Faculty of Science at Bethlehem University. Palestine.

99. Rahn, K., De Grandis, S. A., Clarke, R. C., Curtiss, R. & Gyles, C. L. (1992). Amplification of an invA gene sequence of *Salmonalla typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. Mol. Cell. Probes., 6: 271- 279.

100. Rasschaert, G., Houf, K., Godard, C., Wildemauwe, C., Pastuszczak-Frak, M. & De Zutter L. (2008). Contamination of carcasses with *Salmonella* during poultry slaughter. J Food Prot. 71:146–152.

101. Runa J. A., Lijon M. B. & Rahman M. A. (2018). Detection of Multidrug Resistant and Shiga Toxin Producing *Escherichia coli* (STEC) From Apparently Healthy Broilers in Jessore, Bangladesh. Frontiers in Environmental Microbiology. Vol. 4, No. 1, pp. 16-21. doi: 10.11648/j.fem.20180401.13.

102. Ryan, K.J. & Ray, C.G. (editors) (2004). Sherris Medical Microbiology (4th ed.). McGraw Hill. pp. 362–8. ISBN 0-8385-8529-9.

103. Sanchez, S., Hofacre, C.L., Lee, M.D., Maurer, J.J. & Doyle, M.P. (2002). Animal sources of salmonellosis in humans.J.Am.Vet.Med.Assoc.221:492–497.

104. Scallan, E., Hoekstra R.M., Angulo F.J., Tauxe R.V., Widdowson M.-A., Roy S.L., Jones J.L. & Griffin P.M. (2011). Foodborne illness acquired in the United States—major pathogens. Emerg. Infect. Dis. 17:7-15.

105. Schneder, A., Gronewald, C., Fandke, M., Kurth, B., Barkowski, S. & Berghof – ager, K.(2002). Real-time detection of the genus Salmonella with the Light Cycler system. Biochemica., 4: 19-21.

106. Selim, S. A., Ahmed, S. F., Abdel Aziz, M. H., Zakaria, A.M., Klena, J. D. & Pangallo D. (2013). Prevalence and Characterization of Shiga-Toxin O157:H7 and Non-O157:H7 Enterohemorrhagic *Escherichia coli* Isolated from Different Sources. Biotechnology & Biotechnological Equipment. 27(3), 3834-3842.

107. Smith, H. R., & S. M. Scotland. (1988). Vero cytotoxin- producing *Escherichia coli*. J. Med. Microbial. 26:77-85.

108. Smith, H.W. (1965) The development of the flora of the alimentary tract in young animals. J Pathol Bacteriol 90:495-513.

109. Su, LH. & Chiu, CH. (2007). "*Salmonella*: clinical importance and evolution of nomenclature.". Chang Gung medical journal 30 (3): 210–9. PMID 17760271.

110. Tabatabaei, M., Mokarizadeh, A. & Foad-Marashi, N. (2011). Detection and Molecular Characterization of Sorbitol Negative Shiga Toxigenic *Escherichia Coli* in Chicken from Northwest of Iran. Veterinary Research Forum. 183 – 188.

111. Talukdar ,P.K., Rahman, M., Rahman, M., Nabi, A., Islam ,Z., Hoque, M., Endtz, H.P. & Islam, M.A. (2013). Antimicrobial resistance, virulence factors and genetic diversity of Escherichia coli isolates from household water supply in Dhaka, Bangladesh. PloS ONE 8: e61090.

112. Tejada, T. S., Silva, C. S. J., Lopes, N. A., Silva, D. T., Agostinetto, A., Silva, E. F., Menezes, D. B. & Timm, C. D. (2016, Oct - Dec) DNA Profiles of *Salmonella* Spp. Isolated from Chicken Products and From Broiler and Human Feces. Brazilian Journal of Poultry Science. ISSN 1516-635X.V.18 / n.4 / 693-700.

113. Toze, S. (1999). PCR and the detection of microbial pathogen in water and waste water. Wat. Res. Vol. 33, No. 17, pp. 3545±3556.

114. Wang, R. F., Cao, W.W. & Cerniglia ,C.E. (1996) . PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. Appl. Environ. Microbiol. 62:1242–1247.

115. Wani, S.A., Samanta, I., Bhat, M.A. & Nishikawa, Y. (2004). Investigation of Shiga toxin-producing *Escherichia coli* in avian species in India. Lett Appl Microbiol. 39:389–94.

116. White, P., Meglli, K., Collins, D. & Gormely, E. (2002). The prevalence and PCR detection of Salmonella contamination in raw poultry. Vet Microbiol. 89: 53-60.

117. Zhao, X., Gao, Y., Ye, C., Yang, L., Wang, T. & Chang, W. (2016). Prevalence and Characteristics of *Salmonella* Isolated from Free-Range Chickens in Shandong Province, ChinaBiomed Res Int. 8183931.

118. Ziebell, K.A., Read, S.C., Johnson,RP. & Gyles, C.L. (2002).Evaluation of PCR and PCRRFLP protocols for identifying Shiga toxins. Research in Microbiology. 153:289-300.

Appendix

Sample number	Farm	Sample type	On XLD	On Hicrome	InvA PCR	On EMB	On Hicrome	EC PCR	STX1 PCR	STX2 PCR
1	A	Cloacal sample	-	-	-	+	+	+	+	-
2	A	Cloacal sample	+	-	-	+	-	+	-	-
3	A	Cloacal sample	+	+	-	+	-	+	-	-
4	A	Cloacal sample	-	-	-	+	+	+	-	-
5	A	Cloacal sample	+	+	-	+	-	+	-	-
6	A	Cloacal sample	+	-	-	+	-	+	-	-
7	A	Cloacal sample	+	-	-	+	-	+	-	-
8	A	Cloacal sample	-	-	-	+	-	+	-	-
9	A	Cloacal sample	+	-	-	+	-	+	-	-
10	A	Cloacal sample	+	-	-	+	+	+	-	-
11	A	Cloacal sample	-	-	-	+	+	+	-	-
12	A	Cloacal sample	+	-	-	+	-	+	-	-
13	A	Cloacal sample	+	+	+	+	-	+	-	-
14	A	Cloacal sample	+	+	+	+	-	+	-	-
15	A	Cloacal sample	+	-	-	+	-	+	-	-
16	A	Cloacal sample	+	-	-	+	+	+	+	-
17	A	Cloacal sample	-	-	-	+	+	+	-	-
18	A	Cloacal sample	-	-	-	+	+	+	-	-
19	A	Cloacal sample	-	-	-	+	+	+	-	-
20	A	Cloacal sample	-	-	-	+	-	+	-	-
21	A	Cloacal sample	-	-	-	+	-	+	-	-
22	A	Cloacal	+	-	+	+	-	+	-	-

		sample								
23	A	Cloacal sample	+	+	-	+	-	+	-	-
24	A	Cloacal sample	+	+	-	+	+	+	-	-
25	A	Cloacal sample	-	-	-	+	+	+	-	-
26	A	Cloacal sample	-	-	-	+	-	+	-	-
27	A	Cloacal sample	+	+	-	+	+	+	-	-
28	A	Cloacal sample	-	+	-	+	+	+	-	-
29	A	Cloacal sample	+	-	+	+	+	+	-	-
30	A	Cloacal sample	+	+	+	+	+	+	-	-
31	A	Cloacal sample	-	-	-	+	+	+	-	-
32	A	Cloacal sample	+	-	-	+	+	+	-	-
33	A	Cloacal sample	+	-	-	+	-	+	-	-
34	A	Cloacal sample	+	+	-	+	-	+	-	-
35	A	Cloacal sample	+	+	-	+	-	+	-	-
36	A	Cloacal sample	-	-	-	+	-	+	-	-
37	A	Cloacal sample	+	+	-	+	-	+	-	-
38	A	Cloacal sample	+	+	-	+	-	+	-	-
39	A	Cloacal sample	+	+	+	+	-	+	-	-
40	A	Cloacal sample	+	+	+	+	-	+	-	-
41	A	Cloacal sample	+	+	-	+	-	+	-	-
42	A	Cloacal sample	-	-	-	+	+	+	-	-
43	A	Cloacal sample	-	-	-	+	-	+	-	-
44	A	Cloacal sample	-	-	-	+	-	+	-	-
45	A	Cloacal sample	+	+	+	+	-	+	-	-
46	A	Cloacal sample	+	+	-	+	-	+	-	-
47	A	Cloacal	-	-	-	+	+	+	-	-

		sample								
48	A	Cloacal sample	-	-	-	+	+	+	-	-
49	A	Cloacal sample	+	+	+	+	-	+	-	-
50	A	Cloacal sample	+	+	+	+	-	+	-	-
51	B	Cloacal sample	-	-	-	+	+	+	-	-
52	B	Cloacal sample	-	-	-	+	-	+	-	-
53	B	Cloacal sample	-	-	-	+	-	+	-	-
54	B	Cloacal sample	-	-	-	+	-	+	-	-
55	B	Cloacal sample	-	-	-	+	-	+	-	-
56	B	Cloacal sample	-	-	-	+	-	+	-	-
57	B	Cloacal sample	-	-	-	-	-	-	-	-
58	B	Cloacal sample	+	-	-	+	-	+	-	-
59	B	Cloacal sample	-	-	-	+	-	+	-	-
60	B	Cloacal sample	-	-	-	+	-	+	-	-
61	B	Cloacal sample	-	-	-	+	+	+	-	-
62	B	Cloacal sample	-	-	-	+	-	+	-	-
63	B	Cloacal sample	-	-	-	+	-	+	-	-
64	B	Cloacal sample	-	-	-	+	+	+	+	-
65	B	Cloacal sample	-	-	-	+	+	+	+	-
66	B	Cloacal sample	+	-	-	+	+	+	-	-
67	B	Cloacal sample	-	-	-	-	+	-	-	-
68	B	Cloacal sample	-	-	-	+	+	+	+	-
69	B	Cloacal sample	-	-	-	+	+	+	-	-
70	B	Cloacal sample	-	-	-	+	-	+	-	-
71	B	Cloacal sample	-	+	-	+	+	+	-	-
72	B	Cloacal	-	-	-	+	+	+	-	-

		sample								
73	B	Cloacal sample	-	-	-	+	-	+	-	-
74	B	Cloacal sample	-	-	-	+	+	+	-	-
75	B	Cloacal sample	-	-	-	+	-	+	-	-
76	B	Cloacal sample	-	-	-	+	+	+	-	-
77	B	Cloacal sample	-	-	-	-	-	-	-	-
78	B	Cloacal sample	-	+	-	+	-	+	-	-
79	B	Cloacal sample	-	-	-	+	-	+	-	-
80	B	Cloacal sample	-	-	-	+	-	+	-	-
81	B	Cloacal sample	-	-	-	+	-	+	-	-
82	B	Cloacal sample	-	-	-	+	+	+	+	-
83	B	Cloacal sample	+	-	+	+	-	+	-	-
84	B	Cloacal sample	-	-	-	+	-	+	-	-
85	B	Cloacal sample	+	-	+	+	-	+	-	-
86	B	Cloacal sample	+	-	-	+	-	+	-	-
87	B	Cloacal sample	+	-	-	+	+	+	-	-
88	B	Cloacal sample	+	+	-	+	-	+	-	-
89	B	Cloacal sample	+	-	-	+	-	+	-	-
90	B	Cloacal sample	-	+	-	+	-	+	-	-
91	B	Cloacal sample	+	-	+	+	-	+	-	-
92	B	Cloacal sample	-	-	-	-	-	-	-	-
93	B	Cloacal sample	+	+	+	+	-	+	-	-
94	B	Cloacal sample	-	-	-	+	+	+	-	-
95	B	Cloacal sample	-	-	-	+	+	+	-	-
96	B	Cloacal sample	+	-	-	+	-	+	-	-
97	B	Cloacal	+	+	-	+	-	+	-	-

		sample								
98	B	Cloacal sample	+	+	-	+	-	+	-	-
99	B	Cloacal sample	-	-	-	+	+	+	-	-
100	B	Cloacal sample	+	-	-	+	-	+	-	-
101	C	Cloacal sample	+	-	+	+	+	+	-	-
102	C	Cloacal sample	+	-	+	+	-	+	+	-
103	C	Cloacal sample	+	-	-	+	-	+	-	-
104	C	Cloacal sample	-	-	-	+	+	+	-	-
105	C	Cloacal sample	-	-	-	+	+	+	-	-
106	C	Cloacal sample	-	-	-	+	+	+	-	-
107	C	Cloacal sample	+	-	-	+	+	+	-	-
108	C	Cloacal sample	-	-	-	+	-	+	-	-
109	C	Cloacal sample	+	-	-	+	+	+	-	-
110	C	Cloacal sample	+	-	-	+	+	+	-	-
111	C	Cloacal sample	-	-	-	+	-	+	-	-
112	C	Cloacal sample	+	-	-	+	+	+	-	-
113	C	Cloacal sample	+	-	-	+	+	+	+	-
114	C	Cloacal sample	-	-	-	+	+	+	-	-
115	C	Cloacal sample	-	-	-	+	+	+	-	-
116	C	Cloacal sample	-	-	-	+	-	+	-	-
117	C	Cloacal sample	+	-	-	+	-	+	-	-
118	C	Cloacal sample	-	-	-	+	-	+	-	-
119	C	Cloacal sample	-	-	-	+	-	+	-	-
120	C	Cloacal sample	+	-	-	+	+	+	-	-
121	C	Cloacal sample	-	-	-	+	+	+	-	-
122	C	Cloacal	+	-	+	+	-	+	-	-

		sample								
123	C	Cloacal sample	+	-	+	+	+	+	-	-
124	C	Cloacal sample	+	-	-	+	+	+	-	-
125	C	Cloacal sample	-	-	-	+	+	+	-	-
126	C	Cloacal sample	-	-	-	+	+	+	-	-
127	C	Cloacal sample	-	-	-	+	+	+	-	-
128	C	Cloacal sample	-	-	-	+	+	+	-	-
129	C	Cloacal sample	+	-	-	+	+	+	-	-
130	C	Cloacal sample	+	-	-	+	+	+	-	-
131	C	Cloacal sample	-	-	-	+	+	+	-	-
132	C	Cloacal sample	-	-	-	+	+	+	-	-
133	C	Cloacal sample	-	-	-	+	+	+	-	-
134	C	Cloacal sample	+	-	-	+	+	+	-	-
135	C	Cloacal sample	+	-	+	+	+	+	-	-
136	C	Cloacal sample	-	-	-	+	-	+	-	-
137	C	Cloacal sample	+	-	-	+	+	+	-	-
138	C	Cloacal sample	+	-	-	+	+	+	-	-
139	C	Cloacal sample	+	-	-	+	-	+	-	-
140	C	Cloacal sample	-	-	-	+	-	+	-	-
141	C	Cloacal sample	-	-	-	+	-	+	+	-
142	C	Cloacal sample	+	+	+	+	-	+	-	-
143	C	Cloacal sample	+	-	+	+	-	+	-	-
144	C	Cloacal sample	-	-	-	+	-	+	-	-
145	C	Cloacal sample	+	-	+	+	+	+	-	-
146	C	Cloacal sample	-	-	-	+	+	+	-	-
147	C	Cloacal	+	-	+	+	-	+	-	-

		sample								
148	C	Cloacal sample	-	-	-	+	+	+	-	-
149	C	Cloacal sample	+	-	+	+	-	+	-	-
150	C	Cloacal sample	-	-	-	+	+	+	+	-
151	D	Cloacal sample	+	-	-	+	-	+	-	-
152	D	Cloacal sample	-	-	-	+	-	+	-	-
153	D	Cloacal sample	-	-	-	+	-	+	-	-
154	D	Cloacal sample	-	-	-	+	+	+	-	-
155	D	Cloacal sample	+	-	-	+	+	+	-	-
156	D	Cloacal sample	-	-	-	+	+	+	-	-
157	D	Cloacal sample	+	-	-	+	-	+	-	-
158	D	Cloacal sample	-	-	-	+	+	+	-	-
159	D	Cloacal sample	-	-	-	+	+	+	-	-
160	D	Cloacal sample	+	-	-	+	+	+	-	-
161	D	Cloacal sample	-	-	-	+	+	+	-	-
162	D	Cloacal sample	-	-	-	+	+	+	-	-
163	D	Cloacal sample	-	-	-	+	-	+	+	-
164	D	Cloacal sample	-	-	-	+	-	+	-	-
165	D	Cloacal sample	+	-	-	+	+	+	-	-
166	D	Cloacal sample	-	-	-	+	+	+	-	-
167	D	Cloacal sample	-	-	-	+	-	+	-	-
168	D	Cloacal sample	-	-	-	+	-	+	-	-
169	D	Cloacal sample	-	-	-	+	-	+	-	-
170	D	Cloacal sample	-	-	-	+	-	+	-	-
171	D	Cloacal sample	+	-	-	+	-	+	-	-
172	D	Cloacal	+	-	-	+	+	+	-	-

		sample								
173	D	Cloacal sample	-	-	-	+	+	+	-	-
174	D	Cloacal sample	-	-	-	+	+	+	-	-
175	D	Cloacal sample	+	-	-	+	-	+	-	-
176	D	Cloacal sample	-	-	-	+	+	+	-	-
177	D	Cloacal sample	-	-	-	+	+	+	+	-
178	D	Cloacal sample	-	-	-	+	+	+	-	-
179	D	Cloacal sample	+	-	-	+	-	+	-	-
180	D	Cloacal sample	-	-	-	+	+	+	-	-
181	D	Cloacal sample	+	-	-	+	-	+	-	-
182	D	Cloacal sample	-	-	-	+	+	+	-	-
183	D	Cloacal sample	-	-	-	+	-	+	-	-
184	D	Cloacal sample	-	-	-	+	+	+	-	-
185	D	Cloacal sample	-	-	-	+	-	+	+	-
186	D	Cloacal sample	-	-	-	+	+	+	-	-
187	D	Cloacal sample	+	-	-	+	-	+	-	-
188	D	Cloacal sample	-	-	-	+	+	+	-	-
189	D	Cloacal sample	-	-	-	+	+	+	-	-
190	D	Cloacal sample	-	-	-	+	-	+	-	-
191	D	Cloacal sample	-	-	-	+	+	+	-	-
192	D	Cloacal sample	+	-	-	+	+	+	-	-
193	D	Cloacal sample	-	-	-	+	+	+	-	-
194	D	Cloacal sample	+	-	+	+	-	+	-	-
195	D	Cloacal sample	+	-	-	+	+	+	-	-
196	D	Cloacal sample	-	-	-	+	-	+	-	-
197	D	Cloacal	-	-	-	+	-	+	-	-

		sample								
198	D	Cloacal sample	+	-	-	+	-	+	-	-
199	D	Cloacal sample	-	-	-	+	-	+	-	-
200	D	Cloacal sample	-	-	-	+	-	+	-	-