The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

Avian Pathogenic *Escherichia coli* (APEC) in Palestine: Characterization of Virulence Factors and Antibiotic Resistance Profile

By
Mohammad Hassan Qabajah

In partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

Graduate Advisory Committee:

Dr. Yaqoub Ashhab, Palestine Polytechnic University	Date
Dr. Robin Abu Ghazaleh, Palestine Polytechnic University	Date
Dr. Sameer Barghouthi, Al-Quds University	Date

Approved for the Faculties

Dean of Faculty of Scientific Research and Higher studies Palestine Polytechnic University	Dean of Faculty of Science Bethlehem University
Date	Date

ABSTRACT

Avian Pathogenic *Escherichia coli* (APEC) in Palestine: Characterization of Virulence Factors and Antibiotic Resistance Profile

By Mohammad Qabajah

Escherichia coli bacterium is common to many environments and there are over 150 different strains. Avian pathogenic *E. coli* (APEC) strains cause diseases in birds at various ages. The introduction of such strains to chicken respiratory tract causes invasive infections, collectively known as colibacillosis. It can cause extensive mortality in poultry flocks leading to great economic losses.

Recent reports showed that the APEC pathogenicity is associated with certain virulence genes (*papC*, *astA*, *vat*, and *irp2*) are located within the bacterial genome and/or their ColV plasmids (*tsh*, *iucD*, *iss*, and *cvi*). Identification and characterization of these genes are essential to implementing efficient disease control and prevention systems. The aim of this study is to identify the virulence associated genes and the antibiotic resistance profiles of APEC strains in Palestine.

Internal organ samples from 83 infected flocks were collected and tested for presence of the mentioned virulence genes using an adapted and improved multiplex PCR protocol. The resistance of the isolated strains to 10 commonly used antibiotics in Palestine was analyzed using the disc diffusion method.

The multiplex PCR of the tested samples revealed a high prevalence of the following genes: *iss* and *cvi* 100%, *astA* 98.48% and *iucD* 78.79%. The genes *vat* and *papC* have a prevalence of 34.85% and 31.81%, respectively. To a lesser extent *irp2* 19.70% and *tsh* 10.61% were identified. The study of antibiotic susceptibility profiles showed high resistance levels against Tetracycline 100%, Ampicillin 83.33%, Amoxicillin 83.33%, Kanamycin

80.3%, Ciprofloxacin 72.72% and Neomycin 69.70%, while the lowest resistance levels were against Nitrofurantoin 18.18% and Cephalexin 12.12%.

The improved multiplex PCR has proven to be a useful and rapid assay to identify virulence factor profiles of APEC. In Palestine, the indiscriminate use of antibiotics should be avoided. It may increase the risk of development of drug-resistant E. *coli* strains that constitute a human risk due to zoonose potential reservoir of Extended-spectrum b-lactamases resistance genes. Therefore, programs are recommended to increase farmer's awareness about the devastating effects of antibiotic misuse. In addition, the authorities must take a responsible role through imposition a set of regulations to ensure safe poultry products.

ABSTRACT IN ARABIC

موضوع البحث

البكتيريا المعوية الممرضة في الدواجن: تحديد خصائها الامراضية وقدرتها على مقاومة المضادات الحيوية

تعد البكتيريا القولونية (*Escherichia coli*) من الاصناف البكتيرية الشائعة حيث يوجد أكثر من 150 سلالة مختلفة منها. العترات المسببة للمرض في الدواجن يطلق عليها اسم (APEC). تتسبب هذه العترات في نفوق اعداد كبيرة من الدواجن بسبب الممرض المسمى ب(Colibacillosis) مما يؤدي إلى خسائر اقتصادية هائلة.

أظهرت التقارير الحديثة أن امراضية مثل هذه العترات مرتبط بوجود بعض الجينات الممرضة في محتوياتها الجينية. ان تحديد هذه الجينات ضروري لتنفيذ خطط مكافحة ونظم الوقاية ضد هذه الامراض. الهدف من هذه الدراسة هو تحديد الجينات المرتبطة بالقدرة الامراضية لعترات ال(APEC) في فلسطين وتحديد مقاومة هذه العترات للمضادات الحيوية الاكثر استخداما.

تم جمع ٨٣ عينة من دواجن متوقع اصابتها ب(Colibacillosis)، وتم فحص وجود الجينات المرتبطة بالمرض باستخدام بروتوكول ال(Multiplex PCR)، كما تم دراسة مقاومة العترات ل ١٠ من المضادات الحيوية الاكثر استخداما في فلسطين. كشفت الدراسة عن ارتفاع معدل انتشار الجينات الممرضة التالية: 100% (*iss, cvi*) و 98.48% *astA* و 78.79% *iucD* . بينما تواجدت الجينات *vat* و papC بنسب 34.85% و 31.81% على التوالي. وبنسب أقل، تم الكشف عن irp2 19.70% و 10.61% *tsh*. وأظهرت الدراسة المقاومة العالية للعزلات التي تم دراستها ضد Tetracycline 100% و Ampicillin 83.33% و Amoxicillin 83.33% و Kanamycin 80.3% و Ciprofloxacin 72.72% و Neomycin 69.70%، في حين كانت أدنى مستويات المقاومة ضد Nitrofurantoin 18.18% و Cephalexin 12.12% .

اظهر بروتوكول ال(Multiplex PCR) قدرة جيدة على الكشف عن ٨ من الجينات الممرضة في العينات التي تم عزلها. في فلسطين، ينبغي تجنب الاستخدام العشوائي للمضادات الحيوية لانه يزيد من خطر تطوير سلالات مقاومة ضد هذه المضادات مما يهدد صحة الانسان. لذلك، ينصح بتطوير برامج لزيادة الوعي لدى المزارعين من الآثار المدمرة لسوء استخدام المضادات الحيوية .وبالإضافة إلى ذلك، يجب على السلطات أن تتخذ دورا مسؤولا من خلال فرض مجموعة من القواعد لضمان سلامة منتجات الدواجن.

DECLARATION

I declare that the Master Thesis entitled "**Avian Pathogenic *Escherichia coli* (APEC) in Palestine: Characterization of Virulence Factors and Antibiotic Resistance Profile**" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

Mohammad Hassan Qabajah

Date: 24th May 2011

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for the joint master degree in biotechnology at Palestine Polytechnic University and Bethlehem University, I agree that the library shall make it available to borrowers under rules of the library. Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgement of the source is made.

Permission for extensive quotation from, reproduction, or publication of this thesis may be granted by my main supervisor, or in [his/her] absence, by the Dean of Higher Studies when, in the opinion of either, the proposed use of the material is for scholarly purposes. Any copying or use of the material in this thesis for financial gain shall not be allowed without my written permission.

Mohammad Hassan Qabajah

Date: 24th May 2011

DEDICATION

I would like to dedicate this thesis to my parents who have been a major influence in my life, who have never failed to give me their support, who provided me with all needs during the time I developed my system and for teaching me that even the largest task can be accomplished if it is done one step at a time. My parents, thank you very much, without you I would not be here. Thank you for all you have given me without asking for anything in return.

I would also like to dedicate the thesis to my brother and my sisters who have been among my best friends and whose encouragement has kept me going throughout my life as a student.

Also, I dedicate this work to my wife for standing with me step-by-step and supporting me. Thank you Asmaa for your patience and encouragement, and for the sacrifice that you have made along the way to complete this work.

ACKNOWLEDGMENT

I wish to thank my supervisor, Dr. Yaqoub Ashhab, for his support and direction that he has given me.

I would like to express my appreciation to the team of Biotechnology Research Center of Palestine Polytechnic University.

My special thanks to Dr. Elena Awwad, Central Veterinary Laboratories (CVLs), Palestine for providing APEC isolates.

Many thanks to Dr. Francis Dziva, Insitutue of Animal Health, Compton, UK for his collaboration and provision of controls.

I am also grateful to Dr. Sameer Barghouthi and Dr. Ahmad Amro for being good friend during working in this study.

I also thank Center for Infection Biology and Immunity of the Humboldt-University (ZIBI) for hosting me in International Summer School of Pathogen-Host-Interplay 2009.

ABBREVIATIONS

AML	Amoxicillin
AMP	Ampicillin
APEC	Avian pathogenic *E. coli*
astA	Enteroaggregative heat-stable toxin gene
CL	Cephalexin
CLSI	Clinical and Laboratory Standards Institute
ColV	Colicin V plasmid
CIP	Ciprofloxacin
CvaA	Colicin V secretion protein
cvi	Colicin-V immunity protein gene
Cvi	Colicin-V immunity protein
CVLs	Central Veterinary Laboratories
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides triphosphate
ESBL	Extended spectrum b-lactamases
ETEC	Enterotoxigenic *E. coli*
EIEC	Enteroinvasive *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EPEC	Enteropathogenic *E. coli*
EAggEC	Enteroaggregative *E. coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
EMB	Eosin Methylene Blue
EC-MUG	*E. coli* Medium with 4-methylumbelliferyl- β-D-glucuronide
F	Nitrofurantoin
GSSH	Genomic Suppression Subtractive Hybridization
iss	Increased serum survival gene
Iss	Increased serum survival protein
irp2	High molecular weight protein 2 gene
iucD	L-lysine 6-monooxygenase gene
iutA	Ferric aerobactin receptor gene
K	Kanamycin
N	Neomycin
PAI	Pathogenicity island
papC	Outer membrane usher protein papC gene
PCR	Polymerase Chain Reaction
STs	Secretory enterotoxins

TBE	Tris-Borate EDTA buffer
TE	Tetracycline
tsh	Temperature-sensitive hemagglutinin gene
Tsh	Temperature-sensitive hemagglutinin protein
vat	Vacuolating autotransporter gene
Vat	Vacuolating autotransporter toxin
uidA	beta-D-glucuronidase gene
UK	United Kingdom
USA	United States of America
UTI	Urinary tract infection
UV	Ultra Violet

LIST OF FIGURES

Figure	Description	Page
1.1	Pathomorphological changes of the internal organs of a chicken that died from colibacillosis	5
1.2	Chicken infected with APEC showing swollen head syndrome	6
1.3	Circular genetic map of pAPEC-O2-ColV plasmid	13
3.1	West-Bank map showing the sources of the 83 isolates	18
3.2	Visible blue fluorescence under UV-light (366nm) results from (MUG) cleavage by *E. coli*	20
4.1	APEC isolates screened for antibiotic resistance	26
4.2	Results obtained for APEC reference strain O78χ7122 with the gene-specific PCR reaction and multiplex PCR	28
4.3	Compatibility of the multiplex PCR protocol with gene-specific PCR results for the eight virulence genes of 031NS isolate	29
4.4	Compatibility of genetic profile of 6 random colonies of the same isolate	30
4.5	Agarose gel electrophoresis of the multiplex PCR products with representative APEC isolates carrying various combinations of virulence determinants	30
4.6	Number of virulence associated genes included in different isolates	31

LIST OF TABLES

Table	Description	Page
1.1	Summary of the virulence determinants of pathogenic *E. coli*	3
1.2	*E. coli* virotypes that are related to diarrheal diseases	4
1.3	The virulence related genes of APEC strains	11
3.1	EC-MUG broth formula	19
3.2	Reference zone of inhibition of antibiotics used in this study	21
3.3	Gene specific PCR primers used in this study	22
4.1	Antimicrobial sensitivity of *E. coli* isolates	25
4.2	Resistance pattern of *E. coli* isolates for the six most six common antibiotics	26
4.3	Prevalence of virulence-associated genes in APEC isolates included in this study	31
5.1	Antibiotic resistance results in relation to previous works	38

TABLE OF CONTENTS

ABSTRACT ii
ABSTRACT IN ARABIC iv
DECLARATION v
STATEMENT OF PERMISSION TO USE vi
DEDICATION vii
ACKNOWLEDGMENT viii
ABBREVIATIONS ix
LIST OF FIGURES xi
LIST OF TABLES xii
TABLE OF CONTENTS xiii
CHAPTER ONE 1
INTRODUCTION 1
1.1 Human Pathogenic *E. coli* 2
1.2 Pathogenesis of *E. coli* among mammals 2
1.3 Avian Pathogenic *E. coli* 4
CHAPTER TWO 15
PROBLEM STATEMENT AND OBJECTIVES 15
2.1 Problem statement 15
2.2 Objectives 16
CHAPTER THREE 17
MATERIALS AND METHODS 17
3.1 Materials 17
3.2 Methods 19
CHAPTER FOUR 24
RESULTS 24
4.1 Identification of *Escherichia coli* isolates 24
4.2 Antibiotic susceptibility testing 24
4.3 Multiplex PCR 27

4.4 Genetic profile of the Isolates 29
CHAPTER FIVE 32
DISCUSSION 32
5.1 Samples Identification 32
5.2 Enrichment step 33
5.3 Multiplex protocol 34
5.4 Molecular identification 34
5.5 Antibiotic susceptibility 37
CHAPTER SIX 41
CONCLUSIONS AND RECOMMENDATIONS 41
6.1 Conclusions 41
6.2 Recommendations 41
APPENDICES 43
REFERENCES 56

CHAPTER ONE

INTRODUCTION

Escherichia coli belongs to the Enterobacteriaceae family. It is a rod-shaped, Gram-negative, facultative anaerobic bacterium that can live on a wide variety of substrates. *E. coli* uses aerobic or anaerobic respiration. In anaerobic conditions, it uses the mixed-acid fermentation, producing lactate, succinate, ethanol, acetate and carbon dioxide. The optimal growth of most *E. coli* strains occurs aerobically at 37°C and some exceptional strains can grow at temperatures up to 49°C (Fotadar *et al.*, 2005). Various *E. coli* strains possess flagella and hence are classified as motile bacteria. The average genome size of *E. coli* bacteria is $4.6x10^6$ base pairs, which contains more than 4000 protein coding genes (Blattner *et al.*, 1997). *E. coli* can transfer its DNA via bacterial conjugation, transduction, or transformation allowing horizontal spreading of genetic material through an existing population (Brussow *et al.*, 2004).

E. coli usually colonizes the gastrointestinal tract of most warm-blooded animals (Dho-Moulin and Fairbrother, 1999, Lamarche *et al.*, 2005). Most *E.*

coli strains are considered a part of the normal intestinal flora where they produce vitamin K2 (Menaquinone) which acts as a competitive inhibitor preventing the establishment of pathogenic bacteria within the intestine (Bentley and Meganathan, 1982).

1.1 Human Pathogenic *E. coli*

E. coli strains are harmless to their hosts, however, some strains can be highly pathogenic and may cause serious problems especially in immuno-compromised individuals (Nataro and Kaper, 1998). Pathogenic *E. coli* are associated with intestinal and extraintestinal human infections (Nataro and Kaper, 1998) including: pyelonephritis, cystitis, septicemia, and some strains are associated with meningitis in neonatal infants (Germon *et al.*, 2005).

Usually, colibacillosis starts with severe abdominal cramp; within a few hours, it is followed by a watery diarrhea causing loss of fluids and electrolytes. Diarrhea lasts for about one day, then, intestinal sores will change this diarrhea to bright red bloody stools. Bloody diarrhea usually lasts for 2 to 5 days. In some cases, the disease may cause damage to the central nervous system.

1.2 Pathogenesis of *E. coli* among mammals

In humans, *E. coli* is responsible for three types of infections: urinary tract infections (UTI), neonatal meningitis, and intestinal diseases. The virulence determinants of *E. coli* that are related to these infections are summarized in (Table 1.1)

Table 1.1. Summary of the virulence determinants of pathogenic *E. coli* (Todar, 2007)

Adhesins
CFAI/CFAII
Type 1 fimbriae
P fimbriae
S fimbriae
Intimin (non-fimbrial adhesin)
Invasins
Hemolysins
Siderophores and siderophore uptake systems
Shigella-like "invasins" for intracellular invasion and spread
Motility
Flagella
Toxins
LT toxin
ST toxin
Shiga-like toxin
Cytotoxins
Endotoxin (LPS)
Antiphagocytic, surface properties
Capsules**
K antigens*/**
Lipopolysaccharides (LPS)*/**

* Defense against serum bactericidal reaction
** Defense against immune responses

Five virotypes of *E. coli* that cause diarrheal diseases are now recognized (Table1.2):

- Enterotoxigenic *E. coli* (ETEC)
- Enteroinvasive *E. coli* (EIEC)
- Enterohemorrhagic *E. coli* (EHEC)
- Enteropathogenic *E. coli* (EPEC)
- Enteroaggregative *E. coli* (EAggEC)

Table1.2: *E. coli* virotypes that are related to diarrheal diseases (Todar, 2007)

Name	Hosts	Description
Enterotoxigenic *E. coli* (ETEC)	Humans, pigs, sheep, goats, cattle, dogs, and horses	• Non-invasive strains. • Cause diarrhea in children, as well as traveler's diarrhea. • Produces a heat-stable (ST) enterotoxin • 200 million cases of diarrhea and 380,000 deaths each year.
Enteropathogenic *E. coli* (EPEC)	Humans, rabbits, dogs, cats and horses	• Has an array of virulence factors similar to Shigella toxin • Moderately invasive and elicit an inflammatory immune response.
Enteroinvasive *E. coli* (EIEC)	found only in humans	• Causes a syndrome that is identical to Shigellosis, with profuse diarrhea and high fever.
Enterohemorrhagic *E. coli* (EHEC)	Humans, cattle, and goats	• The most famous member of this virotype is strain O157:H7, which causes bloody diarrhea and no fever. • Can cause hemolytic-uremic syndrome and sudden kidney failure. • Moderately invasive and possesses a phage-encoded Shiga toxin that can elicit an intense inflammatory response.
Enteroaggregative *E. coli* (EAEC)	found only in humans	• EAEC bind to the intestinal mucosa to cause watery diarrhea without fever. EAEC are non-invasive. • They produce a hemolysin and an ST enterotoxin similar to that of ETEC.

1.3 Avian Pathogenic *E. coli*

Pathogenic *E. coli* strains are also related to extraintestinal infections for other animals (Nakazato *et al.*, 2009). Among birds, it was proved that the pathogenic strains of *E. coli* cause respiratory diseases (Ewers *et al.*, 2004, Stehling *et al.*, 2007, Saberfar *et al.*, 2008).

Ten to fifteen percent of the intestinal coliforms in chickens have a potential to be pathogenic (Tabatabaei and Nasirian, 2003). Avian pathogenic *E. coli* strains are known as APEC and are mainly associated extraintestinal diseases

(Dho-Moulin and Fairbrother, 1999, Stehling *et al.*, 2007). Avian diseases are responsible for great losses in the avian industry (Ewers *et al.*, 2004, Skyberg *et al.*, 2006, Saberfar *et al.*, 2008).

Colibacillosis is the most important disease caused by APEC strains and is characterized by multi-extraintestinal disorders (Tabatabaei and Nasirian, 2003, Yaguchi *et al.*, 2007) including: respiratory tract infection, septicemia, omphalitis, enteritis, and cellulitis (Herren *et al.*, 2006, Stehling *et al.*, 2007). This infectious disease is considered to be initiated in the avian upper respiratory tract; air sacs being the first organs infected (Germon *et al.*, 2005, Saberfar *et al.*, 2008), followed by septicemia and organ colonization (Figure1.1) (Ewers *et al.*, 2004, Saberfar *et al.*, 2008).

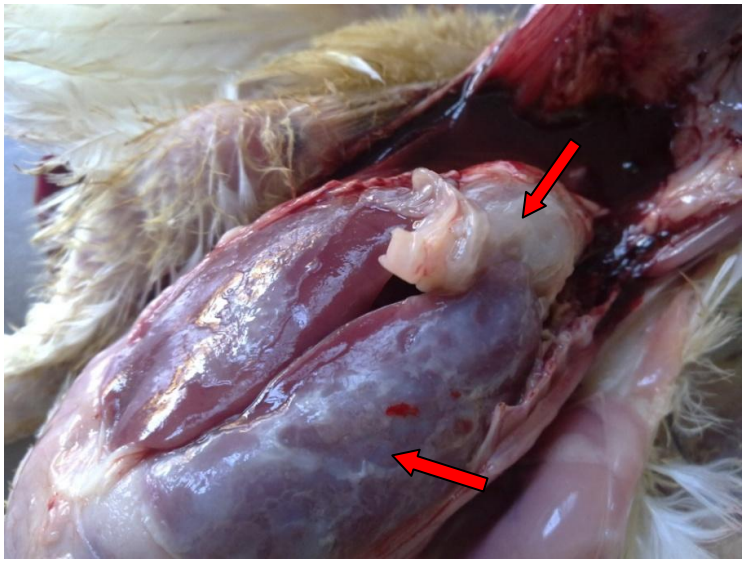

Fig. 1.1: Pathomorphological changes of the internal organs for a chicken that died from colibacillosis caused by APEC strains. The white layer shown is the result of APEC proliferation at colonization level of colibacillosis infection. The presence of this layer around internal organs, heart and lungs mainly, is considered as a strong evidence for colibacillosis infection.

Colibacillosis is initiated after a primary infection caused by *Mycoplasma gallisepticum* or different viruses such as the *Infectious bronchitis* virus or *Newcastle* virus (Karch *et al.*, 1999, Germon *et al.*, 2005). The respiratory infection caused by APEC strains, is considered to be the initial step for colibacillosis development in birds (Nakazato *et al.*, 2009). Birds from 2 to 12 weeks of age are more susceptible to disease and mortality rates may reach as high as 20% in birds within the 4 to 9 weeks age range (Dho-Moulin and Fairbrother, 1999).

In broilers and hatchers chickens, swollen head syndrome is one of the common syndromes caused by APEC strains (Figure1.2) (Nunoya *et al.*, 1991). This syndrome is responsible for mortality of 3 to 4% of total birds and for reduction in egg production of 2 to 3 % (Morley and Thomson, 1984). Swollen head syndrome usually begins after an acute rhinitis caused by pneumovirus being followed by the invasion of the subcutaneous skin tissues by *E. coli*, which causes the characteristic edema (Hafez and Lohren, 1990).

Fig. 1.2: Chicken infected with APEC showing swollen head syndrome. In chicken, swollen head syndrome may cause ocular discharge and conjunctivitis progressing to periorbital swelling. Terminally, eyes will be closed and the enlargement of the head is a prominent sign in severely depressed or recumbent broilers

In broilers, APEC strains are also associated with cellulitis that causes a necrotic dermatitis of the abdomen and thighs (Dho-Moulin and Fairbrother, 1999). Epidemiological data about this syndrome are not available but the lesions associated with cellulitis have a role in causing economic losses in the avian industry (Elfadil *et al.*, 1996, Knobl *et al.*, 2006).

1.3.1 APEC Infection Model

APEC pathogenesis consists of four stages: colonization of the respiratory tract; penetration of the epithelium into the respiratory organs mucosa; survival and multiplication in the blood stream and in the internal organs causing different pathomorphological change; finally, production of poisonous effects on the eukaryotic cells and tissues leading to lesions followed by clinical signs (Dho-Moulin and Fairbrother, 1999).

For APEC, there is only little information about the virulence genotypes for strains involved in pathogenicity (Dho-Moulin and Fairbrother, 1999, Dozois *et al.*, 2000, Janben *et al.*, 2001). However, certain virulence factors have been described to be positively linked with APEC pathogenicity including: epithelial adherence and invasion, flagella, iron sequestering systems, toxins and cytotoxins, temperature-sensitive hemagglutinin, serum resistance, colicin production, and outer membrane proteins. (Schouler *et al.*, 2004, Tivendale *et al.*, 2004, Amabile de Campos *et al.*, 2005, Stehling *et al.*, 2007, Saberfar *et al.*, 2008). Indeed, not a single virulence gene is found in any of the APEC strains that is absent from all non-pathogenic strains. This might indicate the use of

different virulence mechanisms by different putative pathotypes (La Ragione and Woodward, 2002, Germon *et al.*, 2005). This virulence capacity is acquired by horizontal transmission of certain genes located on plasmids, bacteriophages or particular regions of DNA called pathogenicity islands (Kilic *et al.*, 2007, Moulin-Schouleur *et al.*, 2007).

1.3.2 APEC and food safety

Due to the low cost of production as well as the relatively cheap prices, poultry meat is very important in the consumer market including the Palestinian one. However, epidemiological reports showed that the presence of pathogenic and spoilage microorganisms in poultry meat and its by-products remains a significant concern. *E. coli* has been consistently associated with food-borne illnesses in most countries of the world (Lutful Kabir, 2010).

Recent studies have suggested that some APEC strains are considered as potential zoonotic agents (Ewers *et al.*, 2007, Moulin-Schouleur *et al.*, 2007, Johnson *et al.*, 2008). Earlier works had shown APEC strains to be easily transmitted to humans (Linton *et al.*, 1977, Ojeniyi, 1989). Indeed, studies have shown that some APEC strains could belong to the same clones as human ExPEC strains (Achtman *et al.*, 1986, White *et al.*, 1993b). Recently, it has been reported that very closely related clones of serotype O18:K1:H7 could be recovered from extra-intestinal infections in humans and chickens and that isolates from both species were virulent for chicks (Moulin-Schouleur *et al.*, 2006). PCR-based phylotyping and multi-locus sequence typing have revealed a

link between APEC and human ExPEC (Johnson *et al.*, 2007, Moulin-Schouleur *et al.*, 2007), further suggesting the potential food-borne source of human ExPEC. Consistent with these observations, whole genome sequence analysis has revealed a high degree of similarity between APEC and ExPEC, with only 4.5% of the APEC O1:K1:H7 genome not found in three ExPEC genomes (Johnson *et al.*, 2007).

1.3.3 Identification Methods of Pathogenic E. coli

APEC identification has passed through a number of developments, beginning with conventional methods of identification based on chemical characterization and the nature of selective media. A more specific identification approach is the serotyping analysis that aims at classifying pathogenic strains based on their surface antigens. The DNA based identification methods are considered as extremely sensitive approaches that aim at the identification of *E. coli* virulence genes.

1.3.3.1 Conventional Identification

E. coli strains isolated from internal organs of chicken that have died from colibacillosis are cultured and enriched so as to be identified via morphological and routine biochemical tests. Using microscopy, the enriched bacteria show gram negative rods, with no particular cell arrangement. On MacConkey agar, as the *E. coli* is lactose-positive, deep red colonies are produced as a result of fermentation of lactose sugar that cause the medium's pH to drop, leading to

darkening of the medium. Growth on EMB agar produces greenish-black metallic sheen colonies.

1.3.3.2 Serotyping Identification

Based on O, H, and K antigens, more than 700 serotypes of *E. coli* were recognized. O Serotyping is one of the basic diagnostic methods for the classification of pathogenic strains of *E. coli*. It is suggested that some strains (O1, O2 and O78) could be classified as avian pathogenic strains (Kawano *et al.*, 2006, Yaguchi *et al.*, 2007, Giovanardi *et al.*, 2005, Ozawa *et al.*, 2008). Recent researches mentioned that this method is not efficient enough for APEC classification (Ewers *et al.*, 2004, Kawano *et al.*, 2006, Yaguchi *et al.*, 2007) especially as some of the available commercial kits even they are very expensive, do not include all O antisera which lose the ability to classify all the O serotypes. In some cases, O serotyping was not able to classify around 50% of total APEC strains (Yaguchi *et al.*, 2007, Ozawa *et al.*, 2008). Thus, serotyping identification will not be able to give us clear information about the pathogenic strains of *E. coli* and it is not able to classify all of these strains.

1.3.3.3 Molecular Identification

The limited knowledge about the molecular epidemiology as well as about virulence-associated genes of avian pathogenic *E. coli* (APEC) hinders the application of essential and efficient epidemiological control measures for the prevention of colibacillosis. However, several studies have identified genes encoding virulence factors of APEC. The according genes encode adhesion-

related factors such as P-fimbriae (*papC*) and a temperature-sensitive hemagglutinin (*tsh*); iron-acquisition systems (*fyuA*/*irp2*) and aerobactin (*iutA*/*iucD*); a protein for increased serum survival (*iss*); a colicin V plasmid (*cva*/*cvi*); an enteroaggregative heat-stable toxin (*astA*); as well as a vacuolating autotransporter toxin (*vat*). The most related virulence genes of APEC are listed in (Table 1.3) the function and the location of each gene (Dozois *et al.*, 2000, Delicato *et al.*, 2003, Dozois *et al.*, 2003, Mellata *et al.*, 2003, Parreira and Gyles, 2003).

Table 1.3: The virulence related genes of APEC strains

Adhesins/Miscellaneous		**Location**
papC	Outer membrane usher protein PapC gene	Chromosome
tsh	Temperature-sensitive hemagglutinin Tsh autotransporter gene	Plasmid
Iron-Related		
irp2	Iron repressible gene	Chromosome
iucD	Involved in aerobactin synthesis	Plasmid
Structural gene		
cvi	colicin V immunity protein gene	Plasmid
Toxins		
astA	Heat-stable enterotoxin-1gene	Chromosome
vat	Vacuolating autotransporter toxin gene	Chromosome
serum survival		
iss	Increased serum survival gene	Plasmid and Chromosome

In the early stages, molecular identification of APEC strains was based on screening of few genes expected to be related to the pathogenicity. Previous investigations have indicated that the prevalence of some virulence genes among isolates from chickens with colibacillosis were useful markers for the detection and characterization of avian pathogenic *E. coli*, and could, therefore, be used in the diagnosis of colibacillosis in poultry (Ewers *et al.*, 2005). At this level, gene inactivation experiments confirmed a role for type 1 and P fimbrial adhesions, aerobactin iron transport system and the temperature-sensitive hemagglutinin in APEC pathogenicity (Lafont *et al.*, 1987, Dozois *et al.*, 2000, La Ragione *et al.*, 2000).

Later on, researchers focused on studying the virulence profile of APEC, many studies showed that the virulence profile is quite important, while the presence of virulence genes in combination to each other will increase the pathogenicity of APEC (Ngeleka *et al.*, 2002). New studies started to establish protocols are useful to identify the combinations between virulence genes to determine the effect of such combinations on APEC pathogenicity. These studies started to screen bacterial genes on the large scale to identify the most related pathogenic genes; and they were able to identify some of expected pathogenic families of virulence genes. Parallel to that, a lot of studies have found that many APEC isolates carry certain plasmids (e.g. ColV plasmids) some of which encode potential virulence factors (Ewers *et al.*, 2004); several studies have demonstrated a link between APEC virulence and the possession of ColV plasmids which have different genes islands are showing the relation to

pathogenicity (Ginns *et al.*, 2000, Ewers *et al.*, 2004, Tivendale *et al.*, 2004) (Figure 1.3).

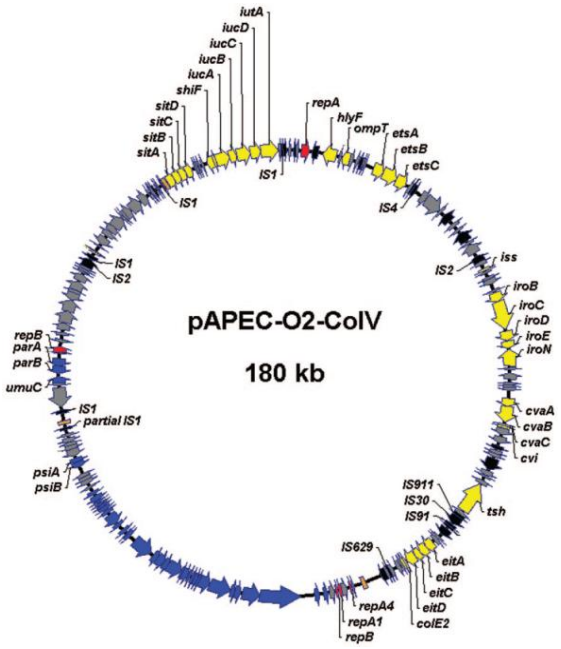

Fig. 1.3: Circular genetic map of pAPEC-O2-ColV plasmid. Arrows indicate predicted genes and their directions of transcription. Yellow arrows indicate virulence-associated genes.

1.3.3.4 Other useful techniques

Different virulence assays or pathogenicity tests in vivo using a chicken infection model employed to study the virulence of a particular APEC strain, as well as to compare the wildtype strain with mutant strains lacking a certain gene or function (Antao *et al.*, 2008).

Molecular typing methods have also been employed to study APEC including consensus-PCR typing (Carvalho de Moura *et al.*, 2001), random amplification of polymorphic DNA (Maurer *et al.*, 1998), multilocus enzyme electrophoresis (White *et al.*, 1993a), genomic suppression subtractive hybridization (GSSH)

(Schouler *et al.*, 2004, Mokady *et al.*, 2005). Genome analyses have led to a better understanding of APEC pathogenicity. Selective capture of transcribed sequence studies conducted by (Dozois *et al.*, 2003) identified pathogen-specific transcripts in an APEC strain corresponding to putative adhesins, lipopolysaccharide core synthesis, iron transport systems, plasmid and phage encoded genes, and genes of unknown function.

CHAPTER TWO

PROBLEM STATEMENT AND OBJECTIVES

2.1 Problem statement

The poultry sector has a great importance in Palestine at the economic and health levels. Economically, many farmers depend on this sector as one of the main sources for their income, thus it is important to maintain this sector and help it to grow. These motives aim to enhance Palestinian economy. Health wise, chickens are one of the most important protein sources among Palestinians and there is a great need to ensure the health safety of poultry.

In Palestine, colibacillosis is a common disease responsible for hundreds of thousands shekels losses for Palestinian economic sector every year (CVLs). At farmer level, there is unregulated and uncontrolled use of antibiotics to control this disease irrespective of any negative consequences that may result of antibiotic misuse. Conventional identification is used to identify APEC responsible for colibacillosis, which is not able to describe the genetic criteria of these strains, and there is no research conducted to screen APEC strains present

in Palestine, so the genetic profile of APEC strains present in Palestine is still unknown.

Our belief is that it is important to characterize APEC strains present in Palestine to give a background, especially for farmers, about these strains and their antibiotic resistance profiles; this will contribute to determining appropriated antibiotic for protection against these strains. Screening the APEC genetic traits in Palestine is important as we can determine the virulence genes involved in APEC pathogenicity, while genetic description can give a clear picture to explain and control pathogenic strains.

2.2 Objectives

- To establish a multiplex PCR protocol to screen the eight virulence genes related to APEC pathogenicity
- To study the genetic composition of isolates included in this study to establish a virulence gene profile of these isolates
- To study the antibiotic resistance profile of APEC strains in Palestine for commonly used antibiotics.

This study represents the first description of the genetic criteria and the antibiotic profile of the APEC strains present in Palestine.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Materials

3.1.1 Samples

Samples from 83 broiler farms suspected to have *Escherichia coli* infection were collected between Feb-Jun 2009 through the Central Veterinary Laboratory, Ramallah, Palestine. The farms were distributed over 56 locations from 11 governates in the West-Bank, Palestine (Figure 3.1). The international APEC reference genomic DNA of the strain O78χ7122 was provided by Dr. Francis Dziva from the Institute of Animal Health, Compton, UK. This strain contains the virulence genes: *astA, ireA, iss, irp2, papC, iucD, tsh, vat* and *cvaC*. Therefore, it was used as a positive control to establish the multiplex PCR protocol.

Governates	Isolates	Villages
Jenin	13	6 villages
Tobas	2	1 village
Tulkarm	4	3 villages
Nablus	6	6 villages
Qalqelyah	2	2 villages
Salfet	3	3 villages
Ramallah	28	19 villages
Jericho	5	4 villages
Jerusalem	1	1 village
Bethlehem	4	3 villages
Hebron	15	8 villages
Total	83 Isolates	56 Villages

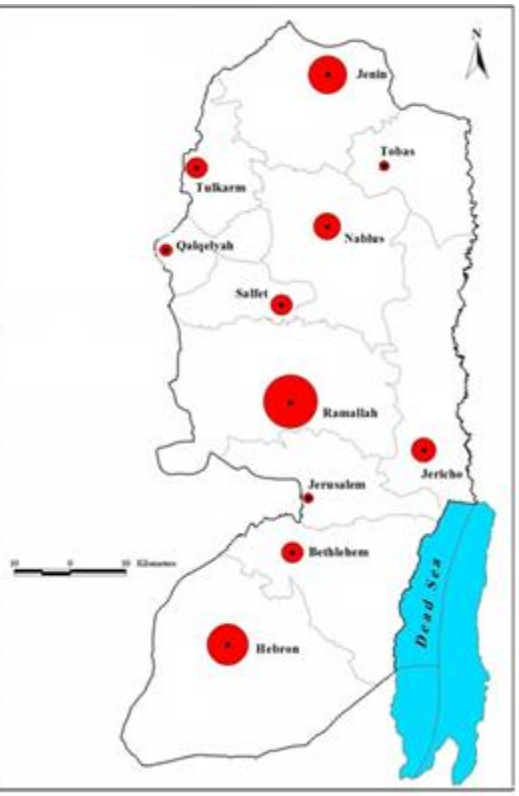

Fig 3.1: A map of West-Bank showing the sources of the 83 isolates collected from 56 locations distributed over 11 governates. The sizes of the red circles reflect the number of the samples obtained from each governorate.

3.1.2 Media

EC-MUG medium (Oxoid, UK) catalogue number-CM0979A, supplied as 100g powder to be mixed with 2.70 liter distilled water. EC-MUG Medium was used to improve *E. coli* detection. This medium consists of lactose, with the addition of 0.15% bile salts, tryptone, dipotassium phosphate and monopotassium phosphate, sodium chloride and 4-methylumbelliferyl- β-D-glucuronide (MUG) (Table 3.1). Blood agar, MacConkey agar, Eosin -Methylene Blue agar and Muller-Hinton agar (Oxoid, UK) were used for *E. coli* identification and characterization.

Table 3.1: EC-MUG broth formula

Typical Formula*	gm/liter
Tryptone	20.0
Lactose	5.0
Bile salts No. 3	1.5
Di-potassium phosphate	4.0
Mono-potassium phosphate	1.5
Sodium chloride	5.0
4-methylumbelliferyl-ß-D-glucuronide (MUG)	0.05
pH 6.9 ± 0.2 (25°C)	

3.1.3 Chemicals and reagents

All antimicrobial diffusion discs used in this study were from (Oxoid, UK). Molecular grade agarose was purchased from (SEAKEM LE, USA). Enzymes were procured from (Hylabs, Israel) and the deoxynucleoside triphosphates were from Sigma-Aldrich Co. (St. Louis MI, USA). Oligonucleotides used in this study were synthesized by (Metabion, Germany)

3.2 Methods

3.2.1 Culture and biochemical characterization

Internal origins of suspected liver were inoculated in *E. coli* Broth with MUG (EC-MUG broth) at 44.5ºC for 22 hours to 26 hours as an *E. coli* specific pre-enrichment step. In the presence of 4-methylumbelliferyl- β-D-glucuronide (MUG), *E. coli* produces the enzyme glucuronidase that hydrolyzes MUG to yield a fluorogenic product which is detectable under long-wave (366 nm) UV light (Figure 3.2).

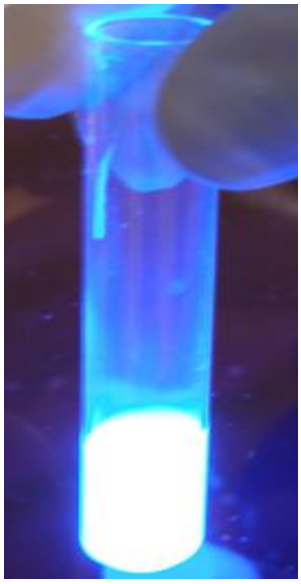

Fig. 3.2: Visible blue fluorescence under long-wave ultra-violet light (366nm) results from 4-methylumbelliferyl-ß-D-glucuronide (MUG) cleavage by beta-glucuronidase enzyme of 24h enriched *E. coli* at 44.5ºC in the EC-MUG broth.

Isolates were then cultured onto 7% sheep blood, MacConkey and eosin - methylene blue agar. The identification of *E. coli* was based on the results of diagnostic tests, which included Gram stain, colony characterization (flat, grayish, with spreading edges colonies on blood agar, red/pink colonies on MacConkey agar, and green metallic sheen on EMB agar), gas production and ability to be enriched in the EC-MUG broth at 44.5ºC. All confirmed strains were kept at -80ºC in EC-MUG medium containing 15% glycerol. Specific primers were designed for beta-D-glucuronidase gene (*uidA*) for further molecular confirmation of *E. coli* identity. uidA-F: CTGAACTGGCAGACTATCCC forward primer and uidA-R: CAGCACATCAAAGAGATCGC reverse primer were designed using PerlPrimer v1.1.19, which is an open-source primer design software (perlprimer.sourceforge.net).

3.2.2 Antimicrobial sensitivity

A sensitivity test for ten antimicrobial agents was carried out on the isolated strains by the standard disk procedure (Bauer *et al.*, 1966) on Muller-Hinton agar. For each isolate, saline suspension of five colonies from an agar plate culture (18- to 24-hour blood agar plate) were prepared. The top of each colony was touched with a loop to be transferred into 1ml saline making 0.5 McFarland standard suspension (0.008 to 0.10 absorbance at 625 nm). The suspension was streaked on 4 mm depth Mueller-Hinton agar plate. Streaking was repeated three times, rotating the plate approximately 60° each time. After a period of five minutes, Ampicillin, Tetracycline, Amoxicillin, Neomycin, Gentamycin, Nitrofurantoin, Ciprofloxacin, Kanamycin, Chloramphenicol and Cephalexin standard paper disks were laid on the medium. The plates were incubated for 24h at 37ºC and inhibition zones were measured. Using reference (Table 3.1) the size of zones were related to the zone of inhibition so as to decide whether the examined strain is susceptible (S), intermediately susceptible (I), or resistant (R) to the tested antibiotic. The susceptibility results for the sixty six isolates are shown in (Appendix 1).

* Table 3.2: Reference zone of inhibition of antibiotics used in this study

Antibiotic (Disc identifier)	**Disk potency**	**Inhibition zone diameter to nearest mm**		
		Resistant ≤	Intermediate	Susceptible ≥
Amoxicillin (AML)	25 ug	13	14-17	18
Ampicillin (AMP)	10 ug	13	14–16	17
Cephalexin (CL)	30 ug	14	15-17	18
Chloramphenicol (C)	30 ug	12	13-17	18
Ciprofloxacin (CIP)	5 ug	15	16-20	21
Gentamycin (CN)	10 ug	12	13–14	15
Kanamycin (K)	5 ug	13	14-17	18
Neomycin (N)	30 ug	12	13-16	17
Nitrofurantoin (F)	300 ug	14	15-16	17
Tetracycline (TE)	30 ug	14	15-18	19
* (Lammert, 2007)				

3.2.3 Multiplex PCR analyses

A multiplex PCR protocol was adapted and improved to detect the presence of the following virulence genes: *papC*, *astA*, *vat*, *irp2*, *tsh*, *iucD*, *iss*, and *cvi*. The gene specific primers (Table 3.2) were designed using PerlPrimer v1.1.19. Each primer was tested for the following criteria: internal stability, melting temperatures, cross dimerization with the other primers, and non-specific binding either to *E. coli* or to the host genome.

Table 3.3: Gene specific PCR primers used in this study

Gene	Primer	Primer sequence (5'- 3')	Localization within gene	$T_{melting}$ (C^o)	Amplicon size (bp)
cvi	*cvi*(F)115	CCATGCATACATTTTGCTTCTCTG	115 – 138	63	114
	cvi(R)229	AGTCAGAGTTCTCATATGATCTCC	229 – 206	61	
iss	*iss*(F)102	GCAGTAACACCAAAGGAAACC	102 – 122	62	184
	iss(R)286	CTTCCAGCGGAGTATAGATGC	286 – 266	63	
astA	*astA*(F)249	GATCCCTGGTACAACTATCGC	249 – 269	62	266
	astA(R)515	TAGCCGTGTTCGTCAATCAC	515 – 496	63	
iucD	*iucD*(F)315	GCTGCTGAAGATATGAATAACC	315 – 336	60	431
	iucD(R)746	CGAATATCTTCCTCCAGTCC	746 – 727	60	
papC	*papC*(F)857	CTATGCACCGCAGATTACC	857 – 875	61	538
	papC(R)1395	GAACGTAATGTCGGCATCC	1395 – 1377	60	
vat	*vat*(F)824	ACTGGTCGGTGTTTACTCG	824 – 842	62	682
	vat(R)1506	GTCATTCCCGTTAACATCCAG	1506 – 1486	61	
tsh	*tsh*(F)1341	GTTGTACTGAACCAGCAGG	1341 – 1359	61	786
	tsh(R)2127	GTTCTTCAGTGACAGCCTG	2127 – 2109	61	
irp2	*irp2*(F)2048	GTCAGACGATATTCCCGTCC	2048 – 2067	62	886
	irp2(R)2934	CAGCTCGATGCGATATCCTC	2934 – 2815	63	

Bacterial colonies from overnight MacConkey agar at 37ºC were picked using a sterile pipette tip and aseptically suspended in 100 µl of sterile distilled water in an eppendorf tube. The suspension was boiled for 15 min. After centrifugation

for 5 min at 13000 r.p.m, 2µl of the supernatant were taken as template DNA and added to the PCR reaction mixture (50µl) containing 0.5µl of each primer (10 pmol concentration), 4µl of the four deoxynucleoside triphosphates (10mM solution), 5µl of 10X PCR buffer, 8 µl of 20 mM Magnesium Chloride, and 5 units of *Taq*-Polymerase. The samples were subjected to 30 cycles of amplification. The cycling conditions were as following: step1, 5 min at 94ºC; step2, 1 min at 94ºC; step3, 45 sec at 55ºC; step4, 2 min at 72ºC (step2–step4, repeated 30 times); step5, 10 min at 72ºC.

The amplification products were analyzed by electrophoresis on 2.0 % agarose gel, in 1x TBE buffer for 90 min at 90V. The amplicons were stained with ethidium bromide, and photographed under illumination (UV box from UVP, United States.

CHAPTER FOUR

RESULTS

4.1 Identification of *Escherichia coli* isolates

Out of the eighty three field samples, sixty six were positive for *E. coli* based on morphological and biochemical characteristics. Using gram stain method, the sixty six positive isolates showed short-rod gram negative bacteria. The sixty six isolates showed pink colonies on MacConky agar and green metallic sheen colonies on EMB agar, which are typical for *E. coli*. In addition, all of positive sixty six isolates were enriched in EC-MUG broth as represented by their capacity to produce fluorogenic product which is characteristic of *E. coil*.

4.2 Antibiotic susceptibility testing

Antibiotic resistance is a pandemic feature of APEC. There is no data about antibiotic resistance in poultry farms in Palestine. Therefore, it was important to assess the resistance of the isolated strains to a group of antimicrobial drugs that

are frequently used by farmers. The susceptibility patterns of the tested antimicrobial agents are shown (Table 4.1). The first group included the antibiotics to which there were high levels of resistance (69% to 100%); these were Tetracycline (TE): 100%, Ampicillin (AMP): 83.33%, Amoxicillin (AML): 83.33%, Kanamycin (K): 80.3%, Ciprofloxacin (CIP): 72.72% and Neomycin (N): 69.70%. The second group included the antibiotics to which there were moderate levels of resistance (30% to 69%); these were Gentamycin (CN): 50% and Chloramphenicol (C): 39.39%. The third group included the antibiotics to which there were low levels of resistance (0% to 30%); these were Nitrofurantoin (F): 18.18% and Cephalexin (CL): 12.12%.

Table 4.1: Antimicrobial sensitivity of *E. coli* isolates

Resistant level	Antibiotics	Number of resistant isolates (%)
High level	Tetracycline (TE)	66 (100)
	Ampicillin (AMP)	55 (83.33)
	Amoxicillin (AML)	55 (83.33)
	Kanamycin (K)	53 (80.30)
	Ciprofloxacin (CIP)	48 (72.72)
	Neomycin (N)	46 (69.70)
Moderate level	Gentamycin (CN)	33 (50.00)
	Chloramphenicol (C)	26 (39.39)
Low level	Nitrofurantoin (F)	12 (18.18)
	Cephalexin (CL)	8 (12.12)

Each of the sixty six *E. coli* isolates showed resistance to two antibiotics at least (Figure 4.1).

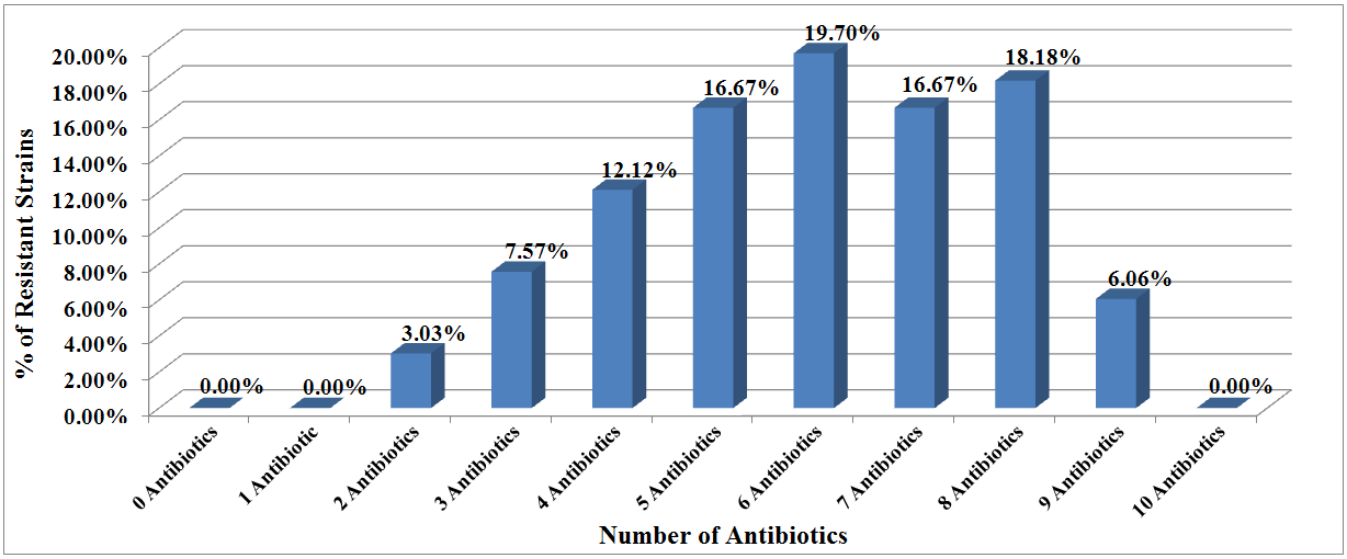

Fig. 4.1: APEC isolates resistance for antibiotic screened

Further exploration the association between resistance to the used antibiotics to reveal possible resistance patterns. Table 4.2 shows nine different patterns of the sixty six *E. coli* isolates. Pattern number 1 (TE/AMP/AML/N/CIP/K) was the most common pattern (43.94%) and pattern number 8 and 9 (TE/AML/N/CIP/K) and (TE/AMP) showed the least common patterns (3.03%).

Table 4.2: Resistance pattern of *E. coli* isolates for the most six common antibiotics

Antimicrobial agent							
Resistance pattern ID	**AML**	**AMP**	**CIP**	**K**	**N**	**TE**	**Number of isolates (%)**
1	R	R	R	R	R	R	**29 (43.94)**
2	R	R	S	R	R	R	**10 (15.15)**
3	R	R	S	S	S	R	**6 (9.1)**
4	R	R	R	S	S	R	**5 (7.57)**
5	S	S	R	R	R	R	**5 (7.57)**
6	S	S	R	R	S	R	**4 (6.06)**
7	R	R	R	R	S	R	**3 (4.55)**
8	R	S	R	R	R	R	**2 (3.03)**
9	S	R	S	S	S	R	**2 (3.03)**
Number of resistant isolates (%)	**55 (83.33)**	**55 (83.33)**	**48 (72.72)**	**53 (80.30)**	**46 (69.70)**	**66 (100.00)**	

4.3 Multiplex PCR

In order to setup and optimize a useful multiplex PCR reaction, initial experiments we performed on the APEC reference strain O78χ7122, which is known to harbor different APEC virulence genes including: *astA, ireA, iss, irp2, papC, iucD, tsh, vat* and *cvaC.* In the first experiment, gene-specific PCR reactions for each virulence factor were performed separately to determine the optimum conditions most appropriate for these reactions, to reach the most appropriate condition for multiplexing. Through several experiments and after testing different conditions and concentrations, the optimum conditions for these reactions were as following: 25µl PCR reaction mixtures containing 1µl of each primer in a 10 pmol concentration, 2µl 10mM of dNTPs, 3µl of 10X PCR buffer, 3 µl of 20 mM $MgCl_2$, and 1 unit of Taq-polymerase. The samples were subjected to 30 cycles of amplification including five steps: step1, 5 min at 94ºC; step2, 45 sec at 94ºC; step3, 40 sec at 57ºC; step4, 1 min at 72ºC (step2–step4, repeated 30 times); step5, 10 min at 72ºC.

For multiplex protocol, it was more difficult to optimize the reaction conditions, the conditions should be optimized to be compatible for all gene-specific primers to work at the same time. For the multiplex PCR protocol, the eight virulence genes could not be simultaneously under the given conditions; concentrations and annealing temperature that equal to (T_a = 57ºC) of gene-specific PCR reactions. So the challenge was to determine the concentrations and volumes of the PCR reaction that are suitable for the multiplex protocol.

Several experiments led to the optimum conditions that can be adopted to detect the eight target virulence genes. The conditions were as follows: PCR reaction mixture 50µl containing 0.5µl of each primer in a 10 pmol concentration, 4µl 10mM of dNTPs, 5µl of 10X PCR buffer, 8 µl of 20 mM MgCl2, and 5 units of Taq-Polymerase, with 30 cycles of amplification including five steps: step1, 5 min at 94ºC; step2, 1 min at 94ºC; step3, 45 sec at 55ºC; step4, 2 min at 72ºC (step2–step4, repeated 30 times); step5, 10 min at 72ºC. Figure 4.2 shows the results obtained with the gene-specific PCR reaction are consistent with the multiplex PCR.

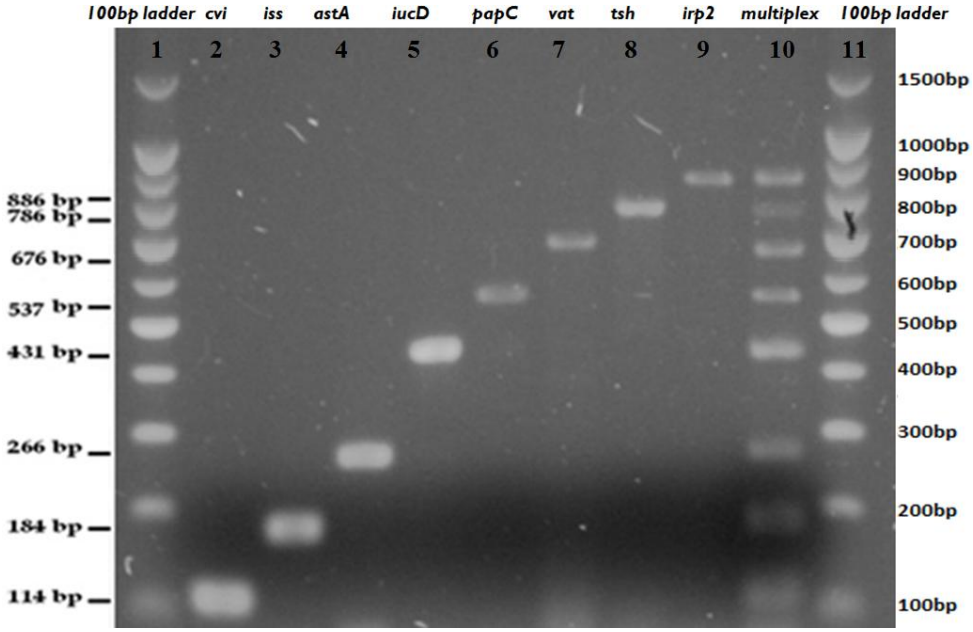

Fig 4.2: Results obtained for APEC reference strain O78χ7122 with the gene-specific PCR reaction in comparison to the multiplex PCR. Lanes 1&11: 100pb DNA ladder; lane 2: *cvi* 114bp; lane 3: *iss* 184bp; lane 4: *astA* 266bp; lane 5: *iucD* 431bp; lane 6: *papC* 537bp; lane 7: *vat* 676bp; lane 8: *tsh* 786bp; lane 9: *irp2* 886bp; lane 10: multiplex PCR for (*cvi*, *iss*, *astA*, *iucD*, *papC*, *vat*, *tsh* and *irp2*) genes

The efficiency of the multiplex PCR versus the gene-specific PCR reactions was further assessed using 12 isolates selected randomly from the 66 positive

samples. The results of this comparison were confirmatory and they showed almost identical pattern of virulence genes in both PCR protocols (Figure 4.3). This gave a clear indication that the new multiplex PCR protocol can be considered as an effective method for screening of these groups of virulence factors.

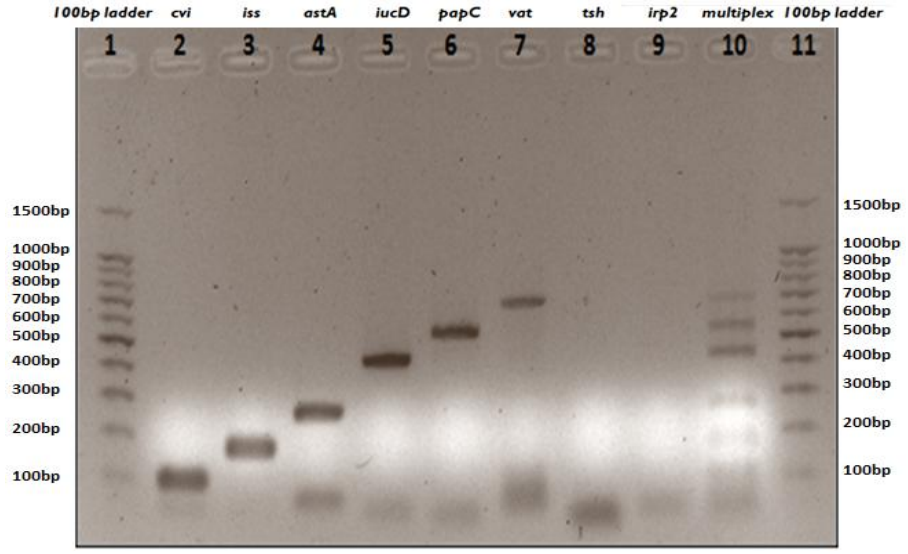

Fig. 4.3: Isolate 031NS Compatibility of the multiplex PCR protocol with gene-specific PCR results for the eight virulence genes. Lane1&11: 100pb DNA ladder; lane 2: *cvi* 114bp; lane 3: *iss* 184bp; lane 4: *astA* 266bp; lane 5: *iucD* 431bp; lane 6: *papC* 537bp; lane 7: *vat* 676bp; lane 8: negative *tsh*; lane 9: negative *irp2*; lane 10: multiplex PCR for (*cvi*, *iss*, *astA*, *iucD*, *papC* and *vat*) genes.

4.4 Genetic profile of the Isolates

In order to rule out the mixed infection by more than *E. coli* strain, a pilot experiment was performed for 15 isolates. For each isolate, six colonies were picked from the enriched culture and were tested individually by the multiplex PCR. The results showed that the 6 colonies of each isolate have the same genetic profile for the 8 virulence factors. Figure 4.4 shows a typical result of the genetic-profile compatibility of 6 colonies from two different isolates.

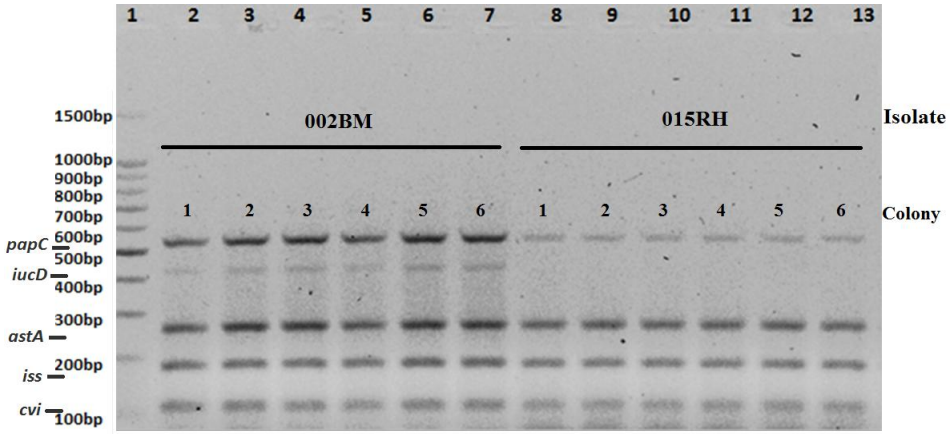

Fig. 4.4: Compatibility of genetic profile of 6 random colonies of the same isolate of origin. Lane1: 100bp DNA ladder; lanes 2-7: Individual colonies of isolate 002BM; lanes 8-13: Individual colonies of isolate 015RH

The optimized multiplex PCR reaction was used to screen the whole panel of the 66 isolates. (Figure 4.5) represents typical gel electrophoresis results of the multiplex PCR for a group of isolates used in this study.

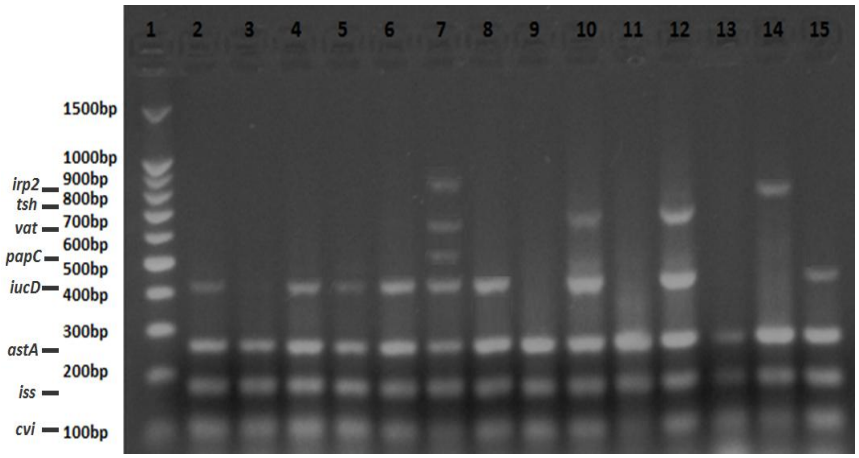

Fig. 4.5: Agarose gel electrophoresis of the multiplex PCR products with representative APEC isolates carrying various combinations of virulence determinants. Lane 1: 100bp DNA ladder; lane 2: Isolate 022QH; lane 3: Isolate 021HN; lane 4: Isolate 024JN; lane 5: Isolate 027BM; lane 6: Isolate 028RH; lane 7: Isolate 014RH; lane 8: Isolate 030RH; lane 9: Isolate 023RH; lane 10: Isolate 016HN; lane 11: Isolate 029HN; lane 12: Isolate 020ST; lane 13: Isolate 054NS; lane 14: Isolate 052HN; lane 15: Isolate 033BM.

The prevalence of the eight virulence genes in the whole panel of isolates is shown in (Table 4.3). Two virulence genes; *iss* and *cvi* showed the highest prevalence (100%), while *astA* (98.48%) and *iucD* (78.79%) were less

prevalent. The genes *vat* (34.85%) and *papC* (31.81%) were present in about one third of the tested strains. To a lesser extent multiplex PCR identified *irp2* (19.70%) and *tsh* (10.61%). The profiles of the virulence genes for the sixty six isolates are shown in (Appendix 2).

Table 4.3: Prevalence of virulence-associated genes in APEC isolates included in this study as detected by multiplex PCR

Tested strains	**Immunity**	**Adhesion**		**Toxin**		**Serum survival**	**Iron uptake**	
	cvi	***papC***	***tsh***	***astA***	***vat***	***iss***	***iucD***	***irp2***
n = 66	66	21	7	65	23	66	52	13
%	100	31.81	10.61	98.48	34.85	100	78.79	19.70

More than 91% of the isolates showed four virulence genes at least, four virulence associated genes are considered as minimal cut-off number of genes for avian *E. coli* to be considered pathogenic strain (Ewers *et al.*, 2005); all strains possessed two virulence genes at least. Data obtained are presented in (Figure 4.6).

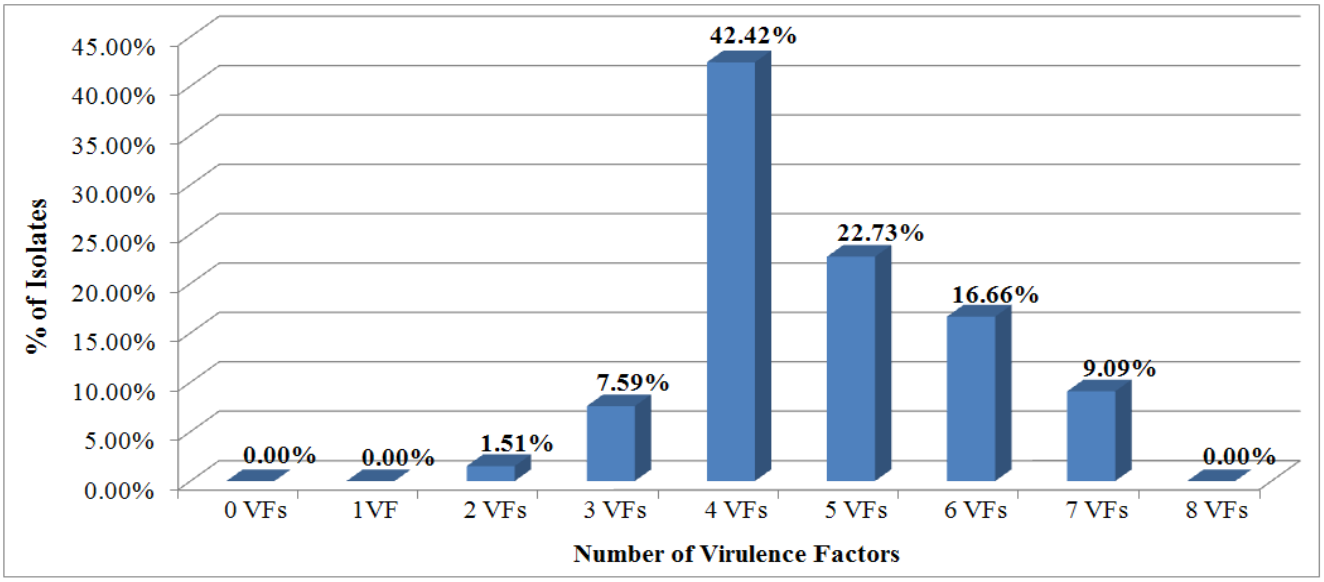

Fig. 4.6: Number of the virulence associated genes detected in isolates

CHAPTER FIVE

DISCUSSION

5.1 Samples Identification

In the present study 83 samples of colibacillosis suspected broiler chicken were used to study and characterize avian pathogenic *E. coli*. 66 samples showed positive for *E. coli*, while the other 17 samples were negative. The lack of *E. coli* in these samples can be due to different reasons. After a recheck of the farms' history, it was noted that many of these samples were obtained from farms that had been subjected to antibiotic treatment directly after the onset of the disease, it is known that antibiotic treatment prevents the isolation of pathogens in most cases. On the other hand, it is known that the pathogenicity of *E. coli* passes through several stages and the last stage is the colonization of the internal organs, since internal organs were the source of isolates, it is possible that the disease itself has not reached the advanced stages, this means that the bacteria did not reach the colonization level at the internal organs. This may have contributed to inability to obtain the bacteria due to its

absence from the sample tissue. Also, other pathogens rather than APEC may have caused similar diseases which explain the absence of APEC.

5.2 Enrichment step

In this study, the specimens that were initially obtained from internal organs were inoculated in a special EC Broth medium that contains 4-methylumbelliferyl- β-D-glucuronide (MUG). The EC-MUG medium was used for its ability to selectively enrich *E. coli* bacteria due to the special additives that it contains. This medium consists of lactose as carbon source, 0.15% bile salts as a selective agent against gram positive bacteria, and 4-methylumbelliferyl- β-D-glucuronide (MUG) compound which can be hydrolyzed by glucuronidase enzyme produced by *E. coli* to yield a fluorogenic product which is detectable under long-wave (366 nm) UV light. While *E. coli* bacterium is a gas producer bacteria and has the ability to grow at 44.5ºC, the enrichment of *E. coli* in EC-MUG at 44.5ºC for 22 to 26 hours with fluorescence and gas production is considered as specific indicator for *E. coli* enrichment.

Selection may have affected the results by eliminating other similar bacteria such as shigella which may share genetic markers with APEC. The specific enrichment and selection of *E. coli* in our study was necessary to reduce the false positive results that may appear from other gram negative bacterial species such as *Salmonella Typhimurium*, and *Shigella flexneri* which may harbor the same ColV plasmid.

5.3 Multiplex protocol

In order to ensure good performance of multiplex PCR, PerlPrimer v1.1.19 software was used to examine essential primer criteria. All primers were analyzed carefully to optimize the melting temperatures, secondary structures, self and cross dimerization, and homology to non-specific genomic template either from *E. coli* itself or from host genome. Using primers that were used in other similar studies was deliberately avoided unless they were carefully analyzed for the mentioned criteria. Some primer pairs that were used in other studies have dissimilar annealing temperature reaching 13ºC and some of these primers suffer from a strong cross dimerization which might affect amplification efficiency (Ewers *et al.*, 2005, Wen-Jie *et al.*, 2008).

5.4 Molecular identification

This study targeted 8 virulence genes that are typically associated with APEC and they are grouped into five functional classes; genes essential for adhesion (*papC* and *tsh*), toxin genes (*astA* and *vat*), serum resistance (*iss*), iron uptake (*irp2* and *iucD*), and *cvi* gene which protect *E. coli* against bacteriocin (Ewers *et al.*, 2005).

The eight virulent genes were present in different combinations ranging from two genes in some isolates to seven genes in other isolates. It is interesting to notice that the pattern of virulent genes in each isolate tends to represent the different functional classes. This might indicate that these genes work in integrated manner to ensure the ability of bacteria to survive and to move from one stage to the next until a full bloom colibacillosis.

The *cvi* gene was detected samples that were *E. coli* positive. The importance of this gene is that it confers the immunity to bacteria against a specific bacteriocin as it encodes the colicin V immunity protein (Fath *et al.*, 1991). In ColV plasmid containing bacteria, colicin V immunity protein can protect the cell against colicin V encoded and secreted by a dedicated export system (Skvirsky *et al.*, 1995).

Adhesion to the lung cells of the bird is essential to APEC especially during the early stages of infection. Pilus is the main virulence factor involved in adhesion of pathogenic *E. coli* to the host cells including: type 1, P, and curli pilus (Dozois *et al.*, 1992). The subunit C of type 1 pilus is encoded by *fimC* gene, and *papC* gene. *papC* is the main functional gene of P pilus. This gene was identified in 31.81% of the sixty six *E. coli* isolates. This frequency is in agreement with previous work which showed (30.0%) (Janben *et al.*, 2001). Compatible to a previous work by Ngeleka et al which showed (15.4%) (Ngeleka *et al.*, 2002); only 10.61% of isolates in this study were positive for *tsh* gene, which is another adhesion- related factor (Tivendale *et al.*, 2004). This might indicate that the presence of *tsh* gene is not necessary to increase the level of pathogenicity of APEC. It is interesting to notice that the 62% of the examined isolates neither have *papC* nor *tsh* adhesion genes. This does not mean that these isolates do not contain any gene coding for adhesion factor. In fact there are several adhesion associated genes that were not examined in this study. Therefore, it is, possible that such isolates contain one or more of the

unexamined adhesion factors. This opens a new avenue for investigating the adhesion factors and tissue tropism association.

Iron acquisition systems have been recognized to be associated with bacterial virulence especially in bacteria causing septicemia (Headley *et al.*, 1997, Gophna *et al.*, 2001, Barghouthi *et al.*, 1989). 80.30% of isolates have at least one of the two examined iron acquisition encoding genes *iucD* and *irp2*.This result demonstrates, indeed, the importance of iron acquisition systems during pathogenesis. In fact, bacteria depend on these systems to get the heme molecule to survive in their host and in aquatic habitats. Remarkably, *iucD* gene that belongs to the aerobactin iron acquisition system stands as the major factor for regulating iron uptake in *E. coli*. It was also described as part of the PAI *Shigella* island-2 (Vokes *et al.*, 1999). It was found that 79% of the examined APEC isolates were positive for *iucD* gene. This result is in agreement with several reports which demonstrated that most APEC strains (63% – 98%) express the *iucD* aerobactin iron acquisition system (Ngeleka *et al.*, 1996, Ewers *et al.*, 2004).

In this study, all isolates were positive for increased serum survival gene (*iss*). Johnson et al demonstrate that *iss* is significantly associated ($p < 0.0001$) with APEC strains than nonpathogenic strains and may be an indicative of its ability to cause disease (Johnson *et al.*, 2002, Johnson *et al.*, 2008). Three alleles of *iss* gene have been identified; one of them is harbored in the ColV virulence plasmids and the others two are chromosomal (Johnson *et al.*, 2008). Thus, *iss*

has a vital role in *E. coli* pathogenicity and could be a potential target for developing novel therapeutics and prevention strategies.

Secretory toxins play key role in enabling pathogenic *E. coli* to influence the biological processes of the host (Qadri *et al.*, 2005). In *E. coli* samples, 98.48% were positive for the *astA* toxin gene. This gene encodes for a peptide enterotoxin-1sequence that is heat-stable, which is a member of heat-stable secretory enterotoxins (STs). STs remain active at temperatures as high as 100^{o}C while they can keep their 3D structure in such high temperatures (Whipp *et al.*, 1975, Kapitany *et al.*, 1979). STs can recognize different receptors on the surface of host cells and affect different intracellular signaling pathways (Hasegawa and Shimonishi, 2005).

About 35% of the isolates were positive for *vat* gene. Vacuolating autotransporter toxin (Vat), the product of *vat* gene, which plays a major role in protein hydrolysis as a result of serine-type endopeptidase activity (Parreira and Gyles, 2003). Vat has been shown as a factor involved in the pathogenicity of APEC strains (Parreira and Gyles, 2003, Ewers *et al.*, 2004, Ewers *et al.*, 2005). In addition to its adhesion related function, Tsh protein which is the product of *tsh* gene, shows a serine-type endopeptidase function similar to Vat (Parreira and Gyles, 2003).

5.5 Antibiotic susceptibility

Comparing data with studies performed in Europe, United States and Japan (Blanco *et al.*, 1997, David and Burch, 2000, ROY *et al.*, 2006) Palestinian

isolates were relatively more resistant for antibiotics than European, United States, and Japan isolates (Table 5.1).

Table 5.1: Comparing antibiotic resistance results to previous works

Antibiotics	Blanco *et al.*, 1997	David and Burch, 2000	Roy *et al.*, 2006	This study
Tetracycline (TE)	94	56	31	100
Ampicillin (AMP)	35	62	-	83.3
Amoxicillin (AML)	-	15	-	83.3
Kanamycin (K)	19	-	-	80.3
Ciprofloxacin (CIP)	17	-	21	72.7
Neomycin (N)	14	83	-	69.7
Gentamycin (CN)	14	-	19	50.0
Chloramphenicol (C)	25	-	31	39.4
Nitrofurantoin (F)	49	-	16	18.2
Cephalexin (CL)	-	-	-	12.1

In Palestine, the Central Veterinary Laboratory confirmed that there was an exaggerate misuse of antibiotics in the poultry sector, which may have enhanced antibiotic selection of resistant strains in various zoonotic pathogens. Unfortunately, there were no studies about the actual consumption rates of antibiotics in the Palestinian poultry sector. However, comparing obtained results of antibiotic resistance with the information available at the Central Veterinary Laboratory, showed a positive correlation between the consumption and resistance frequency. For example, farmers rarely use Nitrofurantoin and Cephalexin which show a good antibacterial activity, whereas the majority of isolates were fairly resistant to Tetracycline, Ampicillin, Amoxicillin,

Kanamycin, and Ciprofloxacin, which are themselves or their closely related antibiotics are commonly used (CVLs).

The indiscriminate use of antibiotics in Palestine may have a major role in increasing resistance in *E. coli*, which has a distinctive ability to adapt with its surrounding environment. Through field trips to more than 15 poultry farms it was observed that all visited farms were routinely subjected to various types of antibiotics as "a preventive prophylactic measure" as they said.

In addition to the misuse of antibiotics, the lack of biosafety standards during rearing and transporting birds can play a major role in accelerating the development of resistance in *E. coli* as well as many other pathogenic organisms. For example, the lack of proper sterilization for the farm between the consecutive breeding cycles, the poor hygienic conditions, and malpractices during preslaughter handling and transportation can create an ideal environment for the genetic transfer of antibiotic resistant genes within and across species (Rule *et al.*, 2008).

E. coli is one the main reservoirs of moveable elements of antibiotic resistance. The high capacity of these bacteria for horizontal gene transfer poses a clear danger for the antibiotics future (Warren *et al.*, 2008). The ability of these bacteria is not limited to transfer of the genetic contents into other *E. coli* strains; in fact, *E. coli* is a common member of biofilms where many species of bacteria exist in close proximity to each other allowing genetic exchange of antibiotic resistance including multidrug resistant plasmids to other bacteria.

Subsequently recipient bacteria gain the ability to resist many kinds of antibiotics (Salyers *et al.*, 2004, Perfeito *et al.*, 2007, Summers, 2006).

Recently, many of extended-spectrum beta-lactamases (ESBL) producing *E. coli* have become a world-wide problem. The ESBL-positive *E. coli* strains are highly resistant to a broad range of antibiotics. Controlling such strains with commonly used antibiotics is ineffective; currently there are very few antibacterial alternatives that remain effective against these multi-resistant pathogens (Paterson and Bonomo, 2005, Yang *et al.*, 2004).

It is very important to control APEC because it represents a grave danger to domestic animals and is a potential source of transferring multi-drug resistance genes to human specific *E. coli* or other bacteria such as *Staphylococcus aureus* and *shigella* strains (Salyers *et al.*, 2004, Elena *et al.*, 2005, Wolf *et al.*, 1979). The fact that this pathogen is naturally present in daily consumed food should be considered as a serious public health and food biosafety issue.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

- The improved multiplex protocol is able to detect eight of APEC pathogenicity related genes to give a picture regarding the virulence factors profile
- The minimal cutoff number of virulence factors is four factors; 91% of the identified isolates had 4 or more virulence factors.
- Most of the identified isolates are multidrug resistance to different types of antibiotics screened in the study

6.2 Recommendations

Because the poultry sector in Palestine is a very important sector on the health and economic levels, there is a vital need to monitor and develop this sector through concerted efforts, especially between the involved responsible authorities, farmers, and consumers.

- Authorities can enact different roles to support such areas economically and to meet the farmers' needs. Authorities can plan research studies on the national level, which puts Palestine on the map and determine the level of agriculture sector in Palestine relative to international standards.

- Farmers have to follow rules based upon scientific consultation in poultry farming and antibiotic use.

- Consumers have the right to obtain healthy food sources that are certified by the Ministry of Health.

APPENDICES

Appendix 1: Antibiotic susceptibility results for the sixty six isolates

Isolate	Antibiotic	Zone of inhibition				Susceptibility
		First reading mm	Second reading mm	Third reading mm	Mean mm	
001RH	Amoxicillin	20	20	19	20	S
	Ampicillin	19	20	19	19	S
	Cephalexin	19	19	19	19	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	14	14	14	14	**R**
	Gentamycin	18	18	18	18	S
	Kanamycin	12	12	11	12	**R**
	Neomycin	17	17	17	17	S
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
002BM	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	18	17	18	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	14	14	14	14	**R**
	Gentamycin	9	10	9	9	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	NZ	NZ	NZ	NT	**R**
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
003JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	17	18	18	S
	Chloramphenicol	10	11	10	10	**R**
	Ciprofloxacin	13	13	13	13	**R**
	Gentamycin	10	10	10	10	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	11	11	11	11	**R**
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
004RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	19	19	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	23	23	23	23	S
	Gentamycin	NZ	NZ	NZ	NT	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	NZ	NZ	NZ	NT	**R**
	Nitrofurantoin	٢٠	٢٠	٢٠	٢٠	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
005TM	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	18	18	18	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	NZ	NZ	NZ	NT	**R**
	Gentamycin	8	8	8	8	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	12	12	12	12	**R**
	Nitrofurantoin	١٩	١٩	١٩	١٩	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
006RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	12	12	12	12	**R**
	Chloramphenicol	10	10	10	10	**R**
	Ciprofloxacin	23	23	23	23	S
	Gentamycin	20	20	21	20	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	17	17	17	17	**R**
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
007JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	19	19	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	24	24	24	24	S
	Gentamycin	19	19	19	19	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	8	8	8	8	**R**
	Nitrofurantoin	17	17	17	17	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
008RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	18	19	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	10	10	10	10	**R**
	Gentamycin	22	22	21	22	S
	Kanamycin	19	19	19	19	S
	Neomycin	18	18	18	18	S
	Nitrofurantoin	17	17	17	17	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
009NS	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	20	20	20	20	S
	Chloramphenicol	10	10	10	10	**R**
	Ciprofloxacin	NZ	NZ	NZ	NT	**R**
	Gentamycin	7	7	7	7	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	9	9	9	9	**R**
	Nitrofurantoin	17	17	17	17	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
011TS	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	20	18	19	S
	Chloramphenicol	21	21	21	21	S
	Ciprofloxacin	25	25	25	25	S
	Gentamycin	19	18	19	19	S
	Kanamycin	18	18	18	18	S
	Neomycin	17	17	17	17	S
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
013RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	11	11	10	11	**R**
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	24	24	24	24	**R**
	Gentamycin	10	10	10	10	**R**
	Kanamycin	18	19	19	19	S
	Neomycin	8	8	8	8	**R**
	Nitrofurantoin	18	18	18	18	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
014RH	Amoxicillin	10	10	11	10	**R**
	Ampicillin	8	9	8	8	**R**
	Cephalexin	18	18	18	18	S
	Chloramphenicol	11	11	11	11	**R**
	Ciprofloxacin	13	13	13	13	**R**
	Gentamycin	9	9	9	9	**R**
	Kanamycin	11	11	11	11	**R**
	Neomycin	12	11	11	11	**R**
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
015RH	Amoxicillin	12	11	11	11	**R**
	Ampicillin	7	6	8	7	**R**
	Cephalexin	9	9	9	9	**R**
	Chloramphenicol	9	8	9	9	**R**
	Ciprofloxacin	21	22	22	22	S
	Gentamycin	18	18	18	18	S
	Kanamycin	20	20	20	20	S
	Neomycin	18	18	18	18	S
	Nitrofurantoin	١٧	١٧	١٧	١٧	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
016HN	Amoxicillin	19	19	19	19	S
	Ampicillin	18	18	18	18	S
	Cephalexin	21	21	21	21	S
	Chloramphenicol	23	22	22	22	S
	Ciprofloxacin	10	10	10	10	**R**
	Gentamycin	18	18	18	18	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	19	19	19	19	S
	Nitrofurantoin	١٣	١٣	١٣	١٣	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
017TM	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	20	20	20	20	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	11	10	11	11	**R**
	Gentamycin	10	10	10	10	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	11	11	11	11	**R**
	Nitrofurantoin	١١	١١	١١	١١	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
018RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	19	19	S
	Chloramphenicol	19	19	19	19	S
	Ciprofloxacin	12	12	12	12	**R**
	Gentamycin	21	21	21	21	S
	Kanamycin	10	10	10	10	**R**
	Neomycin	11	11	11	11	**R**
	Nitrofurantoin	١٢	١٢	١٢	١٢	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
019JN	Amoxicillin	10	10	10	10	**R**
	Ampicillin	8	8	9	8	**R**
	Cephalexin	18	19	19	19	S
	Chloramphenicol	18	18	19	18	S
	Ciprofloxacin	24	23	24	24	S
	Gentamycin	19	19	19	19	S
	Kanamycin	19	19	19	19	S
	Neomycin	19	19	19	19	S
	Nitrofurantoin	١٨	١٨	١٨	١٨	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
020ST	Amoxicillin	12	12	13	12	**R**
	Ampicillin	12	10	11	11	**R**
	Cephalexin	18	18	18	18	S
	Chloramphenicol	21	21	20	21	S
	Ciprofloxacin	8	8	8	8	**R**
	Gentamycin	18	18	18	18	S
	Kanamycin	20	19	20	20	S
	Neomycin	18	18	18	18	S
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
021HN	Amoxicillin	26	25	25	25	S
	Ampicillin	22	22	22	22	S
	Cephalexin	18	19	19	19	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	9	9	8	9	**R**
	Gentamycin	19	18	19	19	S
	Kanamycin	11	10	10	10	**R**
	Neomycin	21	20	20	20	S
	Nitrofurantoin	١٧	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
022QH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	21	21	21	21	S
	Chloramphenicol	19	19	19	19	S
	Ciprofloxacin	10	9	10	10	**R**
	Gentamycin	NZ	NZ	NZ	NT	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	10	10	10	10	**R**
	Nitrofurantoin	١٢	١٢	١٢	١٢	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
023RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	22	22	22	22	S
	Chloramphenicol	21	21	21	21	S
	Ciprofloxacin	24	24	23	24	S
	Gentamycin	16	16	15	16	S
	Kanamycin	10	10	10	10	**R**
	Neomycin	12	11	11	11	**R**
	Nitrofurantoin	٢٠	٢٠	٢٠	٢٠	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
024JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	18	18	18	S
	Chloramphenicol	17	18	18	18	S
	Ciprofloxacin	NZ	NZ	NZ	NT	**R**
	Gentamycin	12	11	11	11	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	NZ	NZ	NZ	NT	**R**
	Nitrofurantoin	١٤	١٣	١٣	١٣	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
025RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	18	19	S
	Chloramphenicol	24	24	24	24	S
	Ciprofloxacin	12	11	12	12	**R**
	Gentamycin	19	18	19	19	S
	Kanamycin	11	11	11	11	**R**
	Neomycin	10	10	10	10	**R**
	Nitrofurantoin	٢٢	٢٣	٢٢	٢٢	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
026JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	21	21	21	21	S
	Chloramphenicol	23	23	23	23	S
	Ciprofloxacin	13	12	12	12	**R**
	Gentamycin	18	18	18	18	S
	Kanamycin	21	20	20	20	S
	Neomycin	١٩	١٩	١٩	١٩	S
	Nitrofurantoin	٢٠	٢٠	٢٠	٢٠	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
027BM	Amoxicillin	23	23	23	23	S
	Ampicillin	20	19	19	19	S
	Cephalexin	20	19	20	20	S
	Chloramphenicol	24	24	23	24	S
	Ciprofloxacin	12	12	12	12	**R**
	Gentamycin	16	16	16	16	S
	Kanamycin	8	8	8	8	**R**
	Neomycin	٨	٩	٩	٩	**R**
	Nitrofurantoin	١٠	١٠	١٠	١٠	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
028RH	Amoxicillin	10	10	10	10	**S**
	Ampicillin	10	10	10	10	**R**
	Cephalexin	18	18	18	18	S
	Chloramphenicol	24	24	24	24	S
	Ciprofloxacin	25	25	24	24	S
	Gentamycin	17	16	16	16	S
	Kanamycin	18	18	18	18	S
	Neomycin	١٨	١٨	١٨	١٨	S
	Nitrofurantoin	٢٠	٢٠	٢٠	٢٠	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
029HN	Amoxicillin	9	8	7	8	**R**
	Ampicillin	7	7	8	7	**R**
	Cephalexin	8	8	8	8	**R**
	Chloramphenicol	22	22	22	22	S
	Ciprofloxacin	14	14	14	14	**R**
	Gentamycin	12	11	11	11	**R**
	Kanamycin	12	12	12	12	**R**
	Neomycin	١٢	١٢	١٢	١٢	**R**
	Nitrofurantoin	٢٠	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
030RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	11	11	10	11	**R**
	Chloramphenicol	22	22	22	22	S
	Ciprofloxacin	7	7	8	7	**R**
	Gentamycin	10	10	10	10	**R**
	Kanamycin	11	11	11	11	**R**
	Neomycin	١١	١٠	١١	١١	**R**
	Nitrofurantoin	١٧	١٧	١٧	١٧	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
031NS	Amoxicillin	20	20	19	20	**R**
	Ampicillin	18	18	18	18	**R**
	Cephalexin	18	17	18	18	S
	Chloramphenicol	22	22	22	22	S
	Ciprofloxacin	11	11	11	11	**R**
	Gentamycin	18	18	18	18	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	١٢	١٢	١٢	١٢	**R**

	Tetracycline	NZ	NZ	NZ	NT	**R**
032JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	18	18	18	S
	Chloramphenicol	28	27	28	28	S
	Ciprofloxacin	12	11	12	12	**R**
	Gentamycin	8	8	8	8	**R**
	Kanamycin	10	10	10	10	**R**
	Neomycin	١٩	١٩	١٩	١٩	S
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
033BM	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	11	10	10	10	**R**
	Chloramphenicol	21	22	21	21	S
	Ciprofloxacin	10	10	10	10	**R**
	Gentamycin	10	10	9	10	**R**
	Kanamycin	11	12	11	11	**R**
	Neomycin	١١	١١	١١	١١	**R**
	Nitrofurantoin	١٨	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
034NS	Amoxicillin	12	11	11	11	**R**
	Ampicillin	12	10	10	11	**R**
	Cephalexin	9	10	10	10	**R**
	Chloramphenicol	24	23	24	24	S
	Ciprofloxacin	12	11	11	11	**R**
	Gentamycin	11	11	11	11	**R**
	Kanamycin	13	13	13	13	**R**
	Neomycin	١٠	١٠	١٠	١٠	**R**
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
035JO	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	18	19	S
	Chloramphenicol	23	23	23	23	S
	Ciprofloxacin	11	11	11	11	**R**
	Gentamycin	21	21	21	21	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٩	٩	٩	٩	**R**
	Nitrofurantoin	١٨	١٧	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
036RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	18	19	18	S
	Chloramphenicol	20	20	20	20	S
	Ciprofloxacin	26	26	26	26	S
	Gentamycin	19	18	19	19	S
	Kanamycin	12	12	12	12	**R**
	Neomycin	١١	١١	١١	١١	**R**
	Nitrofurantoin	١٩	٢٠	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
037JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	20	21	20	20	S
	Chloramphenicol	7	8	8	8	**R**
	Ciprofloxacin	7	7	7	7	**R**
	Gentamycin	16	17	16	16	S
	Kanamycin	12	11	11	11	**R**
	Neomycin	١٢	١٢	١٢	١٢	**R**
	Nitrofurantoin	٢١	٢١	٢١	٢١	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
038RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	20	22	20	21	S
	Chloramphenicol	21	21	21	21	S
	Ciprofloxacin	9	8	8	8	**R**
	Gentamycin	17	17	17	17	S
	Kanamycin	19	19	19	19	S
	Neomycin	١٧	١٨	١٨	١٨	S
	Nitrofurantoin	١٧	١٧	١٧	١٧	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
039RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	19	19	19	S
	Chloramphenicol	22	23	23	23	S
	Ciprofloxacin	24	24	23	24	S
	Gentamycin	17	17	17	17	S
	Kanamycin	18	18	18	18	S
	Neomycin	١٨	١٨	١٨	١٨	S
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
041HN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	21	20	20	20	S
	Chloramphenicol	21	20	21	21	S
	Ciprofloxacin	23	23	21	22	S
	Gentamycin	19	19	19	19	S
	Kanamycin	17	18	18	18	S
	Neomycin	١٩	١٨	١٩	١٩	S
	Nitrofurantoin	19	19	18	19	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
042HN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	19	19	S
	Chloramphenicol	23	23	23	23	S
	Ciprofloxacin	20	21	21	21	S
	Gentamycin	18	18	18	18	S
	Kanamycin	18	18	18	18	S
	Neomycin	١٩	١٩	١٩	١٩	S
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
043RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	18	19	S
	Chloramphenicol	21	21	21	21	S
	Ciprofloxacin	9	9	9	9	**R**
	Gentamycin	9	9	9	9	**R**
	Kanamycin	13	12	12	12	**R**
	Neomycin	٢١	٢٠	٢٠	٢٠	S
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
044JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	18	18	18	S
	Chloramphenicol	18	18	18	18	S
	Ciprofloxacin	12	12	12	12	**R**
	Gentamycin	18	18	18	18	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	19	19	18	19	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
045HN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	18	17	18	S
	Chloramphenicol	8	8	8	8	**R**
	Ciprofloxacin	12	11	12	12	**R**
	Gentamycin	19	19	19	19	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	١١	١١	١١	١١	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
046RH	Amoxicillin	10	8	8	9	**R**
	Ampicillin	9	8	9	9	**R**
	Cephalexin	19	18	19	19	S
	Chloramphenicol	19	19	19	19	S
	Ciprofloxacin	22	22	21	21	S
	Gentamycin	19	19	19	19	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	٢٠	٢٠	٢٠	٢٠	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
047TM	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	22	22	22	22	S
	Chloramphenicol	21	22	22	22	S
	Ciprofloxacin	11	11	10	11	**R**
	Gentamycin	10	10	10	10	**R**
	Kanamycin	8	8	8	8	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	٢١	٢٢	٢١	٢١	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
049JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	21	21	21	21	S
	Chloramphenicol	10	10	9	10	**R**
	Ciprofloxacin	21	20	21	21	S
	Gentamycin	19	19	19	19	S
	Kanamycin	10	10	10	10	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
050RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	19	19	S
	Chloramphenicol	10	10	10	10	**R**
	Ciprofloxacin	22	22	21	22	S
	Gentamycin	17	17	17	17	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٩	٩	٩	٩	**R**
	Nitrofurantoin	٢٠	٢٠	٢٠	٢٠	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
051NS	Amoxicillin	21	19	19	20	S
	Ampicillin	19	18	18	18	S
	Cephalexin	21	21	21	21	S
	Chloramphenicol	19	19	18	19	S
	Ciprofloxacin	12	12	12	12	**R**
	Gentamycin	17	18	18	18	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٩	٩	٩	٩	**R**
	Nitrofurantoin	٢٠	٢٠	٢٠	٢٠	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
052HN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	22	21	21	21	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	12	12	12	12	**R**
	Gentamycin	10	10	10	10	**R**
	Kanamycin	10	10	10	10	**R**
	Neomycin	١١	١١	١١	١١	**R**
	Nitrofurantoin	٢٢	٢١	٢٠	٢١	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
053HN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	20	20	20	20	S
	Chloramphenicol	19	19	19	19	S
	Ciprofloxacin	22	21	21	21	S
	Gentamycin	NZ	NZ	NZ	NT	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
054NS	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	19	19	S
	Chloramphenicol	28	28	27	28	S
	Ciprofloxacin	11	11	11	11	**R**
	Gentamycin	21	21	21	21	S
	Kanamycin	14	13	13	13	**R**
	Neomycin	١٨	١٨	١٨	١٨	S
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
055RH	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	18	18	19	18	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	8	8	8	8	**R**
	Gentamycin	11	11	11	11	**R**
	Kanamycin	10	10	10	10	**R**
	Neomycin	١٢	١٢	١١	١٢	**R**
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
056NS	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	17	18	18	18	S
	Chloramphenicol	27	27	27	27	S
	Ciprofloxacin	NZ	NZ	NZ	NT	**R**
	Gentamycin	17	16	17	17	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	١٠	١٠	١٠	١٠	**R**
	Nitrofurantoin	١٩	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
057JO	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	22	22	22	22	S
	Chloramphenicol	26	26	26	26	S
	Ciprofloxacin	14	15	14	14	**R**
	Gentamycin	19	19	19	19	S
	Kanamycin	12	12	12	12	**R**
	Neomycin	١١	١١	١١	١١	**R**
	Nitrofurantoin	١٩	١٩	١٩	١٩	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
058HN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	19	19	19	S
	Chloramphenicol	23	24	24	24	S
	Ciprofloxacin	8	8	8	8	**R**
	Gentamycin	8	8	8	8	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	١٠	١٠	١٠	١٠	**R**
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
059JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	21	21	21	21	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	23	23	22	23	S
	Gentamycin	11	11	11	11	**R**
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	١١	١١	١١	١١	**R**
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
060JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	20	21	20	20	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	14	15	14	14	**R**
	Gentamycin	24	24	24	24	S
	Kanamycin	10	10	10	10	**R**
	Neomycin	١٠	١٠	١٠	١٠	**R**
	Nitrofurantoin	١٨	١٨	١٨	١٨	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
061JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	20	20	20	S
	Chloramphenicol	NZ	NZ	NZ	NT	**R**
	Ciprofloxacin	7	7	7	7	**R**
	Gentamycin	16	16	16	16	S
	Kanamycin	10	10	10	10	**R**
	Neomycin	١٢	١٢	١٢	١٢	**R**
	Nitrofurantoin	١٨	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
062JN	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	19	21	21	20	S
	Chloramphenicol	10	10	10	10	**R**
	Ciprofloxacin	14	14	14	14	**R**
	Gentamycin	23	23	23	23	S
	Kanamycin	19	19	19	19	S
	Neomycin	٢٤	٢٤	٢٤	٢٤	S
	Nitrofurantoin	٢٢	٢٢	٢٢	٢٢	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
066RH	Amoxicillin	20	20	20	20	R
	Ampicillin	19	18	18	18	S
	Cephalexin	18	18	18	18	S
	Chloramphenicol	11	12	12	12	**R**
	Ciprofloxacin	11	11	11	11	**R**
	Gentamycin	9	9	9	9	**R**
	Kanamycin	13	13	13	13	**R**
	Neomycin	٩	٩	٩	٩	**R**
	Nitrofurantoin	١٢	١١	١١	١١	**R**

	Tetracycline	NZ	NZ	NZ	NT	**R**
069JM	Amoxicillin	NZ	NZ	NZ	NT	**R**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	22	22	22	22	S
	Chloramphenicol	20	20	20	20	S
	Ciprofloxacin	NZ	NZ	NZ	NT	**R**
	Gentamycin	20	20	21	20	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	١٠	١٠	١٠	١٠	**R**
	Nitrofurantoin	٢٠	٢١	٢٠	٢٠	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
072QH	Amoxicillin	22	22	21	22	S
	Ampicillin	19	19	19	19	S
	Cephalexin	21	20	20	20	S
	Chloramphenicol	23	22	23	23	S
	Ciprofloxacin	11	11	11	11	**R**
	Gentamycin	22	21	22	22	S
	Kanamycin	12	12	12	12	**R**
	Neomycin	١٢	١٢	١١	١٢	**R**
	Nitrofurantoin	١٩	١٩	١٩	١٩	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
073JO	Amoxicillin	12	12	12	12	**R**
	Ampicillin	11	11	10	11	**R**
	Cephalexin	8	9	9	9	**R**
	Chloramphenicol	12	12	12	12	**R**
	Ciprofloxacin	12	12	11	12	**R**
	Gentamycin	18	18	18	18	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	١١	١٠	١٠	١٠	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
075RH	Amoxicillin	21	21	21	21	R
	Ampicillin	20	20	19	20	S
	Cephalexin	21	21	20	21	S
	Chloramphenicol	20	20	20	20	S
	Ciprofloxacin	8	8	8	8	**R**
	Gentamycin	19	19	19	19	S
	Kanamycin	NZ	NZ	NZ	NT	**R**
	Neomycin	٨	٨	٨	٨	**R**
	Nitrofurantoin	٩	٩	٩	٩	**R**
	Tetracycline	NZ	NZ	NZ	NT	**R**
078RH	Amoxicillin	22	20	20	21	S
	Ampicillin	20	18	19	19	S
	Cephalexin	18	18	18	18	S
	Chloramphenicol	11	11	11	11	**R**
	Ciprofloxacin	14	14	14	14	**R**
	Gentamycin	19	18	19	19	S
	Kanamycin	13	13	13	13	**R**
	Neomycin	١٠	١٠	١٠	١٠	**R**
	Nitrofurantoin	١٩	٢٠	٢٠	٢٠	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
079RH	Amoxicillin	NZ	NZ	NZ	NT	**S**
	Ampicillin	NZ	NZ	NZ	NT	**R**
	Cephalexin	21	20	20	20	S
	Chloramphenicol	23	23	23	23	S
	Ciprofloxacin	24	24	24	24	S
	Gentamycin	18	18	18	18	S
	Kanamycin	18	19	19	19	S
	Neomycin	١٩	١٨	١٨	١٨	S
	Nitrofurantoin	٢١	٢١	٢١	٢١	S

	Tetracycline	NZ	NZ	NZ	NT	**R**
081JO	Amoxicillin	19	19	19	19	S
	Ampicillin	18	18	19	18	S
	Cephalexin	19	19	19	19	S
	Chloramphenicol	20	20	20	20	S
	Ciprofloxacin	9	9	9	9	**R**
	Gentamycin	8	8	8	8	**R**
	Kanamycin	9	9	9	9	**R**
	Neomycin	١٨	١٨	١٧	١٨	S
	Nitrofurantoin	٢٢	٢١	٢٢	٢٢	S
	Tetracycline	NZ	NZ	NZ	NT	**R**
	NZ: No Zone of inhibition **NT: Not Tested**					

Appendix 2: virulence factor profiles for the sixty six isolates

	Chromosomal Genes				**Plasmid Genes**			
Isolate	***papC***	***irp2***	***astA***	***vat***	***cvi***	***iucD***	***tsh***	***iss***
001RH	P	N	P	P	P	P	P	P
002BM	P	N	P	N	P	P	N	P
003JN	N	N	P	N	P	P	N	P
004RH	N	N	P	N	P	P	P	P
005TM	N	N	P	N	P	N	P	P
006RH	P	N	P	N	P	P	P	P
007JN	N	P	P	P	P	P	N	P
008RH	N	P	P	P	P	P	N	P
009NS	P	P	P	P	P	P	N	P
011TS	P	P	P	P	P	P	N	P
013RH	N	N	P	P	P	P	N	P
014RH	P	P	P	P	P	P	N	P
015RH	P	N	P	N	P	N	N	P
016HN	N	N	P	P	P	P	N	P
017TM	P	N	P	N	P	N	N	P
018RH	N	N	P	N	P	P	N	P
019JN	N	N	P	N	P	P	N	P
020ST	N	N	P	P	P	P	N	P
021HN	N	N	P	N	P	N	N	P
022QH	N	N	P	N	P	P	N	P
023RH	N	N	P	N	P	N	N	P
024JN	N	N	P	N	P	P	N	P
025RH	N	N	P	P	P	P	N	P
026JN	P	N	P	N	P	N	N	P
027BM	N	N	P	N	P	P	N	P
028RH	N	N	P	N	P	P	N	P
029HN	N	N	P	N	P	N	N	P
030RH	N	N	P	N	P	P	N	P
031NS	P	N	P	P	P	P	N	P
032JN	N	N	N	N	P	N	N	P
033BM	N	N	P	N	P	P	N	P
034NS	N	N	P	P	P	N	N	P
035JO	N	N	P	P	P	P	N	P
036RH	N	N	P	P	P	P	N	P
037JN	N	N	P	N	P	P	N	P
038RH	P	N	P	P	P	P	N	P
039RH	N	N	P	N	P	P	P	P
041HN	N	N	P	P	P	P	N	P
042HN	N	N	P	P	P	P	N	P

043RH	N	N	P	P	P	N	N	P
044JN	N	P	P	P	P	P	N	P
045HN	P	N	P	P	P	P	N	P
046RH	N	N	P	N	P	P	N	P
047TM	N	N	P	N	P	P	N	P
049JN	P	N	P	P	P	P	N	P
050RH	N	N	P	N	P	P	N	P
051NS	N	N	P	N	P	P	N	P
052HN	N	P	P	N	P	P	N	P
053HN	P	N	P	N	P	P	N	P
054NS	N	N	P	N	P	N	N	P
055RH	N	N	P	N	P	N	P	P
056NS	N	N	P	N	P	P	N	P
057JO	N	N	P	N	P	P	N	P
058HN	N	N	P	N	P	P	N	P
059JN	P	P	P	N	P	P	N	P
060JN	P	N	P	N	P	P	N	P
061JN	P	N	P	N	P	P	N	P
062JN	P	P	P	N	P	P	N	P
066RH	P	P	P	N	P	P	N	P
069JM	N	N	P	N	P	P	N	P
072QH	P	P	P	N	P	P	P	P
073JO	N	N	P	N	P	P	N	P
075RH	P	P	P	P	P	P	N	P
078RH	N	N	P	N	P	N	N	P
079RH	N	P	P	N	P	N	N	P
081JO	N	N	P	P	P	P	N	P
	P: Positive **N: Negative**							

REFERENCES

ACHTMAN, M., HEUZENROEDER, M., KUSECEK, B., OCHMAN, H., CAUGANT, D., SELANDER, R. K., VAISANEN-RHEN, V., KORHONEN, T. K., STUART, S., ORSKOV, F. & ET AL. 1986. Clonal analysis of Escherichia coli O2:K1 isolated from diseased humans and animals. *Infect Immun,* 51**,** 268-76.

AMABILE DE CAMPOS, T., STEHLING, E. G., FERREIRA, A., PESTANA DE CASTRO, A. F., BROCCHI, M. & DIAS DA SILVEIRA, W. 2005. Adhesion properties, fimbrial expression and PCR detection of adhesin-related genes of avian Escherichia coli strains. *Vet Microbiol,* 106**,** 275-85.

ANTAO, E. M., GLODDE, S., LI, G., SHARIFI, R., HOMEIER, T., LATURNUS, C., DIEHL, I., BETHE, A., PHILIPP, H. C., PREISINGER, R., WIELER, L. H. & EWERS, C. 2008. The chicken as a natural model for extraintestinal infections caused by avian pathogenic Escherichia coli (APEC). *Microb Pathog,* 45**,** 361-9.

BARGHOUTHI, S., YOUNG, R., ARCENEAUX, J. E. & BYERS, B. R. 1989. Physiological control of amonabactin biosynthesis in Aeromonas hydrophila. *Biol Met,* 2**,** 155-60.

BAUER, A. W., KIRBY, W. M., SHERRIS, J. C. & TURCK, M. 1966. Antibiotic susceptibility testing by a standardized single disk method. *Am J Clin Pathol,* 45**,** 493-6.

BENTLEY, R. & MEGANATHAN, R. 1982. Biosynthesis of vitamin K (menaquinone) in bacteria. *Microbiol Rev,* 46**,** 241-80.

BLANCO, J. E., BLANCO, M., MORA, A. & BLANCO, J. 1997. Prevalence of bacterial resistance to quinolones and other antimicrobials among avian Escherichia coli strains isolated from septicemic and healthy chickens in Spain. *J Clin Microbiol,* 35**,** 2184-5.

BLATTNER, F. R., PLUNKETT, G., 3RD, BLOCH, C. A., PERNA, N. T., BURLAND, V., RILEY, M., COLLADO-VIDES, J., GLASNER, J. D., RODE, C. K., MAYHEW, G. F., GREGOR, J., DAVIS, N. W., KIRKPATRICK, H. A., GOEDEN, M. A., ROSE, D. J., MAU, B. & SHAO, Y. 1997. The complete genome sequence of Escherichia coli K-12. *Science,* 277**,** 1453-62.

BRUSSOW, H., CANCHAYA, C. & HARDT, W. D. 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiol Mol Biol Rev,* 68**,** 560-602.

CARVALHO DE MOURA, A. C., IRINO, K. & VIDOTTO, M. C. 2001. Genetic variability of avian Escherichia coli strains evaluated by enterobacterial repetitive intergenic consensus and repetitive extragenic palindromic polymerase chain reaction. *Avian Dis,* 45**,** 173-81.

DAVID, G. & BURCH, S. 2000. Antimicrobial sensitivity patterns of UK chicken *E. coli* isolates. *European Association of Veterinary Pharmacology and Toxicology Congress Octagon Services Ltd., Old Windsor, Berks, UK.***,** 73.

DELICATO, E. R., DE BRITO, B. G., GAZIRI, L. C. & VIDOTTO, M. C. 2003. Virulence-associated genes in Escherichia coli isolates from poultry with colibacillosis. *Vet Microbiol,* 94**,** 97-103.

DHO-MOULIN, M. & FAIRBROTHER, J. M. 1999. Avian pathogenic Escherichia coli (APEC). *Vet Res,* 30**,** 299-316.

DOZOIS, C. M., DAIGLE, F. & CURTISS, R., 3RD 2003. Identification of pathogen-specific and conserved genes expressed in vivo by an avian pathogenic Escherichia coli strain. *Proc Natl Acad Sci U S A,* 100**,** 247-52.

DOZOIS, C. M., DHO-MOULIN, M., BREE, A., FAIRBROTHER, J. M., DESAUTELS, C. & CURTISS, R., 3RD 2000. Relationship between the Tsh autotransporter and pathogenicity of avian Escherichia coli and localization and analysis of the Tsh genetic region. *Infect Immun,* 68**,** 4145-54.

DOZOIS, C. M., FAIRBROTHER, J. M., HAREL, J. & BOSSE, M. 1992. pap-and pil-related DNA sequences and other virulence determinants associated with Escherichia coli isolated from septicemic chickens and turkeys. *Infect Immun,* 60**,** 2648-56.

ELENA, S. F., WHITTAM, T. S., WINKWORTH, C. L., RILEY, M. A. & LENSKI, R. E. 2005. Genomic divergence of Escherichia coli strains: evidence for horizontal transfer and variation in mutation rates. *Int Microbiol,* 8**,** 271-8.

ELFADIL, A. A., VAILLANCOURT, J. P., MEEK, A. H., JULIAN, R. J. & GYLES, C. L. 1996. Description of cellulitis lesions and associations between cellulitis and other categories of condemnation. *Avian Dis,* 40**,** 690-8.

EWERS, C., JANSSEN, T., KIESSLING, S., PHILIPP, H. C. & WIELER, L. H. 2004. Molecular epidemiology of avian pathogenic Escherichia coli (APEC) isolated from colisepticemia in poultry. *Vet Microbiol,* 104**,** 91-101.

EWERS, C., JANSSEN, T., KIESSLING, S., PHILIPP, 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**,** 269-73.

EWERS, C., LI, G., WILKING, H., KIESSLING, S., ALT, K., ANTAO, E. M., LATURNUS, C., DIEHL, I., GLODDE, S., HOMEIER, T., BOHNKE, U., STEINRUCK, H., PHILIPP, H. C. & WIELER, L. H. 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing Escherichia coli: how closely related are they? *Int J Med Microbiol,* 297**,** 163-76.

FATH, M. J., SKVIRSKY, R. C. & KOLTER, R. 1991. Functional complementation between bacterial MDR-like export systems: colicin V, alpha-hemolysin, and Erwinia protease. *J Bacteriol,* 173**,** 7549-56.

FOTADAR, U., ZAVELOFF, P. & TERRACIO, L. 2005. Growth of Escherichia coli at elevated temperatures. *J Basic Microbiol,* 45**,** 403-4.

GERMON, P., CHEN, Y. H., HE, L., BLANCO, J. E., BREE, A., SCHOULER, C., HUANG, S. H. & MOULIN-SCHOULEUR, M. 2005. ibeA, a virulence factor of avian pathogenic Escherichia coli. *Microbiology,* 151**,** 1179-86.

GINNS, C. A., BENHAM, M. L., ADAMS, L. M., WHITHEAR, K. G., BETTELHEIM, K. A., CRABB, B. S. & BROWNING, G. F. 2000. Colonization of the respiratory tract by a virulent strain of avian Escherichia coli requires carriage of a conjugative plasmid. *Infect Immun,* 68**,** 1535-41.

GIOVANARDI, D., CAMPAGNARI, E., RUFFONI, L. S., PESENTE, P., ORTALI, G. & FURLATTINI, V. 2005. Avian pathogenic Escherichia coli transmission from broiler breeders to their progeny in an integrated poultry production chain. *Avian Pathol,* 34**,** 313-8.

GOPHNA, U., OELSCHLAEGER, T. A., HACKER, J. & RON, E. Z. 2001. Yersinia HPI in septicemic Escherichia coli strains isolated from diverse hosts. *FEMS Microbiol Lett,* 196**,** 57-60.

HAFEZ, H. M. & LOHREN, U. 1990. Swollen head syndrome: clinical observations and serological examinations in West Germany. *Dtsch Tierarztl Wochenschr,* 97**,** 322-4.

HASEGAWA, M. & SHIMONISHI, Y. 2005. Recognition and signal transduction mechanism of Escherichia coli heat-stable enterotoxin and its receptor, guanylate cyclase C. *J Pept Res,* 65**,** 261-71.

HEADLEY, V., HONG, M., GALKO, M. & PAYNE, S. M. 1997. Expression of aerobactin genes by Shigella flexneri during extracellular and intracellular growth. *Infect Immun,* 65**,** 818-21.

HERREN, C. D., MITRA, A., PALANIYANDI, S. K., COLEMAN, A., ELANKUMARAN, S. & MUKHOPADHYAY, S. 2006. The BarA-UvrY two-component system regulates virulence in avian pathogenic Escherichia coli O78:K80:H9. *Infect Immun,* 74**,** 4900-9.

JANBEN, T., SCHWARZ, C., PREIKSCHAT, P., VOSS, M., PHILIPP, H. C. & WIELER, L. H. 2001. Virulence-associated genes in avian pathogenic Escherichia coli (APEC) isolated from internal organs of poultry having died from colibacillosis. *Int J Med Microbiol,* 291**,** 371-8.

JOHNSON, T. J., GIDDINGS, C. W., HORNE, S. M., GIBBS, P. S., WOOLEY, R. E., SKYBERG, J., OLAH, P., KERCHER, R., SHERWOOD, J. S., FOLEY, S. L. & NOLAN, L. K. 2002. Location of increased serum survival gene and selected virulence traits on a conjugative R plasmid in an avian Escherichia coli isolate. *Avian Dis,* 46**,** 342-52.

JOHNSON, T. J., KARIYAWASAM, S., WANNEMUEHLER, Y., MANGIAMELE, P., JOHNSON, S. J., DOETKOTT, C., SKYBERG, J. A., LYNNE, A. M., JOHNSON, J. R. & NOLAN, L. K. 2007. The genome sequence of avian pathogenic Escherichia coli strain O1:K1:H7 shares strong similarities with human extraintestinal pathogenic E. coli genomes. *J Bacteriol,* 189**,** 3228-36.

JOHNSON, T. J., WANNEMUEHLER, Y. M. & NOLAN, L. K. 2008. Evolution of the iss gene in Escherichia coli. *Appl Environ Microbiol,* 74**,** 2360-9.

KAPITANY, R. A., SCOOT, A., FORSYTH, G. W., MCKENZIE, S. L. & WORTHINGTON, R. W. 1979. Evidence for two heat-stable enterotoxins produced by enterotoxigenic Escherichia coli. *Infect Immun,* 24**,** 965-6.

KARCH, H., SCHUBERT, S., ZHANG, D., ZHANG, W., SCHMIDT, H., OLSCHLAGER, T. & HACKER, J. 1999. A genomic island, termed high-pathogenicity island, is present in certain non-O157 Shiga toxin-producing Escherichia coli clonal lineages. *Infect Immun,* 67**,** 5994-6001.

KAWANO, M., YAGUCHI, K. & OSAWA, R. 2006. Genotypic analyses of Escherichia coli isolated from chickens with colibacillosis and apparently healthy chickens in Japan. *Microbiol Immunol,* 50**,** 961-6.

KILIC, A., ERTAFI, H., MUZ, A., ÖZBEY, G. & KALENDER, H. 2007. Detection of the eaeA Gene in *Escherichia coli* from Chickens by Polymerase Chain Reaction. *Turkish Journal of Veterinary and Animal Sciences,* 31**,** 215-218.

KNOBL, T., GOMES, T., VIEIRA, M., FERREIRA, F., BOTTINO, J. & FERREIRA, A. 2006. Some Adhesins of Avian Pathogenic *Escherichia coli* (APEC) Isolated from Septicemic Poultry in Brazil. *Brazilian Journal of Microbiology,* 37**,** 379-384.

LA RAGIONE, R. M., SAYERS, A. R. & WOODWARD, M. J. 2000. The role of fimbriae and flagella in the colonization, invasion and persistence of

Escherichia coli O78:K80 in the day-old-chick model. *Epidemiol Infect,* 124**,** 351-63.

LA RAGIONE, R. M. & WOODWARD, M. J. 2002. Virulence factors of Escherichia coli serotypes associated with avian colisepticaemia. *Res Vet Sci,* 73**,** 27-35.

LAFONT, J. P., DHO, M., D'HAUTEVILLE, H. M., BREE, A. & SANSONETTI, P. J. 1987. Presence and expression of aerobactin genes in virulent avian strains of Escherichia coli. *Infect Immun,* 55**,** 193-7.

LAMARCHE, M. G., DOZOIS, C. M., DAIGLE, F., CAZA, M., CURTISS, R., 3RD, DUBREUIL, J. D. & HAREL, J. 2005. Inactivation of the pst system reduces the virulence of an avian pathogenic Escherichia coli O78 strain. *Infect Immun,* 73**,** 4138-45.

LAMMERT, J. M. 2007. *Techniques in Microbiology: A student Handbook,* NewYork, Benjamin/Cummings Pub Co.

LINTON, A. H., HOWE, K., BENNETT, P. M., RICHMOND, M. H. & WHITESIDE, E. J. 1977. The colonization of the human gut by antibiotic resistant Escherichia coli from chickens. *J Appl Bacteriol,* 43**,** 465-9.

LUTFUL KABIR, S. M. 2010. Avian colibacillosis and salmonellosis: a closer look at epidemiology, pathogenesis, diagnosis, control and public health concerns. *Int J Environ Res Public Health,* 7**,** 89-114.

MAURER, J. J., LEE, M. D., LOBSINGER, C., BROWN, T., MAIER, M. & THAYER, S. G. 1998. Molecular typing of avian Escherichia coli isolates by random amplification of polymorphic DNA. *Avian Dis,* 42**,** 431-51.

MELLATA, M., DHO-MOULIN, M., DOZOIS, C. M., CURTISS, R., 3RD, LEHOUX, B. & FAIRBROTHER, J. M. 2003. Role of avian pathogenic Escherichia coli virulence factors in bacterial interaction with chicken heterophils and macrophages. *Infect Immun,* 71**,** 494-503.

MOKADY, D., GOPHNA, U. & RON, E. Z. 2005. Extensive gene diversity in septicemic Escherichia coli strains. *J Clin Microbiol,* 43**,** 66-73.

MORLEY, A. J. & THOMSON, D. K. 1984. Swollen-head syndrome in broiler chickens. *Avian Dis,* 28**,** 238-43.

MOULIN-SCHOULEUR, M., REPERANT, M., LAURENT, S., BREE, A., MIGNON-GRASTEAU, S., GERMON, P., RASSCHAERT, D. & SCHOULER, C. 2007. Extraintestinal pathogenic Escherichia coli strains of avian and human origin: link between phylogenetic relationships and common virulence patterns. *J Clin Microbiol,* 45**,** 3366-76.

MOULIN-SCHOULEUR, M., SCHOULER, C., TAILLIEZ, P., KAO, M. R., BREE, A., GERMON, P., OSWALD, E., MAINIL, J., BLANCO, M. & BLANCO, J. 2006. Common virulence factors and genetic relationships between O18:K1:H7 Escherichia coli isolates of human and avian origin. *J Clin Microbiol,* 44**,** 3484-92.

NAKAZATO, G., CAMPOS, T. A., STEHLING, E. G., BROCCHI, M. & SILVEIRA, W. D. 2009. Virulence factors of avian pathogenic *Escherichia coli* (APEC). *Pesquisa Veterinária Brasileira,* 29**,** 479-486.

NATARO, J. P. & KAPER, J. B. 1998. Diarrheagenic Escherichia coli. *Clin Microbiol Rev,* 11**,** 142-201.

NGELEKA, M., BRERETON, L., BROWN, G. & FAIRBROTHER, J. M. 2002. Pathotypes of avian Escherichia coli as related to tsh-, pap-, pil-, and iuc-DNA sequences, and antibiotic sensitivity of isolates from internal tissues and the cloacae of broilers. *Avian Dis,* 46**,** 143-52.

NGELEKA, M., KWAGA, J. K., WHITE, D. G., WHITTAM, T. S., RIDDELL, C., GOODHOPE, R., POTTER, A. A. & ALLAN, B. 1996. Escherichia coli

cellulitis in broiler chickens: clonal relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. *Infect Immun,* 64**,** 3118-26.

NUNOYA, T., TAJIMA, M., IZUCHI, T., TAKAHASHI, K., OTAKI, Y., NAGASAWA, Y. & HAKOGI, E. 1991. Pathology of a broiler disease characterized by the swollen head. *J Vet Med Sci,* 53**,** 347-9.

OJENIYI, A. A. 1989. Direct transmission of Escherichia coli from poultry to humans. *Epidemiol Infect,* 103**,** 513-22.

OZAWA, M., HARADA, K., KOJIMA, A., ASAI, T. & SAMESHIMA, T. 2008. Antimicrobial susceptibilities, serogroups, and molecular characterization of avian pathogenic Escherichia coli isolates in Japan. *Avian Dis,* 52**,** 392-7.

PARREIRA, V. R. & GYLES, C. L. 2003. A novel pathogenicity island integrated adjacent to the thrW tRNA gene of avian pathogenic Escherichia coli encodes a vacuolating autotransporter toxin. *Infect Immun,* 71**,** 5087-96.

PATERSON, D. L. & BONOMO, R. A. 2005. Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev,* 18**,** 657-86.

PERFEITO, L., FERNANDES, L., MOTA, C. & GORDO, I. 2007. Adaptive mutations in bacteria: high rate and small effects. *Science,* 317**,** 813-5.

QADRI, F., SVENNERHOLM, A. M., FARUQUE, A. S. & SACK, R. B. 2005. Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev,* 18**,** 465-83.

ROY, P., PURUSHOTHAMAN, V., KOTEESWARAN, A. & DHILLON, A. S. 2006. Isolation, Characterization, and Antimicrobial Drug Resistance Pattern of *Escherichia coli* Isolated from Japanese Quail and their Environment. *Journal of Applied Poultry Researchs,* 15**,** 442-446.

RULE, A. M., EVANS, S. L. & SILBERGELD, E. K. 2008. Food animal transport: a potential source of community exposures to health hazards from industrial farming (CAFOs). *J Infect Public Health,* 1**,** 33-9.

SABERFAR, E., POURAKBARI, B., CHABOKDAVAN, K. & DOLATSHAHI, F. 2008. Antimicrobial Susceptibility of *Escherichia coli* Isolated from Iranian Broiler Chicken Flocks, 2005-2006. *Journal of Applied Poultry Researchs,* 17**,** 302-304.

SALYERS, A. A., GUPTA, A. & WANG, Y. 2004. Human intestinal bacteria as reservoirs for antibiotic resistance genes. *Trends Microbiol,* 12**,** 412-6.

SCHOULER, C., KOFFMANN, F., AMORY, C., LEROY-SETRIN, S. & MOULIN-SCHOULEUR, M. 2004. Genomic subtraction for the identification of putative new virulence factors of an avian pathogenic Escherichia coli strain of O2 serogroup. *Microbiology,* 150**,** 2973-84.

SKVIRSKY, R. C., REGINALD, S. & SHEN, X. 1995. Topology analysis of the colicin V export protein CvaA in Escherichia coli. *J Bacteriol,* 177**,** 6153-9.

SKYBERG, J. A., JOHNSON, T. J., JOHNSON, J. R., CLABOTS, C., LOGUE, C. M. & NOLAN, L. K. 2006. Acquisition of avian pathogenic Escherichia coli plasmids by a commensal E. coli isolate enhances its abilities to kill chicken embryos, grow in human urine, and colonize the murine kidney. *Infect Immun,* 74**,** 6287-92.

STEHLING, E. G., CAMPOS, T. A., AZEVEDO, V., BROCCHI, M. & SILVEIRA, W. D. 2007. DNA sequencing of a pathogenicity-related plasmid of an avian septicemic Escherichia coli strain. *Genet Mol Res,* 6**,** 331-7.

SUMMERS, A. O. 2006. Genetic linkage and horizontal gene transfer, the roots of the antibiotic multi-resistance problem. *Anim Biotechnol,* 17**,** 125-35.

TABATABAEI, R. R. & NASIRIAN, A. 2003. Isolation, Identification and Antimicrobial Resistance Patterns of *E. coli* Isolated from Chicken Flocks. *Iranian Journal of Pharmacology and Therapeutics,* 2**,** 39-42.

TIVENDALE, K. A., ALLEN, J. L., GINNS, C. A., CRABB, B. S. & BROWNING, G. F. 2004. Association of iss and iucA, but not tsh, with plasmid-mediated virulence of avian pathogenic Escherichia coli. *Infect Immun,* 72**,** 6554-60.

TODAR, K. 2007. Pathogenic E. coli. *Online Textbook of Bacteriology. University of Wisconsin-Madison Department of Bacteriology*.

VOKES, S. A., REEVES, S. A., TORRES, A. G. & PAYNE, S. M. 1999. The aerobactin iron transport system genes in Shigella flexneri are present within a pathogenicity island. *Mol Microbiol,* 33**,** 63-73.

WARREN, R. L., FREEMAN, J. D., LEVESQUE, R. C., SMAILUS, D. E., FLIBOTTE, S. & HOLT, R. A. 2008. Transcription of foreign DNA in Escherichia coli. *Genome Res,* 18**,** 1798-805.

WEN-JIE, J., ZHI-MING, Z., YONG-ZHI, Z., AI-JIAN, Q., HONG-XIA, S., YUE-LONG, L. & JIAO, W., , 2008. Distribution of Virulence-Associated Genes of Avian Pathogenic *Escherichia coli* Isolates in China. *Agricultural Sciences in China,* 7**,** 1511-1515.

WHIPP, S. C., MOON, H. W. & LYON, N. C. 1975. Heat-stable Escherichia coli enterotoxin production in vivo. *Infect Immun,* 12**,** 240-4.

WHITE, D. G., DHO-MOULIN, M., WILSON, R. A. & WHITTAM, T. S. 1993a. Clonal relationships and variation in virulence among Escherichia coli strains of avian origin. *Microb Pathog,* 14**,** 399-409.

WHITE, D. G., WILSON, R. A., EMERY, D. A., NAGARAJA, K. V. & WHITTAM, T. S. 1993b. Clonal diversity among strains of Escherichia coli incriminated in turkey colisepticemia. *Vet Microbiol,* 34**,** 19-34.

WOLF, K., DEL GIUDICE, L. & KAUDEWITZ, F. 1979. Promotion of uniparental inheritance of mitochondrial drug resistance by delayed division of yeast zygotes. *Mol Gen Genet,* 176**,** 301-2.

YAGUCHI, K., OGITANI, T., OSAWA, R., KAWANO, M., KOKUMAI, N., KANESHIGE, T., NORO, T., MASUBUCHI, K. & SHIMIZU, Y. 2007. Virulence factors of avian pathogenic Escherichia coli strains isolated from chickens with colisepticemia in Japan. *Avian Dis,* 51**,** 656-62.

YANG, H., CHEN, S., WHITE, D. G., ZHAO, S., MCDERMOTT, P., WALKER, R. & MENG, J. 2004. Characterization of multiple-antimicrobial-resistant Escherichia coli isolates from diseased chickens and swine in China. *J Clin Microbiol,* 42**,** 3483-9.