An-Najah National University

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Prevalence and Molecular Characterization of ESBL-Producing *Escherichia coli* Isolated from North of Palestine

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This Thesis was Defended Successfully on 10/12/2015 and approved by:

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Dedication

I have to thank my parents, sisters, brothers for their love and support throughout my life. Thank you all for giving me strength to reach for the stars and chase my dream.

To the women and men defending Al -Aqsa mosque, and to the martyrs, the wounded and the prisoners in Israeli jails.

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الإقرار

أنا الموقع أدناه، مقدم الرسالة التي تحمل العنوان:

Prevalence and Molecular Characterization of ESβL-Producing *Escherichia coli* Isolated from North of Palestine

أقر بأن ما شملت عليه هذه الرسالة إنّما هو نتاج جهدي الخاص، باستثناء ما تمّت الإشارة إليه حيثما ورد، وأنّ هذه الرسالة ككل، أو أيّ جزء منها لم يقدّم من قبل لنيل أيّ درجة أو لقب علميّ لدى أيّ مؤسسة تعليمية أو بحثية أخرى.

Declaration

The work provided in this thesis, unless otherwise referenced, is the researcher's own work, and has not been submitted elsewhere for any other degree or qualification.

Student's Name: اسم الطالب: أوس جابر عبد اللطيف الوجار

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Date: التاريخ: 10.12.2015

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List of Abbreviations

ESBL	extended-spectrum β-lactamase
E. coli	*Escherichia coli*
MOX	Moxalactam
OXA	oxacillin hydrolyzing capabilities
SHV	sulfhydryl variable
TEM	Temoneira
ACC	Ambler class C
ACT	AmpC type
CTX-M	cefotaxime hydrolyzing capabilities
DHA	Dhahran Hospital
FOX	Cefoxitin
PCR	polymerase chain reaction
NPHL	National Public health laboratory
bla	β-lactamase
TEST	Tigecycline Evaluation and Surveillance Trial
CLSI	clinical laboratory standards institute
ERIC	Enterobacterial repetitive intergenic consensus
EMB	Eosin Methylene Blue
SIM	Sulfied Indole Motility
MR-VP	Methyl red-Voges Proskauer
MHA	Mueller Hinton agar
NA	Nutrient Agar
NB	Nutrient Broth
TSI	Triple sugar Iron test

IMViC	Indole production, Methyl red test, Voges-Proskauer test and
CRO	Ceftriaxone
CIP	Ciprofloxacin
NOR	Norfloxacin
TE	Tetracycline
K	Kanamycin
SXT	Trimethoprim/Sulfamethoxazole
CTX	Cefotaxime
CAZ	Ceftazidime
NA	Nalidixic acid
DMSO	Dimethyl sulfoxide
CDDT	combination double disk test
EDTA	Ethelen diamine tetra acetic acid
UPGMA	unweighted pair group method for arithmetic averages
SPSS	Statistical Package for the Social Sciences
NCBI	National Center for Biotechnology Information
H2S	Hydrogen Sulfide
MgCl2	Magnesium chloride

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Abstract

Fifty-Two isolates of *E. coli* were recovered from different hospitals and private labs in Jennin district-Palestine, during February-April 2015. Results showed that the prevalences of ESβL and AmpC β-lactamase using conventional techniques were 32.7% and 26.9%, respectively. Whereas, the prevalences using PCR technique were 67.3% and 5.8% for ESβL and AmpC β-lactamase, respectively. *TEM* gene was the dominant (82.9%) among *E. coli* that carried ESβL genes. Other genes were (0.0%), (2.9%) and (15.4%) for *CTX-M*, *SHV* and *OXA* genes, respectively. Whereas, AmpC β-lactamases only *DHA* gene was detected and the prevalence was (5.8%). Molecular analysis by construction phylogenetic tree showed that all sequenced *TEM*, *SHV*, *OXA* and *DHA* genes were belonged to *TEM-1*, *SHV-1*, *OXA-1* and *DHA-1*, respectively. ERIC results showed that these strains were diverse and unrelated clones.

Our results underline the need for continuous monitoring and surveillance of the prevalence, proper control and prevention practices and effective antibiotic use will limit the further spread of *Amp-C* β-lactamases and ESβLs producing isolates within hospitals in Palestine.

Chapter One

Introduction

1.1. General background

Resistant bacteria are emerging world wide as a threat to favorable outcomes of treatment of common infections in community and hospital settings. Urinary tract, gastrointestinal, and pyogenic infections are the common hospital-acquired infections caused by members of *Enterobacteriaceae.* Among *Enterobacteriaceae*, *Escherichia coli* (*E. coli*) has been the most commonly isolated species. *E. coli* strain is very well known to exhibit multidrug resistance. Prolonged antibiotic exposure, overstay in hospitals, severe illness, unprecedented use of third generation cephalosporin, and increased use of intravenous devices or catheters are important risk factors for infection with multidrug resistant *E. coli* (Chaudhary and Aggarwal, 2004).

E. coli possess a naturally occurring chromosomally mediated β-lactamase or plasmid mediated β-lactamases. These enzymes are thought to have evolved from penicillin binding proteins. This development was likely due to selective pressure exerted by β-lactam producing soil organisms found in the environment. The first report of plasmid encoded β-lactamases capable of hydrolyzing the extended spectrum cephalosporins was published in 1983 (Knothe *et al*., 1983). Other β-lactamase were soon discovered which were closely related to (Temoneira), TEM-1 and TEM-2, but which had the ability to confer resistance to the extended-spectrum cephalosporins (Brun-Buisson *et al*., 1987; Sirot *et al*., 1987).

Cephalosporins are bactericidal agents (which means that they kill bacteria) and have the same mode of action as other beta-lactam antibiotics (such as penicillins). cephalosprinas encoded on chromosome of many enteribacteriacia, AmpC enzyme are inducible and can be expressed at high level by mutation, over expression confer resistance to broad spectrum cephalosporins. All bacterial cells have a cell wall that protects them. Cephalosporins disrupt the synthesis of the peptidoglycan layer of bacterial cell walls, which causes the walls to break down and eventually the bacteria die (Ambler, 1980; Bush *et al*., 1995).

Beta-lactamases are commonly classified according to two general schemes: the Ambler molecular classification and the Bush–Jacoby–Medeiros functional classification (Ambler, 1980; Bush *et al*., 1995). The Ambler scheme classifies β-lactamases into four classes according to the protein homology of enzymes. Beta-lactamases of class A, C, and D possess an active site serine called serine β-lactamase, whereas class B beta lactamases are metalloenzymes usually requiring a zinc molecule for their catalytic activities. The Bush–Jacoby–Medeiros functional scheme is based on functional properties of enzymes, i.e. the substrate and inhibitor profiles.

Extended-spectrum β-lactamases (ESβL) are enzymes produced by many Gram-negative bacteria, which have ability to change the susceptibility of different antimicrobial agents (Al-Muharrmi *et al*., 2008). These enzymes have the capability to hydrolyze and inactivate broad spectrum of β-Lactam antimicrobials, including third-generation cephalosporins, penicillins and

aztreonam; but are inhibited by clavulanic acid (Nathisuwan *et al*., 2001; Al-Muharrmi *et al*., 2008). The ESβL-producing organisms are often also able to reduce the susceptibility of other non-β-lactamase antimicrobial classes, such as aminoglycosides, sulphonamides, trimethoprim, tetracyclines, aminoglycosides, chloramphenicol, fluoroquinolones, and nitrofurantoin; (Paterson, 2000; Winokur *et al*., 2001; Wang *et al*., 2004; Mammeri *et al*., 2005). Thus, very broad antibiotic resistance extending to multiple antibiotic classes is now a frequent characteristic of ESβL-producing enterobacterial isolates. As a result, ESβL-producing organisms pose a major problem for clinical therapeutics due to leaving a limited range of therapeutic agents.

Enterobacteriaceae, especially *Klebsiella spp* producing ESβLs such as SHV (sulphydryl variable) and TEM types, have been established since the 1980s as a major cause of hospital-acquired infections. However, during the late 1990s, several community-acquired pathogens that commonly cause urinary tract infections and diarrhea have also been found to be ESβL producers. These include *Escherichia coli, Salmonella, Shigella and Vibrio cholerae* (Paterson and Bonomo, 2005; Pitout *et al*., 2005).

Enterobacterial repetitive intergenic consensus (ERIC) PCR is a PCR-fingerprinting technique but it is not arbitrary. The ERIC sequences are present in many copies in the genomes of different *Enterobacteriaceae*. ERIC elements are highly conserved at the nucleotide level, their positions in enterobacterial genomes varies between different species and has been

used as a genetic marker to characterize isolates within a bacterial species. In ERIC-PCR a band pattern is obtained by amplification of genomic DNA located between successive repetitive ERIC elements or between ERIC elements and other repetitive DNA sequences for subtyping different Gram-negative enteric bacteria (Zulkifli *et al*., 2009).

1.2 Aims of the study

This study conducted to address part of deficient information in molecular antibiotic resistance characterization and their transmissible potential in Palestine.

Therefore, this study aimed:

a. to determine the levels and patterns of antibiotic resistance of human *E. coli* isolates in North of Palestine.

b. to determine the prevalence and molecular epidemiology of ESβLs and AmpC β-lactamases producing *E. coli* isolates using conventional molecular techniques.

c. to study the clonal identity among ESβLs and AmpC β-lactamases producing *E. coli* isolates.

Chapter Two

Literature review

2.1. Prevalence of ESBL strain world wide

The first ESβL to be identified was found in Germany in 1983, and then was in France in 1985 and in the United States at the end of the 1980s and the beginning of the 1990s that the initial nosocomial outbreaks occurred (Rice *et al*., 1990). In recent years, larger nosocomial outbreaks of clonally ESβL strains have been reported: one at a neonatal care unit with ESβL-related mortalities, a large outbreak in Uppsala involving *K. pneumoniae* with CTX-M-15 (predominantly hydrolyze cefotaxime), and in Kristiansand caused by a multi resistant CTX-M-15-producing *E. coli* strain (Alsterlund *et al*., 2009). In Europe, new TEM and the SHV enzymes are still emerging, and distinct epidemic clones have been reported, for example *Salmonella* isolates had TEM-52 enzyme in Spain (Fernandez *et al*., 2006) and *E. coli* and *K. pneumoniae* isolates with SHV-12 enzyme in Italy (Perilli *et al*., 2011). Isolates with the CTX-M-9 group are common in Spain and strains with the CTX-M-3 enzymes have been described chiefly in Eastern Europe, although clones producing the CTX-M group 1 (including the CTX-M-15 type) are the most widespread throughout Europe (Coque *et al*., 2008a,b; Canton *et al*., 2008; Livermore and Hawkey, 2005). Today, *E. coli* and the CTX-M enzymes are common in outpatients. Moreover, the resistance exhibited by *K. pneumoniae* has reached a higher level with emergence of carbapenemases such as OXA-48 (named because of their oxacillin-hydrolyzing abilities), which was first found in Turkey (Aktas *et al*., 2008).

In Kathmandu, Nepal, a study conducted at the National Public health laboratory (NPHL), reported that 31.57% of *E. coli* were confirmed as ESβL producers, these isolates further exhibited co-resistance to several antibiotics (Thakur *et al*., 2013). In Iran, it was found that 26.5% of *E. coli* and 43% of *K. pneumonia* were ESβL positive in a study conducted at the Imam Reza hospital of Mashhad. This indicated the high prevalence of ESβL producing *Enterobacteriaceae* especially in inpatients (Fatemeh *et al*., 2012).

2.2. Prevalence of ESBL strain in middle east

The overall data on ESβL-producing *Enterobacteriaceae* in the countries of the Middle East are extremely worrisome, and this region might indeed be one of the major epicenters of the global ESβL pandemic (Shaikh *et al*., 2014). In North Palestine it was found that 85.4% and 60.1% of *E. coli* were ESβL producers using phenotypic tests and multiplex PCR assay respectively. For these positive with PCR, it was reported that the prevalence for CTX-M and TEM was 100% and 32%, respectively (Adwan *et al*., 2014). In Gaza, Palestine, it was found that 3.7% and 9% of *E. coli* were ESβL producers respectively (Astal *et al*., 2004; El Astal and Ramadan, 2008). In Iraq, It was found that 62.2% of vaginal *E coli* were ESβL producers, the prevalence of CTX-M- (50.8%), SHV- (29.5%), OXA (11.4) and TEM-type (1.6%) (Al-Mayahie, 2013). Investigation carried out in Jordan, it was found that 50.3% of the *E. coli* isolated from outpatients and diagnosed of having urinary tract infections were ESβL-producing,

80.7% had either beta lactamases CTX-M (*bla*CTX-M) or beta lactamases TEM (*bla*TEM), or both (Nimri and Azaizeh, 2012). Investigation conducted in Egypt from patients with urinary tract infections showed that 61% of *E. coli* produced ESβLs of the CTX-M-14, CTX-M 15, and CTX-M 27 types, and all of strains harbored the TEM enzyme (Al-Agamy *et al*., 2006). Investigations conducted in Saudi Arabia in 2004–2005 showed that 10% of clinical urinary *E. coli* isolates from inpatients and 4% of such isolates from outpatients were ESβL producers (Khanfar *et al*., 2009). Another study was conducted in Saudi Arabia, showed that 35.8% of *E. coli* were ESβL producers (Hassan and Abdalhamid, 2014). Moreover, data collected over three years in Kuwait showed that the levels of ESβLs were lower in community isolates of *K. pneumoniae* (17%) and *E. coli* (12%) than in the corresponding hospital isolates (28% and 26%, respectively) (Al Benwan *et al*., 2010). In Kingdome of Bahrain, it was found that 73.9% of *E. coli* isolated produced ESβLs of both CTX-M or TEM in combination or CTX-M alone (Bindayna and Murtadha, 2011).

In China, according to the SENTRY surveillance program there have been rapid increase in ESβL-producing *K. pneumoniae* (up to 60%) and *E. coli* (13–35%), with a predominance of the CTX-M-14 and CTX-M-3 enzymes (Hawkey, 2008; Hirakata *et al*., 2005). It has been found that 66% of third generation Cephalosporin resistant *E. coli* and *K. pneumoniae* from three medical centers in India harbored the CTX-M-15 type of ESβL, which was also the only CTX-M enzyme found (Ensor *et al*., 2006). Recently ESβL production was reported in 50% of *P. aeruginosa*, 44% of *K. pneumoniae*

and 48% of *E. coli* isolates in a tertiary hospital in Patiala, Punjab (Rupinder *et al*., 2013). It was reported that 72% of *E. coli* and 65.8% of *K. pneumoniae* were ESβL producers at the Microbiology laboratory of Shalamar Medical College, Lahore. Antibiotic sensitivity testing showed a multidrug resistance in ESβL producing *E. coli* and *K. pneumoniae*. Maximum resistance was recorded in ESβL producing *E. coli* as cefotaxime (98.9%), Ceftazidime (96.7%) and Cefuroxime (93.4%), while minimum resistance was seen with Imipenem (0.8%), Fosfomycin (1.2%) and (2.2%) for each Nitrofurantoin as well piperacillin/tazobactam. The ESβL producing *Klebsiella* showed maximum resistance to ceftazidime (100%), cefotaxime (89%), and Cefuroxime (84%) while minimum resistance was seen with imipenem (4%), Nitrofurantoin and Piperacillin/Tazobactam (8%) (Majda *et al*., 2013).

In the United States, a large study at a cancer center in Texas performed in 2001, it was demonstrated that about 5.3% of *E. coli* harbored ESβLs (Winokur *et al*., 2001), and an investigation conducted in 2009 showed that 9% of *E. coli* isolates were ESβL producers (Bhusal *et al*., 2011). In India, different study demonstrated the steadily increasing frequency of ESβLs 46% to 79% then to 85.3% in *E. coli* (Varaiya *et al*., 2008; Chaudhuri *et al*., 2011; Chaudhary and Payasi, 2015). Data from 33 centers in Latin America collected over the period 2004–2007 within the Tigecycline Evaluation and Surveillance Trial (TEST) showed ESβLs in 36.7% of *K. pneumoniae* isolates and in 20.8% of 932 *E. coli* isolates (Rossi *et al*., 2008). In Tanzania, a research conducted at a tertiary hospital in Mwanza,

the ESβL prevalence was 64% in *K. pneumoniae* but 24% in *E. coli* (Mshana *et al*., 2009). In Kenya, it was reported that 27% of *E. coli* were ESβLs producers (Kiiru *et al*., 2012).

2.3. prevalence of AmpC β- lactamases strain world wide

It has been reported that isolates producing AmpC β-lactamases raise special concern as these isolates have been responsible for several nosocomial outbreaks and high rate of clinical failure among infected patients (Potz *et al*., 2006; Adler *et al*., 2008).

In Iran, the prevalence bla-AmpC producers among *E. coli* isolates was 5.1% (Dallal *et a*l., 2013). In Turkey, the prevalence of AmpC-producing strains was 10.9% in *E. coli* and 3.6% in *K. pneumoniae* in the tested population by PCR. CIT and Moxalactum (MOX) group genes were predominant type in these strains (Yilmaz *et al*., 2013).

In India, the prevalence of AmpC producers in *E. coli* isolates recovered from a local tertiary care rural hospital was10.5% (Kaur *et al*., 2015). In the same country other studies showed that the prevalence AmpC producers in *E. coli* had a range from 9.1%-70.7% (Subha *et al*., 2003; Ananthan and Subha, 2005; Vandana and Honnavar, 2009). In India, AmpC activity was 63.4% in *K. pneumoniae* and *E. coli* isolates using phenotypic methods, while 38.1% of isolates possessed *AmpC* β- lactamase gene using PCR technique (Mohamudha *et al*., 2013). In other study in the previous country, the prevalence of AmpC β-lactamases among *E. coli*

isolates using PCR and nucleotide sequence analysis was 10%. This study showed that CMY-2 subtype of AmpC β-lactamases to be the predominant type in clinical isolates of *E. coli* and *Klebsiella* spp (Barua *et al*., 2013). In other study in the same country, it was shown high occurrence of CMY-1 AmpC β lactamase gene (56.6%) among AmpC β lactamase producing *E. coli* in cases of complicated UTI (Taneja *et al*., 2012).

In Bangalore, the occurrence rate of AmpC producers in *E. coli* isolates in a Tertiary Care Hospital was 6.3% (Sasirekha and Shivakumar, 2012). In Pakistan, it was found that the frequency of AmpC β-lactamase producing *Escherichia coli was* 12.6% (Noor-ul-Ain *et al*., 2012). Another study in the United States, India, Tanzania, Kenya, Iran, Bangalore and Pakistan carried out at a tertiary care hospital in Rawalpindi reported a high frequency of 45% AmpC β-lactamase producing *E. coli* (Hassan *et al*., 2011). High frequency of 43.6% AmpC producing *E. coli* was also reported in a study at Medical Centers in Taiwan (Yan *et al*., 2006). In study from US veterans medical centers reported the prevalence of AmpC β-lactamases among *E. coli* isolates was1.6% (Coudron *et al*., 2000).

In Eygypt, it was found that the prevalence *AmpC* β- lactamase among *E. coli* isolates was 38.1%, and the most prevalent AmpC gene was that belonging to family CMY-1(El-Hady and Adel, 2015).

Chapter Three

Materials and Methods

3.1. Sample collection and *E. coli* identification

Fifty-Two isolates of *E. coli* were isolated from inpatients and outpatients at The Martyar Dr. Khalil S. Hospital (n=23; 18 from urine samples, 5 from vaginal swabs), Al-Amal Hosptal (n=5; all from urine samples), AL-Razi Hospital (n=14; 11 from urine samples, 3 from vaginal swabs), Al-Shamal Lab (n=7; all from urine samples) and Hi Lab (n=3; all from urine samples), Jennin district-Palestine, during February-April 2015. These isolates were identified in laboratories where they collected as well as confirmed in Microbiology laboratories at An–Najah National University-Nablus, Palesine. In An–Najah National University laboratories, bacterial isolates were cultured on EMB or MacConkey agar, Gram stain and other biochemical tests were conducted such as IMViC Tests (Indole production, Methyl red test, Voges-Proskauer test and Citrate utilization), motility test and H_2S production.

3.2. Media preparation

3.2.1. MacConkey Agar

MacConkey agar (HIMEDIA) was prepared according to the manufacturer's instructions. A 1L flask containing 500 ml deionized water and 25 g MacConkey agar was heated and stirred until the agar dissolved. Then, the agar solution was autoclaved at 121°C for 15 min. After that it was cooled to about 50°C, and poured into sterile Petri dishes to have 25 ml each and left overnight. Next day the Petri dishes were stored at 4°C.

3.2.2. Eosin Methylene Blue (EMB) Agar

EMB medium (HIMEDIA) was prepared according to the manufacturer's instructions. A 1L flask containing 500 ml deionized water and 18.75 g of EMB agar was heated and stirred until the agar dissolved. The flask was plugged tightly with cotton which then covered with a piece of aluminum foil. then was autoclaved at 121°C for 15 min. After that it was cooled to about 50°C, and poured into sterile Petri dishes to have 25 ml each and left overnight. Next day the Petri dishes were stored at 4°C.

3.2.3. Sulfied Indole Motility (SIM) Medium

SIM medium (Acumedia) was prepared according to the manufacturer's instructions. A 0.5L flask containing 250 ml deionized water and 7.5 g of SIM agar was heated and stirred until dissolved. Medium was dispensed into tubes to a give depth of about 4-5 cm. the tube plugged tightly with cotton which then covered with a piece of aluminum foil. Then the medium was autoclaved at 121°C for 15 min, allowed to cool by leaving the tubes on a rack to form deep agar and then stored at 4°C.

3.2.4. Methyl red-Voges Proskauer (MR-VP)

MR-VP medium (HIMEDIA) was prepared according to the manufacturer's instructions. A 1L flask containing 500 ml deionized water and 8.5 g of MR-VP medium was mixed thoroughly, heated to dissolve if necessary. Then, 5 ml of MR-VP broth was dispensed into each tubes and plugged

with cotton, which then covered with a piece of aluminum foil, then was autoclaved at 121°C for 15 min, allowed to cool and then stored at 4°C.

3.2.5. Simmons citrate agar:

Simmons citrate agar (Acumedia) was prepared according to the manufacturer's instructions. A 1L flask containing 500 ml deionized water and 11.25 g of Simmons citrate agar was heated and stirred until dissolved. Ten ml of Simmons citrate agar was dispensed into tubes, the tube plugged tightly with cotton which then covered with a piece of aluminum foil. autoclaved at 121°C for 15 min. The medium was prepared as slant agar in the tubes by leaving the tubes to dry in slant position and then stored at 4°C.

3.2.6. Triple sugar Iron agar

TSI agar (Acumedia) was prepared according to the manufacturer's instructions. A 1L flask containing 500 ml deionized water and 32.5g of TSI agar was mixed thoroughly, heated to dissolve. Ten ml of Triple sugar Iron medium was dispensed into tubes, autoclaved at 121°C for 15min. The medium was prepared as slant agar in the tubes by leaving the tubes to dry in slant position and then stored at 4°C.

3.2.7. Mueller Hinton agar (MHA)

Mueller Hinton agar (Acumedia) was prepared according to manufacturer's instructions. In a 2 L flask, 1 L of deionized water were mixed with 38 g

MHA, heated and stirred until the agar dissolved, then the flask was plugged tightly with cotton which then covered with a piece of aluminum foil. Then the agar solution was autoclaved at 121°C for 15 min. After that it was cooled to about 50°C, and poured into sterile Petri dishes to have 25 ml each and left overnight. Next day the Petri dishes were stored at 4°C.

3.2.8. Nutrient Agar (NA)

Nutrient agar (ACUMEDIA) was prepared according to manufacturer's instructions. In a 1 L flask, 500 ml deionized water were heated and mixed with 11.5 g NA until the agar dissolved. The flask was plugged tightly with cotton which then covered with a piece of aluminum foil before autoclaved at 121°C for 15 min. After that it was cooled to about 50°C, and poured into sterile Petri dishes to have 20 ml each and left overnight. Next day the Petri dishes were stored at 4°C.

3.2.9. Nutrient Broth (NB)

Nutrient broth (ACUMEDIA) was prepared according to manufacturer's instructions. In a 0.5 L flask, 250 ml deionized water were mixed and boiled with 2 g of NB to dissolve. Then, 5 ml of broth was dispensed into each tubes and plugged with cotton and covered with aluminum foil. The tubes were autoclaved at 121 °C for 15 minutes, allowed to cool and then stored at 4°C.

3.3. Sample Identification

3.3.1. Gram staininig

Gram staining was performed to distinguish Gram-positive bacteria from Gram-negative bacteria . A thin smear of bacteria was made on a clean glass slide by picking the isolates from marked colonies after 24 hour incubation and mixed with sterile saline. The smear was heat fixed by passing through a flame. Care was taken to avoid creation of air bubbles and overheating to prevent distortions of glass slides. After cooling of slide, a crystal violet solution was applied for half minute and washed with tap water. Mordant Lugol's iodine solution was poured on smearing spot for half a minute and again washed with tap water, decolorized with absolute acetone/ ethanol for 2-3 seconds and then again washed and flood with safranin, washed and dried. Observed under 100x objective of the microscope. (Cappuccino and Sherman 1996).

3.3.2. Motility test

In SIM deep agar tube, the tested bacteria was inoculated by stabing the butt of SIM, then the tube was inocubated at 35°C for 24 hour, a motile organism, caused a turbidity or diffused growth and the stab line is unclear. In case the organism was nonmotile, the growth was confined to the stab line and the medium showed very clear (Johnson and case, 1998).

3.3.3. Indole Test

Tube (SIM) used for motility test and inoculated by mean of stab method, was also used to detect whether the bacterium have tryptophanase enzyme or not. This was done by adding few drops of Kovac's reagent after 24 hour of inocubation. In Indol positive result, a cherry-red ring appeared on the surface of medium indicate the presence of indol which is an end product of tryptophan metabolism by tryptophanase enzyme, Indol negative result, indicated by absence of red ring from the surface of SIM agar after addition of Kovac's reagent (Cappuccino and Sherman 1996).

3.3.4. MR-VP test

MRVP broth was inoculated with the tested bacterium, then incubated at 35°C for 24 hours, the MRVP broth was divided into two tubes one for MR test and the other for VP test. MR test was carried out by adding 5 drops of methyl red indicator, while the VP test was carried out by adding 10 drops of Barritt's reagent A, the culture was shaken, then immediately 10 drops of Barritt's reagent B was added, then culture was shaken and re-shaken after every 3-4 min. Positive results for MR test showed red color formation after the addition of reagent and negative results for VP test showed no changes in the color after addition the indicators (Cappuccino and Sherman 1996).

3.3.5. Citrate utilization test

Citrate utilization test was carried out by means of stabbing the butt and streaking the slant of Simmons citrate agar. The tube was incubated at 35°C for 24 hour, positive result was indicated by either change the color of the medium from green to blue or growth of bacterium in the media or both. Lack of growth on citrate medium and no change in the color indicated a negative test (Cappuccino and Sherman 1996).

3.3.6. Triple sugar Iron test

TSI test was carried out by inoculation the TSI agar by streaking the slant and stabing the butt and then the tube was incubated at 35°C for 24 hour . *Escherichia coli* sub cultured on TSI agar showed the whole media had a yellow color (acid/acid), cracks and no black color (indicated fermentation of glucose and lactose) (Cappuccino and Sherman 1996).

3.4. Antibacterial resistance

3.4.1. Preparation McFarland turbidity standard No. 0.5

A total of 50 µl of McFarland 0.5 turbidity standard was prepared by adding a 1.175% (wt/vol) barium chloride dihydrate ($BaCl_2.2H_2O$) solution to 9.95 ml of 1% (vol/vol) sulfuric acid in order (Andrews, 2006). Then, the tube sealed with Parafilm to avoid evaporation and stored in the dark at room temperature. The accuracy of the density of a prepared McFarland standard was determined by measuring the absorbance at λ_{625} nm and must

be 0.08 to 0.13. The 0.5 McFarland standard is equivalent to a bacterial suspension of 1.5 X 10^8 colony-forming units (CFU)/ml. A 0.5 McFarland Standard bacterial suspension was used for antibacterial susceptibility test, detection of AmpC phenotype and ESβLs producing isolates.

3.4.2. Antibacterial sensitivity test

Antimicrobial sensitivity was carried out using the disk diffusion method according to guidelines described by the Clinical and Laboratory Standard Institute (CLSI) (CLSI, 2012). Antibiotic disks (Oxoid) used in this study were Ceftriaxone (CRO) 30µg, Trimethoprim/Sulfamethoxazole (SXT) 1.25/23.75µg, Norfloxacin (NOR) 10 µg, Ceftazidime (CAZ) 30µg, Ciprofloxacin (CIP) 5 µg, Tetracycline (TE) 30µg, Nalidixic acid (30µg), Cefotaxime (CTX) 30 µg and Kanamycin (K) 30µg. The plates were incubated at 37°C for 18-24 hrs. Inhibition zones were determined in millimetres. The third generation cephalosporin displayed a reduced susceptibility to *E. coli* were selected for detection of β-lactamases.

3.4.3. Detection of ESβLs and AmpC β-lactamases

3.4.3.1. Detection of AmpC phenotype

Isolates showed resistance to 3rd generation cephalosporins were tested for the presence of AmpC β-lactamases. AmpC phenotype was detected by combined disk method using Cefotaxime (CTX, 30 µg) and Ceftazidime (CAZ, 30 µg) antibiotic disks alone and in combination with boronic acid (400µg). To prepare the combination disks were prepared by adding 20µl

of diluted DMSO-boronic acid solution to each Cefotaxime and Ceftazidime disk (a total of 60 mg phenyl boronic acid was added to 1.5 ml Dimethyl sulfoxide (DMSO), the DMSO-boronic acid solution was diluted with 1.5 ml of sterilized distilled water). Then disks were used after incubation at room temperature for 1 h to dryness. Both disks alone and in combination with boronic acid were placed on MHA plates inoculated with *E. coli*. *Escherichia. coli* strain was considered as AmpC β-lactamases producing organism if inhibition zone diameter around Cefotaxime and/or Ceftazidime disk in combination with boronic acid ≥5mm in comparison with Cefotaxime and Ceftazidime disks alone (Mansouri *et al*., 2014**)**.

3.4.3.2. Detection of ESβLs producing isolates

The isolates showed resistance to 3rd generation cephalosporins were tested for the presence of ESβL by combination double disk test (CDDT). Ceftazidime (CAZ) 30μg and Ceftazidime/Clavulanic acid (30/10 μg), Cefotaxime (CTX) 30 μg and Cefotaxime/Clavulanic acid (30/10 μg) were placed at a distance of 20 mm (centre to centre) on MHA plates streaked from a suspension of the tested *E.coli* in which its turbidity equal to Macfarland standard turbidity. The plates then were incubated for 18-24 hrs at 37°C. *Escherichia. coli* strain was confirmed as ESβL-producing organism if inhibition zone diameter around Cefotaxime and/or Ceftazidime disk in combination with clavulanic acid is ≥5mm in comparison with Cefotaxime and Ceftazidime disks alone (Mansouri *et al*., 2014).

3.5. PCR amplification

3.5.1. DNA extraction

E. coli DNA was prepared for PCR according to the method described by (Adwan *et al*., 2013). Briefly, cells were scraped off an overnight nutrient agar plate with a sterile loop, washed with 1 ml of 1X Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA [pH 8]), then the pellet was resuspended in 0.5 ml of sterile distilled H_2O, and boiled for 10-15 min. The cells then were incubated on ice for 10 min. The debris was pelleted by centrifugation at 11,500 X *g* for 5 min. DNA concentration was determined using a spectrophotometer and the samples were stored at -20ºC until use for further DNA analysis.

3.5.2. Detection of AmpC β-lactamase and ESβL genes

Detection of plasmid mediated AmpC β-lactamase (*ampC*) genes in all isolates were carried out by multiplex PCR (Pérez-Pérez and Hanson, 2002). Primer nucleotide sequences and expected sizes of amplicons for AmpC β-lactamase (*ampC*) genes are presented in Table 3.1. Detection of ESβL gene sequences coding for the TEM, SHV, CTX-M and OXA enzymes were performed also by multiplex PCR. The oligonucleotide primer sets and expected amplicon sizes (bp) specific for the SHV, TEM, CTX-M and OXA genes are presented in Table 3.1. Briefly, PCR reactions were performed in a final volume of 25 µl of the amplification mixture containing 12.5 µL of PCR premix with MgCl2 (ReadyMix^{TM} Taq PCR

Reaction Mix with MgCl2, Sigma), 0.3 µM of each primer, 3 µl (100-150 ng) of DNA template. DNA amplification was carried out with a thermal cycler (Mastercycler Personal, Eppendorf) using the following conditions: 94°C for 3 min; 94°C for 40 s, 64°C (60°C for detection of ESβL genes**)** for 40 s and 72°C for 1 min for 25 cycles; with a final extension at 72°C for 5 min. Amplified PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

3.5.3. ERIC PCR

ERIC-PCR was performed as described by (Adwan et al., 2015). Primer nucleotide sequences are presented in Table 3.1. Each PCR reaction mix (25 µL) was carried out using 12.5 µL of PCR premix with $MgCl_2$ (ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$, Sigma), 1 µM of each primer, 3 µL (100-150 ng) DNA template. In addition, the master mix was modified by increasing the concentration of dNTPs up to 0.4 mM , 3 mM MgCl2 and 2 U of Taq DNA polymerase. Amplification of DNA was carried out using the thermal cycler (Mastercycler personal, Eppendorf, Germany) according to the following conditions: initial denaturation for 2 min at 94ºC was followed by 30 cycles of initial denaturation 94ºC for 50 s, 50ºC for 40 s and 72ºC for 1 min, with a final extension step at 72ºC for 5 min. The amplified PCR products were analyzed by electrophoresis on 1.5 % agarose gel stained with ethidium bromide. The gel images for ERIC-PCR were scored using binary scoring system that recorded the absence and presence of bands as 0 and 1, respectively. Unweighted pair group

method for arithmetic averages (UPGMA) was used to analyz the primary matrix using SPSS statistics software version 21 (IBM).

Table 3.1. Beta-lactamases target genes and ERIC sequences for PCR amplification, amplicon size and primer sequences

Group	Targets	Primer sequence 5′→3′	Expected amplicon size (bp)	Primer mix	References
Plasmid mediated AmpC β-lactamase (Class C)	*MOX-1, MOX-2, CMY-1, CMY-8 to CMY-11*	MOXM F 5-GCT GCT CAA GGA GCA CAG GAT-3 MOXM R 5-CAC ATT GAC ATA GGT GTG GTG C-3	520	1	Péréz-Péréz and Hanson, 2002
	LAT-1 to LAT-4, CMY-2 to CMY-7, BIL-1	CITM F 5-TGG CCA GAA CTG ACA GGC AAA-3 CITM R 5-TTT CTC CTG AAC GTG GCT GGC-3	462	1	Péréz-Péréz and Hanson, 2002
	DHA-1, DHA-2	DHAM F 5-AAC TTT CAC AGG TGT GCT GGG T-3 DHAM R 5-CCG TAC GCA TAC TGG CTT TGC-3	405	1	Péréz-Péréz and Hanson, 2002
	ACC	ACCM F 5-AAC AGC CTC AGC AGC CGG TTA-3 ACCM R 5-TTC GCC GCA ATC ATC CCT AGC-3	346	1	Péréz-Péréz and Hanson, 2002
	MIR-1T, ACT-1	EBCM F 5-TCG GTA AAG CCG ATG TTG CGG-3 EBCM R 5-CTT CCA CTG CGG CTG CCA GTT-3	302	1	Péréz-Péréz and Hanson, 2002

	FOX-1 to FOX-5b	FOXM F 5-AAC ATG GGG TAT CAG GGA GAT G-3 FOXM R 5-CAA AGC GCG TAA CCG GAT TGG-3	190	1	Péréz-Péréz and Hanson, 2002
extended spectrum β-lactamases (Class A)	*SHV*	SHV F 5-ATG CGT TATATT CGC CTG TG-3 SHV R 5-TGC TTT GTT ATT CGG GCC AA-3	747	2	Paterson *et al*., 2003
	TEM	TEM F5-TCG CCG CAT ACA CTA TTC TCA GAA TGA-3 TEM R5-ACG CTC ACC GGC TCC AGA TTT AT-3	445	2	Monstein *et al*., 2007
	CTX-M	CTX-M F 5-ATG TGC AGY ACC AGT AAR GTK ATG GC-3 CTX-M R 5-TGG GTR AAR TAR GTS ACC AGA AYC AGC GG-3	593	2	Boyd *et al*., 2004
extended spectrum β-lactamases (Class D)	*OXA*	OXA F 5-ATT ATC TAC AGC AGC GCC AGT G-3 OXA R 5-TGC ATC CAC GTC TTT GGT G-3	296	2	Kim *et al*., 2009
ERIC	ERIC sequences	ERIC F 5-ATG TAA GCT CCT GGG GAT TCA C-3 ERIC R 5-AAG TAA GTG ACT GGG GTG AGC G-3	-	3	Zulkifli et al., 2009

3.6. Sequence homology and phylogenetic analysis

Amplified PCR products of some β-lactamases genes were purified by Wizard® SV Gel and PCR Clean-Up System kit (Promega) and sequenced by dideoxy chain termination method using ABI PRISM sequencer, model 3130 (Hitachi Ltd, Tokyo, Japan), Bethlehem University, Bethlehem, Palestine. DNA Sequences were further submitted for accession number in primary bioinformatics web servers.

The comparison of the continuous sequences was conducted with previously available sequences of the AmpC β-lactamase and ESβL genes in GenBank using BLAST system. Multiple sequence alignment was carried out using ClustalW of the computer program MEGA software (version 6). The evolutionary distances were computed using the Tajima-Nei method. Phylogenetic analyses were based on alignments obtained from ClustalW of a 282 bp sequence. Phylogenetic tree was constructed using the program Neighbor-Joining in the computer program MEGA software. The robustness of the groupings in the Neighbor Joining analysis was assessed with 1000 bootstrap resamplings.

Chapter Four

Results

4.1. Identification of *E. coli* isolates

The results showed that all *E. coli* strains inoculated on EMB agar had a green metallic sheen while these inoculated on MacConkey agar had bright pink or red colonies. Gram stain showed that *E. coli* is Gram-negative bacteria, short rod, single, pair or in short chain. All isolates were Acid/acid with gas production but no H2S production, positive Methyl Red test, Voges-Proskauer test negative, Indole test positive, citrate utilization negative and motile.

4.2. Antibiotic resistance

The antimicrobial resistance pattern of this research showed that the least resistant *E.coli* strains were against Ceftazidime (11.5%) and Norfloxacin (17.3%), while the most resistant were againts Tetracycline (67.3%) and Trimethoprim /Sulfamethoxazle (55.76%). Data are presented in Table (4.1). In addition, results showed that 40.4% of strains were multidrug resistant.

Table 4.1. Antibiotic resistance of 52 *E. coli* strains recovered from different clinical samples.

Antibiotic	Resistant strains	
	No.	%
Ciprofloxacin	16	30.8%
Trimethoprim/Sulfamethoxazle	29	55.76%
Ceftriaxone	13	25.0%
Tetracycline	35	67.3%
Nalidixic acid	22	42.3%
Norfloxacin	9	17.3%
Kanamycin	13	25.0%
Cefotaxime	16	30.9%
Ceftazidime	6	11.5%

4.3. Detection of ESβL and AmpC β-lactamases

Results of this study showed that the prevalence of ESβL and AmpC β-lactamase using conventional techniques was 32.7% and 26.9%, respectively. Whereas, the prevalence using multiplex PCR technique was 67.3% and 5.8% for ESβLs and AmpC β-lactamases, respectively. In this study it was shown that *TEM* gene was the most common (82.9%) among *E. coli* isolates that carried ESβL genes. While for other genes, *CTX-M*, *SHV* and *OXA* the prevalences were 0.0%, 2.9% and 15.4%, respectively. For AmpC β-lactamases only *DHA* gene was detected and the prevalence was 5.8%. Results of this research are presented in Table 4.2 and Figures 4.1a and 4.1b. In this study, 24 of strains were considered as not ESβL producer strains by conventional methods, but they were detected as ESβL producers using PCR technique. There were 6 strains tested positive for

ESβL using phenotypic tests only, but negative with PCR technique. Also in this study, 13 of *E. coli* strains were considered by conventional methods as AmpC β-lactamases producers, while they were negative by multiplex PCR. (Table 4.2). In addition, all *AmpC* β-lactamases in this study were coexisted with ESβLs and coexistence of two β-lactamases in single strains was observed.

Table 4.2. Prevalence of extended spectrum β-lactamases and AmpC β-lactamase among 52 clinical *E. coli* using conventional technique and PCR technique.

Technique	β-Lactamases									
	extended spectrum β-lactamases No. (%)					AmpC β-lactamase No. (%)				
	Class (A)				Class (D)		Class (C)			
	SHV	TEM	CTX-M	TEM and OXA	OXA	MOX, CMY, BIL	DHA	ACC	MIR-1T, ACT-1	FOX
PCR Technique	1 (1.9)	26 (50)	0 (0)	3 (5.8)	5 (9.6)	0 (0)	3 (5.8)	0 (0)	0 (0)	0 (0)
	Total 35 (67.3)					Total 3 (5.8)				
conventional technique	17 (32.7)					14 (26.9)				
Distribution according to methods	Positive by both techniques: (n=11) Positive by PCR only: (n=24) Positive by conventional only: (n=6)					Positive by both techniques: (n=1) Positive by PCR only: (n=2) Positive by conventional only: (n=13)				

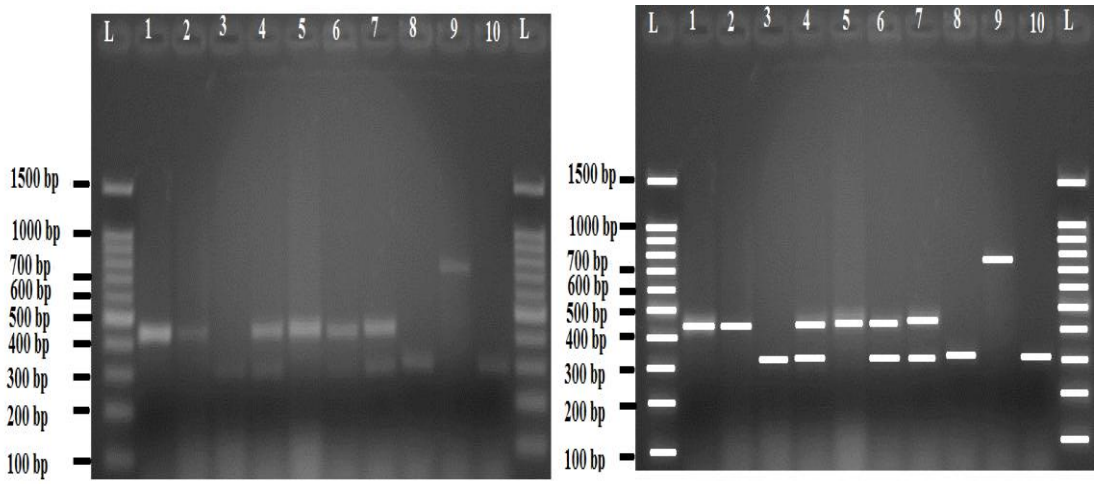

Figure 4.1a. Multiplex PCR profiles specific for extended spectrum β-lactamases genes. L represented the ladder; Lanes1,2 and 5 for *TEM* gene; Lanes 3, 8 and 10 for *OXA* gene, Lanes 4, 6 and 7 for *TEM* and *OXA* genes and Lane 9 for *SHV* gene.

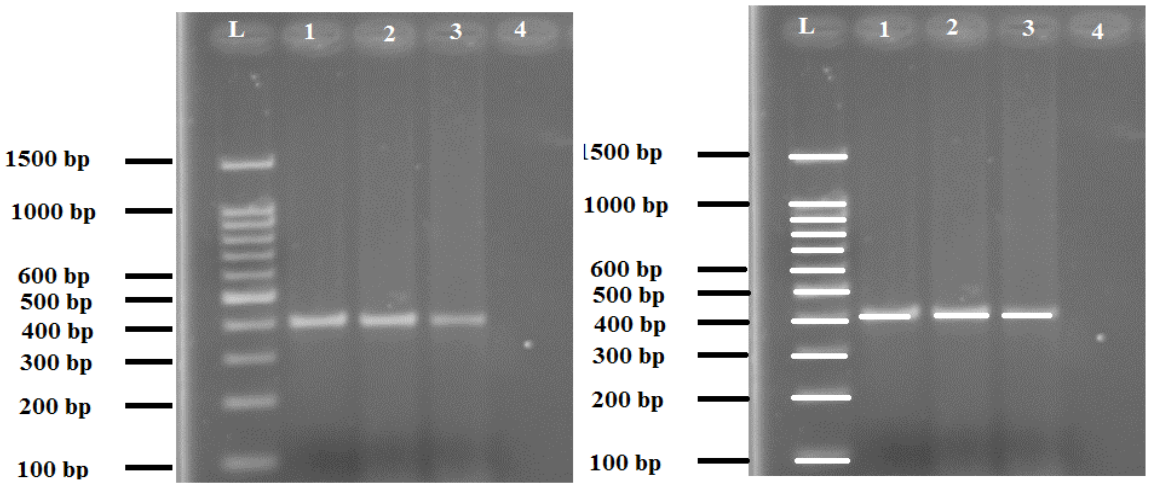

Figure 4.1b. Multiplex PCR profiles specific for AmpC β-lactamase genes. L represented the ladder; Lanes 1, 2 and 3 for DHA gene and Lane 4 for negative control.

Molecular analysis by construction phylogenetic tree showed that all sequenced *TEM*, *SHV*, *OXA* and *DHA* genes were belonged to *TEM-1*, *SHV-1*, *OXA-1* and *DHA-1*, respectively (Figure 4.2). The nucleotide sequences reported in this study were further registered at the GenBank database under the accession numbers (KT336739- KT336757).

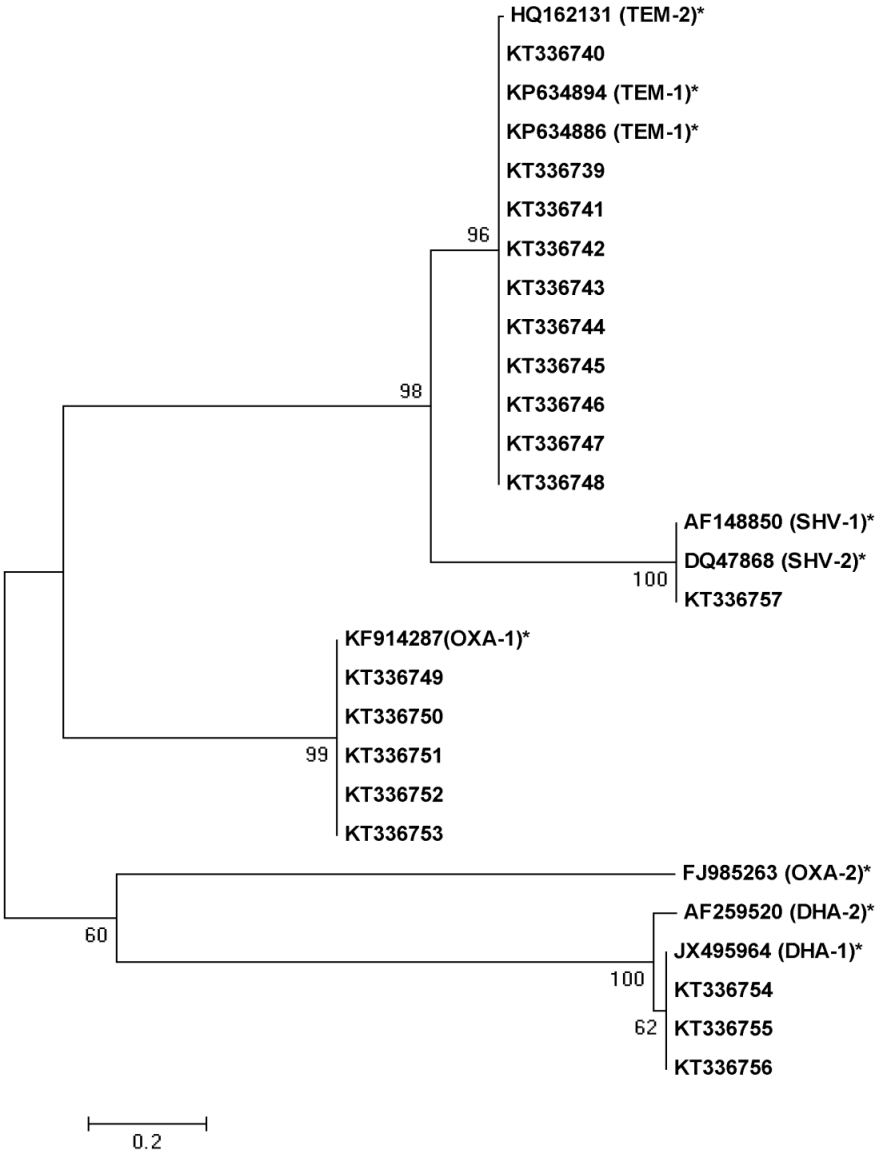

Figure 4.2. Phylogenetic analysis constructed by Neighbor-Joining method based on the partial *OXA-1*, *DHA-1*, *SHV-1* and *TEM-1* gene nucleotide sequences. Reference sequences for the *OXA-1*, *DHA-1*, *SHV-1* and *TEM-1* gene nucleotide sequences were denoted by asterisk. The tree was bootstrapped with 1000 replicate, and the genetic distance corresponding is shown by the bar. There were a total of 282 positions in the final dataset. Evolutionary analyses were conducted in MEGA version 6.

4.4. ERIC-PCR analysis

ERIC-PCR typing of 35 *E. coli* isolates which harbored genes for ESβLs and/or AmpC β-lactamases were genetically diverse and consisted of a heterogeneous population with a total of 16 ERIC PCR profiles (clusters) at a 50% similarity level. Results of ERIC-PCR typing are presented in Figures 4.3 and 4.4.

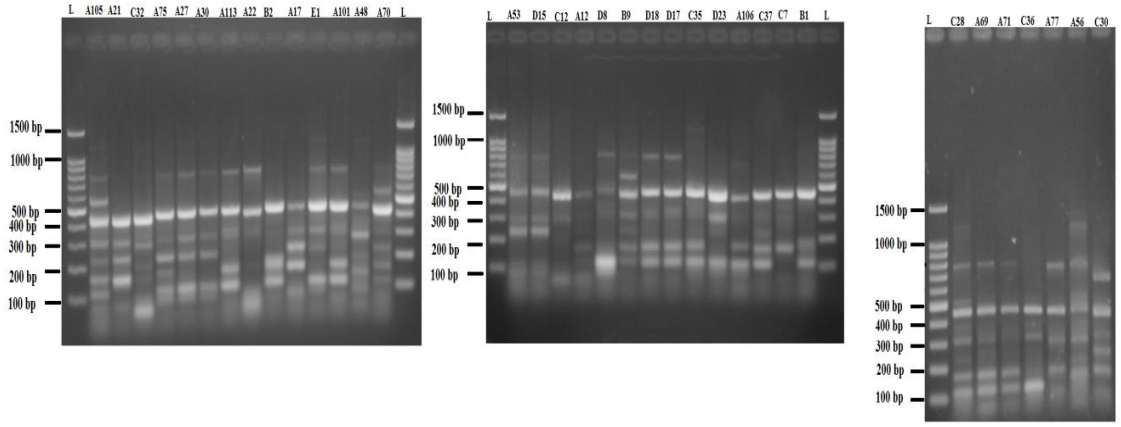

Figure 4.3. DNA fingerprints generated by ERIC PCR typing of 35 clinical *E. coli* isolates carried genes for ESβLs and AmpC β-lactamases recovered on 1.5% agarose gel. Lanes L represented the ladder, while other lanes for ERIC PCR products.

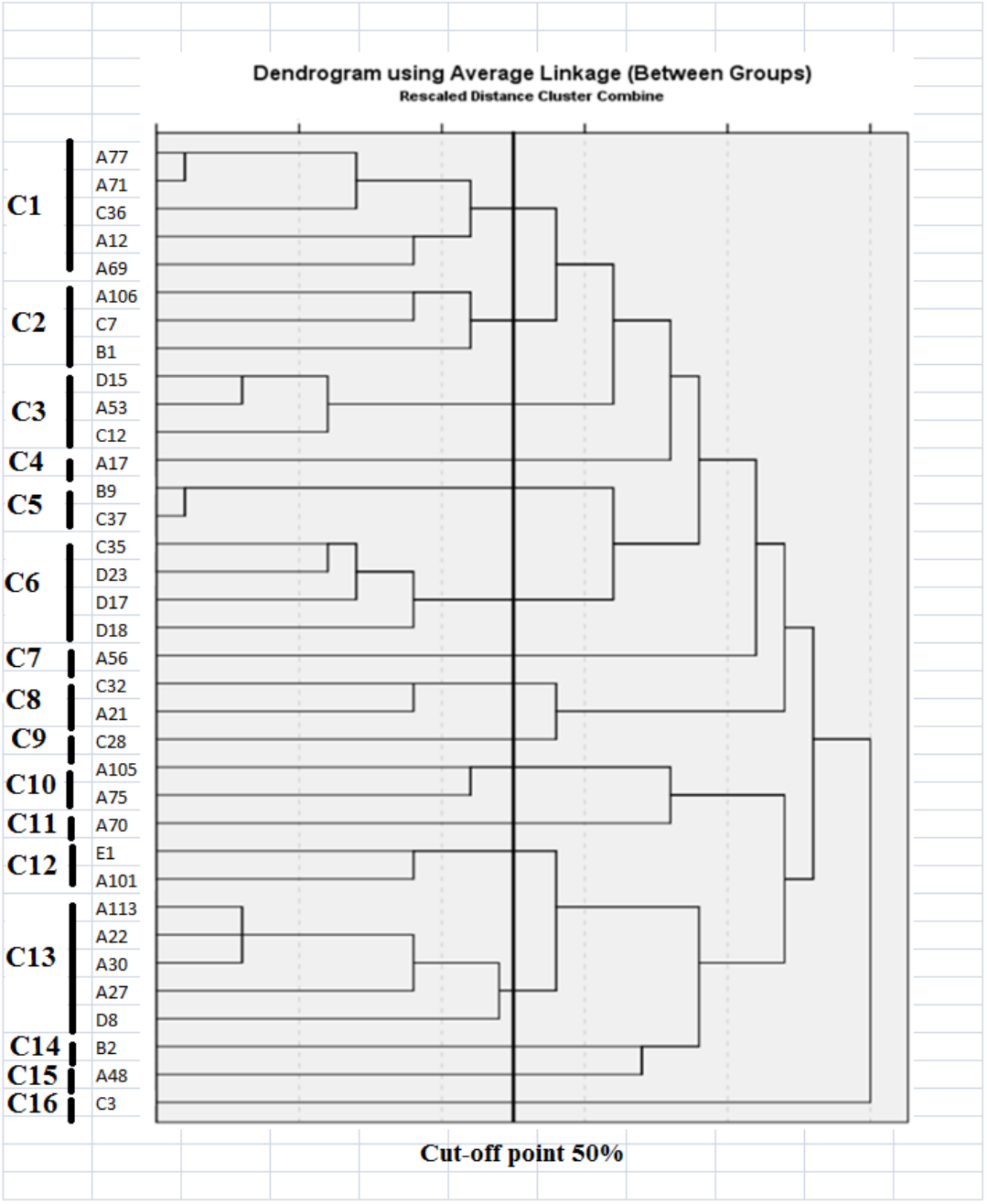

Figure 4.4. Dendrogram of 35 *E. coli* isolates carried genes for ESβLs and/or AmpC β-lactamases based on the UPGMA method derived from analysis of the ERIC-PCR-profiles at a 50% similarity level.

C: Cluster

Chapter Five

Discussion

Beta-lactamases are enzymes expressed either by specific transferable genes located on plasmids or transposons or by certain chromosomal genes (Jesudason *et al*., 2005). *Klebsiella* spp., and *E. coli* were the most common bacteria that have these enzymes, but now these are found in most members of *Enterobacteriaceae* and other Gram negative bacilli (Mathur *et al*., 2002; Kumar *et al*., 2006). Expression of β-lactamases in clinical pathogens including both ESβLs and AmpC of β-lactamases, is one of the most considerable resistance-mechanism that disturbs and prevents the antimicrobial treatment of infections caused by microorganisms. In addition, it forms a major problem for clinical treatments using the currently available antibiotics. A significant increase in the incidents of β-lactamases-associated infections including ESβLs-related infections has been observed throughout the world in may different researches (Shaikh *et al*., 2014).

To our knowledge, this is the first study to document the molecular detection of AmpC β-lactamases genes in isolates of *E. coli* in Palestine. In this study, phenotypic tests and multiplex PCR amplification assay were used to detect both ESβLs and AmpC β-lactamases producing *E. coli* isolates.

Results showed that there were 24 strains were considered as not ESβL producer strains by conventional methods, but they were detected as ESβL producers using PCR technique. It was proposed that the use of three distinct substrates in the combined disk tests will increase the sensitivity of

the test and cefotaxime and cefpodoxime performed the best. The use of cefpodoxime alone was recommended or both as preferred substrates of cefotaxime and ceftazidime for detection ESβL producing clinical isolates of pathogens (Rupp and Fey, 2003; Tofteland *et al*., 2007). However, it has been shown that cefpodoxime susceptibility testing can produce high rate of false-positive ESβL results, this may be explained due to mechanisms other than ESβL expression (Livermoore *et al*., 2006). There were 6 strains tested positive for ESβL using phenotypic tests only, but negative with PCR technique. This negative amplification in these phenotypic positive isolates may be due to these isolates carried other ESβL genes, which could not be detected by these primers or could be chromosomally mediated β-lactamase. Several studies have described or used various molecular approaches for the rapid screening the presence of different ESβL and AmpC beta-lactamases genes. The development of new molecular assays especially using multiplex PCR techniques have been shown to have an evident advantage in comparison with other phenotypic tests, such as, screening large numbers of isolates in short time and the isolated DNA is suitable for further molecular tests if necessary (Monstein *et al*., 2007). Obviously, molecular techniques have strong potential to play an essential role in the laboratory setting for the screening, tracking and controlling of the spread of β-lactamases producing bacteria from both the community and hospital settings (Péréz-Péréz and Hanson, 2002; Monstein *et al*., 2007; Akpaka *et al*., 2010; Kaftandzieva *et al*., 2011; Shaikh *et al*., 2014). Although, molecular techniques such as multiplex PCR method is simple to

use in detecting ESβL producing isolates, but it has become more difficult with the increased number and types of ESβL. The ESβL-producing organisms may appear susceptible to some extended-spectrum cephalosporins (Paterson and Bonomo, 2005). A combination of both conventional tests and molecular techniques for all β-lactamase associated genes is the best way for detection of β-lactamase producing microorganisms (Kaftandzieva *et al*., 2011).

To date, there are several conventional tests used to detect AmpC β-lactamases producing bacteria have been developed. However, currently there are no CLSI approved tests or specific guidelines for detection of *AmpC* β-lactamases producing bacterial isolates (Coşkun and Altanlar, 2012; Barua *et al*., 2013). Results of this research showed that 13 strains were considered *AmpC* β-lactamases producers by conventional methods but negative with multiplex PCR technique. This may be due to these originate from hyperproduction of endogenous or non-plasmid-derived (chromosomal) AmpC activity (Tan *et al*., 2009).

Results of this study showed that the prevalence of ESβL among *E. coli* in Palestine is too high and it was 67.3% using multiplex PCR or 32.7% using phenotypic tests. In countries of the Middle East, the prevalence of ESβL producers *E. coli* ranged from 3.7%-85.4% (Astal *et al*., 2004; Al-Agamy *et al*., 2006; Bindayna and Murtadha, 2011; Nimri and Azaizeh, 2012; Al-Mayahie, 2013; Dallal *et a*l., 2013; Hassan and Abdalhamid, 2014; Adwan *et al*., 2014). In previous studies done in Gaza strip using phenotypic tests,

it showed that the prevalence of ESβL among clinical *E. coli* isolates was 3.7% and 9% (Astal *et al*., 2004; El Astal and Ramadan, 2008). This may indicate that the prevalence of ESβL-producing microorganisms is increasing every year rapidly. In Recent study, It was reported that the prevalence of ESβL-producing *E. coli* was 85.4% and 60.1% using phenotypic tests and multiplex PCR, respectively (Adwan *et al*., 2014).

Finding of this research showed that TEM-type ESβL was most common in *E. coli* isolates and this result is consistent with other studies (Al-Agamy *et al*., 2006; Akpaka *et al*., 2010; Kaftandzieva *et al*., 2011; Chaudhary and Payasi, 2014). In contrast, other studies showed that CTX-M-type ESβL was most common in *E. coli* (Moubareck *et al*., 2005; Ensor *et al*., 2006; Bindayna and Murtadha, 2011; Al-Mayahie, 2013; Pokhrel *et al*., 2014; Adwan *et al*., 2014). Results of this research were in contrast to previous research from North Palestine, which showed that the prevalence of CTX-M-type and TEM-type ESβL was 100% and 32%, respectively, among ESβL-producing *E. coli* detected by PCR (Adwan *et al*., 2014).

In this study, the prevalence of *AmpC* β- lactamases among *E. coli* isolates was 26.9% and 5.8% using conventional technique and multiplex PCR technique, respectively. The prevalence of *AmpC* β- lactamases among *E. coli* isolates reported from various parts of the world ranged from 0.7% to 70.7% (Singhal *et al*., 2005; Woodford *et al*., 2007; Li *et al*., 2008; Vandana and Honnavar, 2009; Singtohin *et al*., 2010, Yilmaz *et al*., 2013; Barua *et al*., 2013; Dallal *et a*l., 2013; Drinkovic *et al*., 2015; El-Hady and

Adel; 2015). This research showed that the only DHA-1 subtype of AmpC β-lactamases detected among clinical isolates of *E. coli*. Whereas, several other studies from various parts of the world reported the presence of different subtypes among isolates of *E. coli* (Yan *et al*., *et al*. 2006; Pitout *et al*., 2007; Woodford *et al*., 2007; Ding *et al*., 2008; Barua *et al*., 2013; Dallal *et a*l., 2013).

Coexistence of two β-lactamases in single isolates was observed in this study. This was an alarming finding, that is, 3 isolates (5.8%) producing both ESβL (Class A and Class D) and only DHA gene was coexisted with them. The coexistence of different classes of β-lactamases in a single bacterial pathogen may pose treatment challenges, and this will seriously restricte treatment options. In addition, may pose diagnostic challenge, such as high-level expression of *AmpC* β-lactamases may prevent the recognition of the ESβLs and it may lead to use an inappropriate antimicrobial therapy and the result may be fatal (Oberoi *et al*., 2013). The expression of both AmpC β-lactamases and ESβLs enzymes in a single isolate decreases the efficacy of the β-lactam/β-lactamase inhibitor combinations (Chelliah *et al*., 2014). Coexistence more than one type of beta-lactamases or multiple ESβLs has been reported from different species of bacterial pathogens belonged to *Enterobacteriaceae* including *E. coli* (Paterson *et al*., 2003; Eckert *et al*., 2004; Jones *et al*., 2009; Dzierzanowska *et al*., 2010; Lin *et al*., 2010; Kaftandzieva *et al*., 2011; Bindayna and Murtadha, 2011; Oberoi *et al*., 2013; Tada *et al*., 2013; Al-Mayahie, 2013; Pokhrel *et al*., 2014; Adwan *et al*., 2014).

The high prevalence of β-lactamases producers among *E. coli* isolates in Palestine may be due to several risk factors such as long term exposure to antibiotics in hospitals, prolonged hospitalization, incorrect therapy, nursing home residency, severe illness, catheterization and movement of health staff in the hospital leading to dissemination of ESβL producers pathogens throughout the hospital (Waiwarawooth *et al*., 2006; Kateregga *et al*., 2015). Recently, it was shown that limited use of cephalosporins considerably decreased the frequency of ESβL producing microorganisms (Murki *et al*., 2010). Geographical variation in the occurrence rate of β-lactamases production have been detected from different countries and even from hospital-to-hospital within the same country (Jain *et al*., 2003; Paterson and Bonomo, 2005; Livermore, 2012).

The ERIC PCR typing of *Amp-C* β-lactamases and ESβL-producing isolates showed various DNA banding profiles. These isolates recovered mostly from urine of patients treated mainly in hospitals, sharing significant patient demographics and isolate characteristics including *Amp-C* β-lactamases and ESβL enzymes differed. It is clearly indicates that multiple clones of *Amp-C* β-lactamases and ESβL-producing isolates were prevalent in these hospitals. This supporting that the high prevalence of *Amp-C* β-lactamases and ESβL-producing *E. coli* isolates may be due to selective pressure imposed by the high rate and extensive incorrect use of antimicrobial agents especially cephalosporins in the country could be the only major cause.

In this study, transformation experiments are needed to make sure that isolates which showed negative β-lactamases by PCR and positive by conventional methods are chromosomal encoded β-lactamases. Our results underline the need for continuous monitoring, surveillance programs, proper control and prevention measures and effective antimicrobials use will restrict the further spread of *Amp-C* β-lactamases and ESβLs producing isolates within hospitals in Palestine.

Conclusion

In conclusion, our results showed high occurrence of ESβLs and AmpC β-lactamases among *E. coli* isolates in Palestine. Based on these results we recommend the continuous monitoring and surveillance of the prevalence, proper control and prevention practices and effective antibiotic use will limit the further spread of AmpC β-lactamases and ESβLs producing isolates within hospitals in this country.

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جامعة النجاح الوطنية

كلية الدراسات العليا

الوصف الجزيئي ومدى انتشار النوع البكتيري اشيريشيا المعوية المنتجة للإنزيمات المحللة للمضادات الحيوية من نوع بيتا لاكتام في شمال فلسطين

إعداد

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إشراف

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قدمت هذه الأطروحة استكمالاً لمتطلبات الحصول على درجة الماجستير في برنامج العلوم الحياتية، بكلية الدراسات العليا، في جامعة النجاح الوطنية، في نابلس- فلسطين.

2015

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الملخص

تم الحصول على 52 عزلة من بكتيريا الاشيريشيا القولونية من مستشفيات و مختبرات خاصة مختلفة من منطقة جنين/ فلسطين خلال الفترة ما بين شباط الى نيسان من العام 2015 . اظهرت النتائج ان انتشار كل من ESβL و AmpC β-lactamase باستخدام طرق الفحص التقليدية كان بنسبة 32.7% و 26.9% على الترتيب. بينما كان انتشارهما باستخدام تقنية PCR بنسبة 67.3% و 5.8 % على الترتيب. كما أظهرت النتائج أن جين *TEM* كان سائدا بنسبة 82.9% بين البكتيريا القولونية التي تحمل جينات ESβL. اما الجينات الاخرى وهي *CTX-M* ، *SHV* و *OXA* فكانت النسب 0.0%، 2.9% و 15.4% على الترتيب. بينما في AmpC β-lactamases فقد تم تحديد جين *DHA* فقط, وكانت نسبة انتشاره 5.8%. اظهر التحليل الجزيئي باستخدام طريقة شجرة النشوء والتطور. ان جميع تسلسلات جينات *TEM*، *SHV*، *OXA*، و *DHA* تعود الى *TEM-1*، *SHV-1* ، *OXA-1*، و *DHA-1* على الترتيب. كما أظهرت نتائج ERIC-PCR أن هذه السلالات متنوعة.

نتائجنا تؤكد على الحاجة إلى المراقبة المستمرة ومراقبة الانتشار والرقابة السليمة وممارسات الوقاية واستخدام المضادات الحيوية الفعالة سيحد من انتشار المزيد من السلالات المنتجة ل AmpC β-lactamases و ESβLs داخل المستشفيات في فلسطين.