Deanship of Graduate Studies

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Detection of Extended Spectrum Beta Lactamases (ESBL) production in *Pseudomonas aeruginosa* from various clinical samples.

Dana Naim Jabra Sam'an

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Thesis Approval

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Declaration

I certify that this thesis submitted for the degree of Master, is the result of my own research, except where otherwise acknowledged, and that this study (or any part of the same) has not been submitted for a higher degree to any other university or institution.

Signed: 

Dana Naim Jabra Sam'an

Date: May 8th, 2022.

Dedication

I dedicate this work to all of my family especially to my husband Mr. Nader Al- Qass for his patience to help me in my duties for so long as well as for supporting me continuously. A special gratitude to my parents, Mr. Naim Sam'an and Mrs. Sophie Sam'an, whom words of satisfaction, encouragement and love pushed me forward and supported me endlessly.

I also dedicate this work to the spirit of my brother and grandfather whom always wanted to see me reaching here. To my teachers, supervisors and friends at Al- Quds University who have been supporting me through the master thesis work. I will always appreciate all what they have done.

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Abstract

Background: Beta Lactamases (β- Lactamases) production is the most common observed bacterial resistance mechanism in many genera of Enterobacteriaceae. These enzymes are encoded by chromosomal genes or genes located on mobile elements. Reporting of Extended Spectrum Beta Lactamase (ESBL) - producing *Pseudomonas aeruginosa* (*P. aeruginosa*) has been increasing recently and they are acquired either from hospitals or from communities.

Aim: The main aim of this study was to detect the prevalence of ESBL-producing *P. aeruginosa* isolated from different clinical samples taken from Palestinian hospitals in the West Bank region. This was done by using the phenotypic double disk synergy test (DDST) and multiplex polymerase chain reaction (PCR) assay.

Methodology: A total of 163 bacterial isolates were collected from five hospitals in West Bank, Palestine including Bethlehem Arab Society for Rehabilitation hospital/ Beit Jala, Beit Jala Governmental Hospital/ Beit Jala, Al-Istishari Al Arabi Hospital/ Ramallah, Al-Ahli Hospital/ Hebron and Al-Makased Islamic Hospital/ Jerusalem. These isolates were identified as *P. aeruginosa* based on colony morphology and by using confirmatory biochemical tests. The DDST was applied for the detection of ESBL-producing isolates. Additionally, screening for AmpC β-lactamase production was done followed by multiplex PCR to detect the occurrence of *bla*CTX-M, *bla*SHV and *bla*TEM genes.

Result: Of the 163 isolates obtained, 131 *P. aeruginosa* isolates were included in the study and tested using DDST, screened for AmpC production and tested by multiplex PCR. When DDST was used, none of the isolates showed a difference of ≥ 5 mm between the zone of diameter of cefotaxime/clavulanic acid and that of cefotaxime alone. However, all isolates were identified as AmpC producers using AmpC screening method. When multiplex PCR was used, 66 isolates (50.38%) harbored at least one ESBL gene. *Bla*CTX-M gene was the most common among the isolates either produced alone (59.1%) or in combination with *bla*SHV gene (37.88%). *bla*TEM gene was the least detected; alone in none of the isolates but only in one isolate with *bla*SHV gene. However, the combination of the three genes was not detected in any of the isolates. *P. aeruginosa* ESBL-producing isolates taken from inpatients were more than those from outpatients. Furthermore, isolates from the surgical wards and sputum samples were the majority. Colistin-Sulphate (COL), Amikacin (AK) and Gentamicin (GENT) were the most effective treatments against infections caused by ESBL-producing *P. aeruginosa* isolates as 100%, 91.9% and 89.18% of ESBL-producing isolates were susceptible to these antibiotics respectively.

Conclusion: The study finding clearly suggest that the rate of ESBL-production by *P. aeruginosa* isolates is increasing and considered an important cause of healthcare associated infection. None of the isolates were detected by DDST; the reason could be due to the production of AmpC β-lactamases by all isolates tested which may obstruct or even conceal the detection of ESBL by phenotypic methods and so, AmpC detection should be included in the routine antibiotic susceptibility testing for *P. aeroginosa* isolates. Multiplex PCR assay was successful in detecting the targeted ESBL genes.

Keywords: ESBL-producing *P. aeruginosa*, DDST, multiplex PCR, *bla*CTX-M, *bla*SHV, *bla*TEM, AmpC gene, Colistin-Sulphate (COL), Amikacin (AK) and Gentamicin (GENT).

الكشف عن انتاج Extended Spectrum Beta Lactamases (ESBL) بين بكتيريا الزائفة الزنجارية المعزولة من العينات السريرية المختلفة.

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ملخص

خلفية الدراسة: يعتبر إنتاج انزيمات البيتا لاكتاميز آلية المقاومة البكتيرية الأكثر شيوعًا في العديد من أجناس عائلة البكتيريا المعوية. يتم ترميز هذه الإنزيمات بواسطة الجينات المحمولة على الكروموسومات أو الجينات الموجودة على العناصر المتحركة. إن الإبلاغ عن بكتيريا الزائفة الزنجارية المنتجة لانزيمات ESBL متزايد مؤخرًا والتي يتم إكتسابها إما من المستشفيات أو من المجتمعات.

هدف الدراسة: كان الهدف الأساسي من هذه الدراسة هو الكشف عن انتشار بكتيريا الزائفة الزنجارية المنتجة لانزيمات ESBL المعزولة من العينات السريرية المختلفة المأخوذة من المستشفيات الفلسطينية في منطقة الضفة الغربية. تم عمل ذلك باستخدام اختبار تآزر القرص المزدوج المظهر ويليه تفاعل البلمرة المتسلسل المتعدد.

منهجية البحث: تم جمع 163 عزلة بكتيرية من خمسة مستشفيات في الضفة الغربية، فلسطين وتشمل مستشفى جمعية بيت لحم العربية للتأهيل/ بيت جالا، ومستشفى بيت جالا الحكومي/ بيت جالا، ومستشفى الاستشاري العربي/ رام الله، والمستشفى الأهلي/ الخليل، ومستشفى المقاصد/ القدس. تم تعريف العزلات البكتيرية على أنها بكتيريا الزائفة الزنجارية بالاعتماد على شكل المستعمرة و باستخدام الاختبارات التأكيدية البيوكيميائية. تم تطبيق اختبار تآزر القرص المزدوج للكشف عن العزلات المنتجة لانزيمات ESBL. إضافة الى ذلك تم فحص انتاج AmpC في العزلات وتلاه عمل تفاعل البلمرة المتسلسل لاكتشاف وجود الجينات *bla*CTX-M ، و *bla*SHV، و *bla*TEM .

النتائج: من 163 عزلة التي تم الحصول عليها، أُقيمت الدراسة على 131 عزلة من بكتيريا الزائفة الزنجارية حيث تم إجراء إختبار تآزر القرص المزدوج ومن ثم تم فحصها لانتاج AmpC وعمل تفاعل البلمرة المتسلسل المتعدد. عند استخدام اختبار تآزر القرص المزدوج، لم تُظهر أي من العزلات فرقاً بمقدار ≤ 5 مم بين القطر الناتج من cefotaxime/clavulanic acid وذلك الناتج من cefotaxime وحده. مع ذلك، تم تحديد جميع العزلات على أنها منتجة ل AmpC باستخدام الفحص الكاشف عنه. عندما تم استخدام تفاعل البلمرة المتسلسل المتعدد، ستة وستون عزلة (50.38%) كانت تؤوي جيناً واحداً على الأقل من ESBL. جين *bla*CTX-M كان الأكثر شيوعاً بين العزلات سواء منتج بمفرده (59.1%) أو بالاشتراك مع الجين *bla*SHV (37.88%). *bla*TEM كان الأقل اكتشافاً؛ لم يتواجد لوحده في أي من العزلات ولكن فقط في عزلة واحدة مع جين *bla*SHV. ومع ذلك، لم يكن هناك تواجد للثلاث جينات مجتمعة في عزلة واحدة. عزلات بكتيريا الزائفة الزنجارية المنتجة لانزيمات ESBL المأخوذة من المرضى المقيمين كانت أكثر من تلك المأخوذة من مرضى العيادات الخارجية. إضافة إلى ذلك، العزلات من أقسام الجراحة وعينات البلغم كانت تحتل الغالبية العظمى. الكوليستين، والأميكاسين، والجينتامايسين كانت العلاجات الأكثر

فعالية ضد عزلات بكتيريا الزائفة الزنجارية المنتجة لانزيمات ESBL حيث أن 100% ، 91.9% و 89.18% من العزلات المنتجة لانزيمات ESBL أظهرت حساسيتها لهذه االمضادات الحيوية على التوالي.

الخلاصة: توحي نتائج الدراسة بوضوح أن معدل بكتيريا الزائفة الزنجارية المنتجة لانزيمات ESBL في تزايد ويعتبر سبب مهم للعدوى المرتبطة بالرعاية الصحية. لم يتم الكشف عن أي من العزلات بواسطة اختبار تآزر القرص المزدوج وقد يكون السبب هو انتاج انزيم البيتا لاكتاميز AmpC من قبل جميع العزلات المُختبرة والذي قد يعيق أو حتى يُخفي الكشف عن انزيمات ESBL باستخدام طرق النمط الظاهرية وهكذا يجب تضمين اكتشاف AmpC في اختبار الحساسية الروتيني للمضادات الحيوية لبكتيريا الزائفة الزنجارية. اما تفاعل البلمرة المتسلسل المتعدد فلقد نجح في الكشف عن جينات ESBL المستهدفة.

الكلمات الدالة: بكتيريا الزائفة الزنجارية المنتجة لانزيمات ESBL، اختبار تآزر القرص المزدوج، تفاعل البلمرة المتسلسل المتعدد، *bla*CTX-M، *bla*SHV، *bla*TEM، جين AmpC ، الكوليستين، والأميكاسين، والجينتامايسين.

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List of Abbreviations

Abbreviation	Term
AK	Amikacin
ATCC	American Type Culture Collection
Bp	Base pair
β- Lactamases	Beta lactamases
BA	Blood agar
BAL	Broncho-alveolar Lavage
BASR	Bethlehem Arab Society for Rehabilitation Hospital
CCU	Cardiac Care Unit
CTX-30	Cefotaxime
CTC-40	Cefotaxime/ Clavulanic acid
CAZ-30	Ceftazidime
Cm	Centimeters
CVP Tip	Central Venous Pressure Tip
CIP	Ciprofloxacin
CLSI	Clinical Laboratory Standard Institute
COL	Colistin-Sulphate
COVID-19	Coronavirus disease 2019
DNA	Deoxyribonucleic acid
DDST	Double Disk Synergy Test
ESBLs	Extended-Spectrum Beta-Lactamases
FOX-30	Cefoxitin
GENT	Gentamicin
IMP	Imipenem
ICU	Intensive Care Unit
I	Intermediate

LB Broth	Luria Broth
MEM	Meropenem
MBLs	Metalo Beta Lactamases
µL	Micro-liter
µM	Micro-molar
Mm	Milli-meters
Min	Minutes
MHA	Muller Hinton Agar
MDR	Multi Drug Resistant
MDRP	Multi Drug Resistant *Pseudomonas aeruginosa*
PCR	Polymerase Chain Reaction
P. aeruginosa	*Pseudomonas aeruginosa*
R	Resistant
Rpm	Revolution per minute
Sec	Seconds
S	Sensitive
TSI	Triple Iron Sugar
TBE buffer	Tris EDTA buffer
TZP	Piperacillin/Tazobactam
UTI	Urinary tract infection

Chapter One

1.1 Introduction

Beta Lactamases (β-Lactamases) are bacterial enzymes encoded by both chromosomal and plasmids genes (Tavajjohi & Moniri, 2011). Their production is one of the most commonly used bacterial resistance mechanisms against β-lactam antibiotics (Shaikh, Fatima, Shakil, Danish Rizvi, & Kamal, 2015). Extended spectrum beta lactamases (ESBLs) is an important cause of resistance in gram negative bacteria (Paterson & Bonomo, 2005). ESBLs are present in many members of the Enterobacteriaceae family and other gram negative bacteria, such as *Pseudomonas aeruginosa* (*P. aeruginosa*) (Falagas & Karageorgopoulos, 2009).

P. aeruginosa is the most common species that cause human infection of the genus (Abbas, El-Ganiny, & Kamel, 2018). *P. aeruginosa* belongs to a group of nosocomial pathogens with the acronym ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) (Laudy et al., 2017). *P. aeruginosa* is a widely spread opportunistic pathogen in human causing serious and lethal infections (Anil & Shahid, 2013). The most significant infection is usually found in healthcare settings (Naas, Poirel, & Nordmann, 2008). In healthcare settings, *P. aeruginosa* was reported to be the second most common cause of pneumonia, the third most common cause of urinary tract infections (UTIs), the eighth most commonly isolated pathogen from the bloodstream (Gaynes & Edwards, 2005) and the seventh most common cause of nosocomial bacteremia (Rossolini & Mantengoli, 2005). *P. aeruginosa* contributes to surgical site and other wound-related morbidity and mortality (Ranjan, Ranjan, Bansal, & Arora, 2010). Recently, there is a growing incidence of *P. aeruginosa* in post-operative wound infections as previous studies reported a high prevalence of this pathogen among others, being isolated from the surgical wound. However, this is getting more serious in developing countries (Masaadeh & Jaran, 2009; Ranjan et al., 2010).

Due to both intrinsic and acquired antibiotic resistances which are complicated and still not fully understood, there is global spread of multi-drug resistant *P. aeruginosa* strains (MDRP) (Rossolini & Mantengoli, 2005; Saberi, Zamani, & Salehzadeh, 2015; Tam et al., 2010). This wide spread resistance causes serious and adverse outcomes making it an increasing public health threat and thus limiting the available therapeutic options (Cao, Wang, Sun, Zhu, & Chen, 2004)

Few studies are carried out on *P. aeruginosa* infection and drug resistance in Palestine. In two studies performed at the burn unit of two hospitals in Gaza Strip, *P. aeruginosa* was the most commonly isolated bacteria in both hospitals (50% and 37.5% respectively) (Al Laham, Elmanama, & Tayh, 2013; Tayh, Al Laham, Elmanama, & Karim, 2016) . Additionally, in a study carried out by Tayh *et al.*, they determined the antimicrobial susceptibility pattern and identified the ESBL-producers among the isolated pathogens. They reported a high frequency of ESBL-producers among gram negative bacteria but a high susceptibility of these isolates except *Acinetobacter baumanni* towards carbapenems including meropenem and imipenem. However, the percentages of ESBL-producing pathogens were as follows: *P. aeruginosa* (13.3%), *Klebsiella pneumoniae* (40%), *Escherichia coli* (55.6%), *Enterobacter cloacae* (50%) and *Acinetobacter baumannii* (100%) (Tayh et al., 2016).

Another study took place in An-Najah National University-Nablus, Palestine during October 2015-April 2016 which investigated the prevalence and molecular characteristics of ESBLs, metallo-beta-lactamases (MBLs) and integrons genes among clinical isolates of *P. aeruginosa* obtained from different health centers. It showed a high prevalence of MBLs and ESBLs among clinical isolates of *P. aeruginosa* (Adwan, Shtayah, Adwan, & Othman, 2016).

However, other studies from the Middle East reported a range of 7.4% - 39.2% for the prevalence of ESBL-producing *P. aeruginosa* (Al Marjani, Al-Ammar, & Kadhem, 2013; Rafiee, Eftekhar, Tabatabaei, & Minaee Tehrani, 2014; Zafer, Al-Agamy, El-Mahallawy, Amin, & Ashour, 2014). So, due to the very limited number of studies which investigated ESBL production by *P. aeruginosa* in Palestine, more research has to be carried out to establish the extent of the problem. This study was designed to detect the ESBL production by *P. aeruginosa* from various clinical specimens of various healthcare centers from West Bank, Palestine using phenotypic as well as genotypic methods.

1.2 Literature review

1.2.1. β-Lactamases:

β-Lactamases are bacterial enzymes that are encoded by chromosomal genes or genes located on mobile elements such as plasmids and integrons (Bush, 2010) (Tavajjohi & Moniri, 2011). The presence of these genes on plasmids helps in the horizontal spread of antibiotics resistance between bacterial strains (El Salabi, Walsh, & Chouchani, 2013). These hydrolytic enzymes have the ability to inactivate beta lactam class of antibiotics by hydrolyzing the β-Lactam rings rendering the drug inactive against its target (Falagas & Karageorgopoulos, 2009) thus protecting the microorganisms (Tavajjohi & Moniri, 2011).

β-Lactamases production is the most common observed bacterial resistance mechanism, as recently, it is commonly observed in many genera of Enterobacteriaceae (Mathur, Kapil, Das, & Dhawan, 2002). However, classification of β-Lactamases is based on molecular structure known as Ambler classification or functional similarities which is known as Bush-Jacoby-Medeiros classification (Kanj & Kanafani, 2011).

Based on their primary structure, β-Lactamases are classified into four classes including A, B, C and D enzymes. The A, C and D classes are serine β-Lactamases while class B enzymes are zinc metallo-enzymes (Bajpai, Pandey, Varma, & Bhatambare, 2017). Those of class A include *bla*TEM, *bla*SHV, *bla*CTX-M, *bla*PER, *bla*VEB and *bla*GES. However, their prevalence differs among different countries and regions (Chen et al., 2015).

1.2.2. ESBLs:

ESBL enzymes are numerous, rapidly growing group of β-Lactamases (Lin, Liu, Lin, & Shi, 2012; Shaikh et al., 2015). They usually arise by point mutations mainly from narrow spectrum β-Lactamases *bla*TEM1, *bla*TEM2, *bla*SHV1 (Bajpai et al., 2017; Falagas & Karageorgopoulos, 2009) which are responsible for the production of TEM β-Lactamases and SHV β-Lactamases respectively (Ojdana et al., 2014). Other clinically relevant ESBL types include the VEB, PER, GES, TLA, IBC, SFO-1, BES-1 and BEL-1 (Falagas & Karageorgopoulos, 2009). Moreover, like TEM and SHV ESBLs, class D enzymes or OXA ESBLs have minor sequence substitutions extending their spectrum of hydrolysis (Livermore, 2002).

ESBL enzymes are capable of conferring bacterial resistance to the penicillins, different generations of cephalosporins such as cefotaxime, ceftriaxone and ceftazidime as well as to the monobactam aztreonam (Tavajjohi & Moniri, 2011). However, cephamycins such as cefoxitin, cefotetan and cefmetazole are not hydrolyzed by these enzymes (Falagas & Karageorgopoulos, 2009). Additionally, β-Lactamases inhibitors such as clavulanic acid, sulbactam and tazobactam inhibit these enzymes (Bajpai et al., 2017) (Tavajjohi & Moniri, 2011). Carbapenems are considered the drug of choice against them (Tavajjohi & Moniri, 2011).

ESBL producing pathogenic bacteria such as *P. aeruginosa* are now universally recognized (Khanfar, Bindayna, Senok, & Botta, 2009) as ESBLs production is not only pertinent to hospital i.e. nosocomial acquired infections but also it is becoming an important community acquired infection making it a public health issue (Falagas & Karageorgopoulos, 2009).

ESBL production is common in patients who lately received broad-spectrum antibiotics especially third generation cephalosporins and quinolones (Kanj & Kanafani, 2011). Additionally, old age, comorbid conditions, recent or prolonged hospitalization, intensive care unit (ICU) admission as well as the presence of invasive devices are considered to be a risk factors for being infected with ESBL-producing pathogens (Kanj & Kanafani, 2011; Khanfar et al., 2009).

1.2.3. *P. aeruginosa*:

P. aeruginosa is a Gram-negative, non-fermenter aerobic bacilli that is found both in nature and human environment (Czekajło-Kołodziej, Giedrys-Kalemba, & Medrala, 2006). Although it is a commensal human microflora in healthy people (Rashid, Chowdhury, Rahman, Begum, & Muazzam, 2016) that seldom causes disease in them (Rossolini & Mantengoli, 2005), it is reported that it can cause wide spectrum of infections (Hirsch & Tam, 2010). It is a leading hospital acquired pathogen causing severe diseases in burn patients, cystic fibrosis as well as immuno-compromised patients (Rossolini & Mantengoli, 2005; Saberi et al., 2015) such as neutropenic or cancer patients (Shaikh et al., 2015) including respiratory tract infections and UTIs (Abbas et al., 2018; Rossolini & Mantengoli, 2005) in addition to sepsis (Tam et al., 2010). Further, it was reported as the third most frequently isolated pathogen from ICU (Czekajło-Kołodziej et al., 2006).

Hospitals often accommodate MDRP which acts as an infection source for patients hospitalized in burn wards (Anvarinejad et al., 2014). Wound infections caused by *P. aeruginosa* are annoying as they are serious in burn patients because they can go rapidly deep into tissues and worsen to *P. aeruginosa* bacteremia and sepsis which are associated with high mortality rates primarily in immuno-compromised patients (Branski et al., 2009; Coetzee, Rode, & Kahn, 2013; Mousa, 1997).

1.2.4. MDRP:

MDRP has been reported world-wide with adverse and severe outcomes (Aloush, Navon-Venezia, Seigman-Igra, Cabili, & Carmeli, 2006) especially in those strains isolated from hospitals (Anil & Shahid, 2013) as an excessive resistance was reported in isolates from hospital patients versus those isolates from outpatients (Livermore, 2002). This spread is often difficult to control due to the intrinsic and acquired resistance properties to many antimicrobial drugs (Naas et al., 2008) (Tavajjohi & Moniri, 2011). *P. aeruginosa* is intrinsically resistant to many antimicrobial agents such as most beta-lactams, fluoroquinolones, chloramphenicol and other antibiotics (Lin et al., 2012; Rossolini & Mantengoli, 2005), severely limiting the effectiveness and availability of therapeutic options (Anil & Shahid, 2013) rendering nosocomial infections caused by MDRP difficult to cure (Cao et al., 2004).

1.2.5. *P. aeruginosa* resistance mechanisms:

Diverse resistance mechanisms against drugs have been reported in *P. aeruginosa* (Tavajjohi & Moniri, 2011), including the increased expression of multi-drug efflux systems especially Mex-AB-oprM that is responsible for expulsion of β-Lactams and quinolones, the production of inactivating enzymes, reduced expression or loss of outer membrane protein such as OprD porin reducing antibiotic permeability and target mutations (Abbas et al., 2018; Hirsch & Tam, 2010).

1.2.6. ESBLs in *P. aeruginosa*:

ESBL genes are not uncommon in *P. aeruginosa* isolates. Class A ESBLs, class B carbapenamases (Metallo-b-betalactamases (MBLs) and class D extended- spectrum oxacillinases (OXAs) have increasingly been reported in *P. aeruginosa* strains (Lin et al., 2012). In addition, class C cephalosporinases (AmpC β-Lactamases) were also reported in *P. aeruginosa* (Zhao & Hu, 2010).

However, on the other hand, there are many risk factors responsible for acquiring MDR infections including the prior use of antibiotics, previous history of infection or colonization with *P. aeruginosa*, duration of hospital stay whether being bedridden or in ICU, mechanical ventilation where there is a formation of resistant biofilms of bacterial communities and patients are given more broad-spectrum antibiotics (Cao et al., 2004), malignant diseases as well as a previous history of chronic obstructive pulmonary disease (Hirsch & Tam, 2010).

1.2.7. Laboratory detection of ESBLs:

Laboratory detection of ESBLs involves the use of phenotypic and genotypic methods. Furthermore, phenotypic methods can be either screening or confirmatory tests (Falagas & Karageorgopoulos, 2009).

1.2.7.1. Phenotypic methods:

The screening method involves testing for resistance to cefpodoxime, cefotaxime, ceftazidime, ceftriaxone or aztreonam while the confirmatory method depends on the double disk synergy test (DDST) between the above mentioned antibiotics and the beta lactamase inhibitor such as clavulanic acid (Falagas & Karageorgopoulos, 2009). In this test, the organism is considered as ESBL producer if there is a $\geq$ 5.0 mm zone of diameter between ceftazidime/clavulanic acid and that of ceftazidime alone and/or between cefotaxime/clavulanic acid and that of cefotaxime alone (Bajpai et al., 2017).

However, due to the poor sensitivity of this test, it cannot detect those ESBL-producing isolates that produce β-Lactamases not inhibited by clavulanic acid such as AmpC- or MBLs which give a negative confirmatory test result. For this, the addition of cloxacillin disc can enhance the ability of DDST to detect ESBL *P. aeruginosa* because it inhibits AmpC enzyme activity (Falagas & Karageorgopoulos, 2009; Lin et al., 2012). However, the addition of boronic acid to the discs has shown a greater ability than cloxacillin in the inhibition of AmpC overproduction (Glupczynski et al., 2010).

1.2.7.2. Genotypic methods:

The genotypic method is the method of choice for the detection of ESBL-producing strains (Bajpai et al., 2017). This method involves the use of polymerase chain reaction (PCR) which is based on amplification of the specific genes (Falagas & Karageorgopoulos, 2009). Although molecular methods are expensive, require specialized equipment and expertise and can only detect genes with known sequences (Bajpai et al., 2017), these methods are sensitive and time-saver (Falagas & Karageorgopoulos, 2009).

1.2.8. Therapy:

The property of resistance in ESBL-producing organisms have greatly limited the antibiotic options used for the treatment which, in turn, cause therapy failure (Abbas et al., 2018; Kanj & Kanafani, 2011) and decreases the probability of appropriate empirical therapy (Hirsch & Tam, 2010).

Infection control measures should work on preventing the transmission of ESBL-producing organisms among patients in hospitals which usually occur due to colonization on the inanimate environment, hands of healthcare workers and the used medical equipment etc. (Paterson & Bonomo, 2005).

The use of combination therapy has been reported with a potential advantage as MDR isolates will be susceptible to at least one agent in this combination. Further, combined therapy can enhance bacterial killing due to the synergistic combination (Yuan et al., 2010).

Antimicrobial resistance seen in clinical isolates of *P. aeruginosa* complicates the treatment options affecting clinical outcomes and treatment costs (Flamm et al., 2004). Furthermore, resistance can develop during therapy (Dundar & Otkun, 2010). However, there is an emphasis towards the use of combined therapy in the treatment of *Pseudomonas* infections to ensure the synergistic action (Anil & Shahid, 2013; Dundar & Otkun, 2010).

The few effective antibiotics that can be used therapeutically against *P. aeruginosa* include anti-pseudomonal penicillins, cephalosporins, carbapenems and fluoroquinolones. However, Aminoglycosides also have the anti-pseudomonal activity but are rarely used alone (Cao et al., 2004).

However, if all options of beta lactams, aminoglycosides and quinolones usage are lost, polymyxins of which colistin and polymyxin B are currently available for clinical use despite their significant toxicity have an excellent antimicrobial activity against ESBL-producing organisms (Falagas & Karageorgopoulos, 2009; Livermore, 2002). Finally, carbapenems are considered as the treatment of choice against the serious ESBL associated infections (Falagas & Karageorgopoulos, 2009; Kanj & Kanafani, 2011).

1.3 Problem statement

*bla*TEM and *bla*SHV are the classic β-Lactamases. However, the occurrence of point mutations in genes encoding them gave rise to a variety of enzymes with an extended spectrum activity (Kanj & Kanafani, 2011). Moreover, pathogens producing ESBL are now globally considered as major causes of both hospital (nosocomial) and community acquired infections (Khanfar et al., 2009) rendering them an important public health issue (Falagas & Karageorgopoulos, 2009).

P. aeruginosa is a versatile, widely distributed opportunistic pathogen among multiple environments (Czekajło-Kołodziej et al., 2006; Shaikh et al., 2015). Although MDRP true prevalence is not well-established (Hirsch & Tam, 2010), it is posing a serious, rapid growing problem globally (Dundar & Otkun, 2010) as changes in its susceptibility to multiple anti-microbial agents is very familiar (Cao et al., 2004). The increase spread and prevalence of MDPR strains especially ESBL-producing *P. aeruginosa* mainly in hospitals made it a difficult issue to treat due to the strictly limited availability of therapeutic options (Aloush et al., 2006; Anil & Shahid, 2013; Chen et al., 2015) making it an acute underlying problem (Czekajło-Kołodziej et al., 2006).

1.4 Aims and Objectives

Aim: To determine the occurrence of ESBLs in *P. aeruginosa* clinical isolates obtained from patients in the West Bank hospitals.

Objectives:

1. To isolate and identify *P. aeruginosa* from various clinical samples.
2. To detect phenotypically ESBL and non-ESBL producers.
3. To detect ESBL genes by multiplex PCR assay.

Chapter Two

Methodology

2.1 Materials

All materials used in this study are listed in Table 2.1. Additionally, all reagents and chemicals were of analytical grade.

(Table 2.1): Instruments and reagents used in the study.

No.	Item	Components	Supplier / Company
1.	Blood agar (BA)	- HM infusion from (500 g/L) - Tryptose (10 g/L) - Sodium chloride (5.0 g/L) - Agar (15 g/L) - Final pH (at 25°C) 6.8±0.2	HIMEDIA Company
2.	Cefotaxime discs – 30 mcg	Vial (50 discs)	Bioline
3.	Cefotaxime/Clavulanic acid discs – 40 mcg	Vial (50 discs)	Bioline
4.	Ceftazidime discs – 30 mcg	Vial (50 discs)	Bioline
5.	Cefoxitin discs – 30 mcg	Vial (50 discs)	Bioline
6.	Controls	*P. aeruginosa* ATCC® 9027 *P. aeruginosa* ATCC® 27853	ATCC
7.	Gel electrophoresis procedure	- Agarose powder - Tris EDTA buffer 50x and 1x - Microwave - Ethidium Bromide solution - DNA ladder - Bio-Rad gel electrophoresis apparatus - UV- Light documentation system	Hylabs
8.	Muller Hinton Agar (MHA)	- Acicase ™ (17.50 g/L) - HM infusion solids B (2 g/L) - Starch Soluble (1.50 g/L) - Agar (17.00 g/L) - Final pH (at 25°C) 7.3±0.1	HIMEDIA Company
9.	PCR	- PCR tubes - Eschohealthcare Swift PCR thermo- cycler device - Larova master-mix - Heat block 95°C	
10.	Primers	- CTX-M Forward and Reverse - SHV Forward and Reverse - TEM Forward and Reverse	Hylabs

The research plan was carried out in two phases. First, the isolation and phenotypic confirmation of ESBL and non-ESBL producing strains based on susceptibility testing. Second, the genotypic detection of resistance genes by multiplex PCR assay.

2.2 Methods

2.2.1 Bacterial Isolates:

A total of 163 samples were collected during 06 June and 16 December 2021 for the study as shown in table 2.2, out of which a total of 131 were included in the study.

Table 2.2 shows hospitals names and the total number (n=163) of samples collected from each hospital.

Pseudomonas isolates were obtained from various clinical samples such as sputum, urine, wound, ear swabs, blood, tissues, trap, body fluids, central venus pressure (CVP) tip, bronchoalveolar lavage (BAL) and others. These isolates were routinely retained and frozen at -30°C in Luria Broth (LB broth) containing 40% glycerol for further analysis.

Although patients identifiers were removed, the species, date of isolation, source of isolation as well as patient gender, age and residency status (i.e. outpatient or inpatient) were recorded. Some patients, however, had *P. aeruginosa* isolated from one, two sites or from the same type of specimens collected at different days of hospitalization.

P. aeruginosa ATCC 27853 and *P. aeruginosa* ATCC 9027 were used as control strains.

(Table 2.2): No. of samples collected from different hospitals.

No.	Hospital name	No. of samples
1.	Al-Makased Islamic Hospital	70
2.	Bethlehem Arab Society for Rehabilitation Hospital (BASR)	42
3.	Al-Istishari Al Arabi Hospital	19
4.	Beit Jala Govermental Hospital	17
5.	Al-Ahli Hospital	15
Total		**163**

2.2.2. Inoculation of samples:

All available isolates were thawed and sub-cultured from frozen vials on 5% human BA plates (HiMedia, India). The plates were routinely incubated at 37°C aerobically and after overnight incubation, they were checked for bacterial growth.

2.2.3. Colony morphology:

Grown organisms were identified based on colonies color, size and shape, structure, odor and pyocyanin pigment production.

2.2.4. Biochemical confirmation tests:

Further biochemical tests including catalase, oxidase test strip (Bioanalyse, Turkey), citrate utilization, nitrate reduction as well as oxidative/fermentative medium tests were performed.

Samples confirmed as *P. aeruginosa* were inclusion criteria while those dead (showing no growth) or confirmed as not *P. aeruginosa* (n=32) were exclusion criteria. Based on this, the total number of samples included in this study was 131.

2.2.5. Antibiotic susceptibility:

Antimicrobial susceptibility testing was performed for all of the collected samples in the five hospitals under the Clinical & Laboratory Standards Institute guidelines (CLSI) by either Kirby-Bauer disk diffusion method on MHA (Bauer, Kirby, Sherris, & Turck, 1966) or Vitek 2 automated system (VITEK®2 ID & AST CARDS-BioMerieux, UK, Ltd.)

Information about antimicrobial susceptibilities was taken from only three out of five laboratories including Al-Makased Islamic clinical laboratory, BASR clinical laboratory and Beit Jala Govermental clinical laboratory. Eight antibiotics were commonly used in the three labs; Ciprofloxacin, Gentamicin, Meropenem, Amikacin, Imipenem, Ceftazidime, Piperacillin/Tazobactam and Colistin-Sulphate. Additional antibiotics were used in some samples such as Levofloxacin, Aztreonam and Cefepime from Beit Jala Govermental lab, Levofloxacin, Aztreonam and Tobramycin from Al-Makased Islamic lab and Cefotaxime, Norfloxacin, Tobramycin, Ticaricillin, Ticaricillin/Clavulanic acid and Piperacillin from BASR lab.

2.2.6. Detection of ESBL by phenotypic tests:

ESBL screening was performed by DDST. In each isolate, ESBL was confirmed phenotypically by Kirby Bauer disc diffusion method on MHA (HiMedia, India). Briefly, normal saline suspensions of *P. aeruginosa* were adjusted to McFarland's 0.5 standard and inoculated heavily on the surface of MHA plate. Then disks containing cefotaxime (30 µg) (CTX-30) (Bioanalyse, Turkey) and cefotaxime plus clavulanic acid (30 µg plus 10 µg) (CTC-40) (Bioanalyse, Turkey) were placed 2 centimeters (cm) apart on the surface of MHA, and plates were incubated overnight aerobically at 37°C.

Zone of diameter around the discs was determined for every isolate and samples were considered to produce an ESBL if there was an increase of ≥ 5.0 millimeters (mm) in zone of diameter on clavulanate containing disks compared with the zone of diameter on the disk containing cephalosporine alone, as recommended by CLSI (CLSI 2021).

2.2.7. Screening of AmpC β-lactamase:

Isolates were screened for the presence of AmpC β-lactamase according to Chika *et al.* (Chika, Carissa, Chijioke, & Ifeanyichukwu, 2017). Briefly, a bacterial suspension was adjusted to 0.5 McFarland's standard then swabbed on MHA plates**.** Afterwards, disks of ceftazidime (30 µg) (CAZ-30), CTX-30 were placed at a distance of 20mm from cefoxitin disk (30µg) (FOX-30). Isolates showing blunting of CTX-30 or CAZ-30 zone of inhibition next to FOX-30 disk or showing reduced susceptibility to either CTX-30 or CAZ-30 and FOX-30 were considered as possible AmpC producers.

2.2.8. Molecular detection of resistant genes:

2.2.8.1. DNA isolation:

DNA extraction was carried out by boiling method according to Adwan *et al.* (Adwan et al., 2016)**.** Briefly, a loopful of an overnight grown culture on BA plates of each test isolate was washed with 800µL distilled water and centrifuged at 5000 revolution per minutes (rpm) for 3 minutes (min) (Biofuge fresco). The supernatant was discarded and the pellet was re-suspended in 400µL of sterile double distilled water and boiled for 15 min at 95°C. Then cells were incubated on ice for 10 min and the precipitate was pelleted at 13'000 rpm for 5 min. Then, the supernatant was used as DNA template for PCR amplification.

2.2.8.2. Detection of ESBL genes by multiplex PCR:

PCR amplifications were performed using specific primers for Ambler class A (*bla*TEM, *bla*SHV, *bl*aCTX-M) (Bajpai et al., 2017) with few modifications on reaction conditions i.e. number of cycles (changed from 32 to 40 cycles) and annealing temperature (changed from 54°C to 56°C). Sequence of primers and size of amplicons are described in Table 2.3.

PCR experiments were carried out in 20µL volume reaction mixtures containing 4 µL of 5x PCR premix (Taq polymerase master mix, Larova), 0.5 µL of each 10 µM primer, 12.5 µL double sterile distilled water and 3 µL of Deoxyribonucleic acid (DNA) template.

Amplifications were carried in the thermo cycler (Escohealthcare swift. Max Pro) and cycling conditions were: initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 1 min and extension at 72°C for 1.5 min. Followed by final extension at 72°C for 7.5 min.

Negative and positive controls were included in all PCR experiments.

(Table 2.3): β- Lactamases target genes for PCR amplification, primers sequences and amplicon size.

Gene	Primers sequences	Amplicon size (bp)
*bla*CTX-M	F: 5-ACCGCCGATAATTCGCAGAT-3 R: 5-GATATCGTTGGTGGTGCCATAA-3	588
*bla*TEM	F: 5-TTGGGTGCACGAGTGGGTTA-3 R: 5-TAATTGTTGCCGGGAAGCTA-3	465
*bla*SHV	F: 5-AGGATTGACTGCCTTTTTG-3 R: 5-ATTTGCTGATTTCGCTCG-3	392

2.2.9. Agarose gel electrophoresis:

PCR products (15 µL) were confirmed by 2% (w/v) agarose gel electrophoresis in Tris EDTA Buffer (TBE buffer) at 90 voltages for 1 hour and 9 min and visualized with ethidium bromide staining. Then, the gel was photographed under Ultraviolet illuminator using gel documentation system.

Further, 100 base-pair (bp) DNA ladder (Gene DireX, hy-labs) was included in each run to check the molecular weight of PCR products.

2.2.10. Ethical consideration:

Ethical clearance was taken from Research Ethics Sub-committee of Health Professions College.

Chapter Three

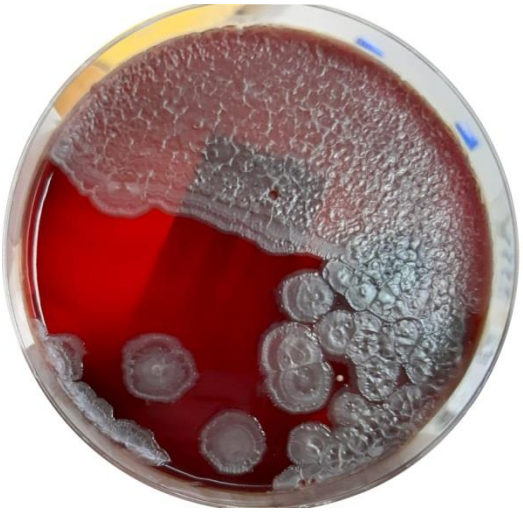
Results

3.1 Samples characteristics

In this study, a total of 163 bacterial culture samples were collected from clinical laboratories of five major hospitals in the West Bank; BASR clinical lab/ Beit Jala, Beit Jala Governmental Hospital, Al-Istishari Al Arabi Hospital/ Ramallah, Al-Ahli Hospital/ Hebron and Al-Makased Islamic Hospital/ Jerusalem.

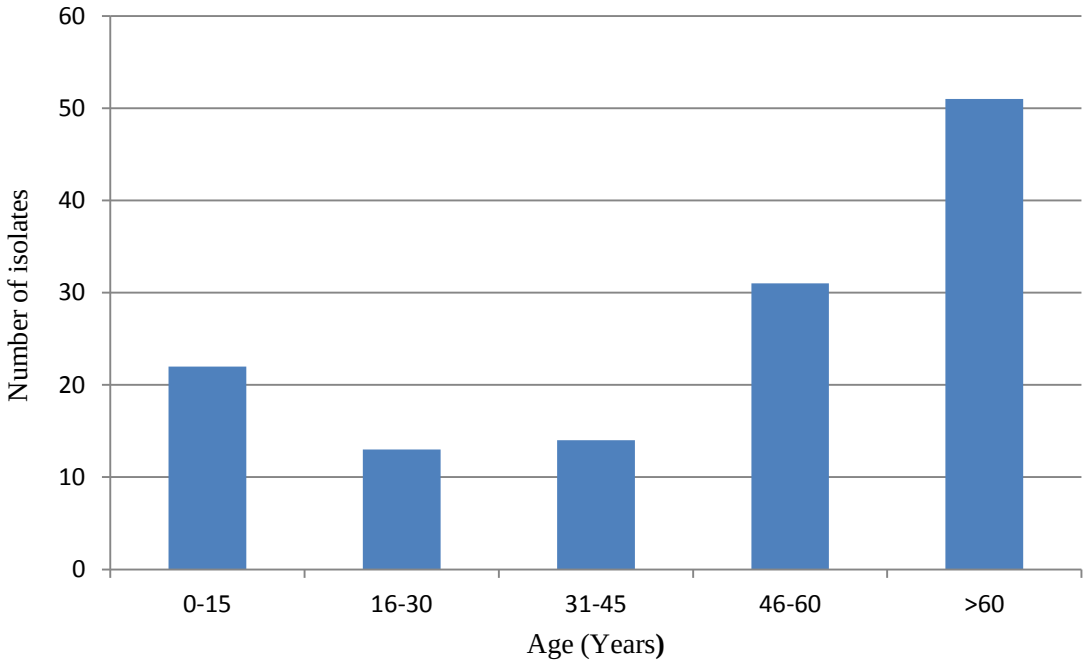
3.2 Identification of strains and demographic characteristics

All of the 163 samples were identified based on colony morphology (Fig.3.1) and further confirmed by biochemical tests. Alive and positive isolates for catalase, oxidase, citrate utilization, nitrate reduction and oxidative using oxidative/fermentative medium test were included in the study (n=131), whereas the rest of the samples were excluded (n=32) because either they were oxidase negative or could not be revived after storage. Furthermore, it was noticed that 79 of the isolates (60.3%) were pigment producers while 52 (39.7%) were non-pigment producers.



(Fig.3.1): *P. aeruginosa* colony morphology.

Eighty two (82) isolates (62.6%) were from males whereas 49 isolates (37.4%) were from females. Most of the isolates were in the age group of > 60 years followed by 46-60 years and 0-15 years as shown in Fig.3.2.



(Fig.3.2): Distribution of *P. aeruginosa* isolates according to age groups

The majority of the isolates were collected from urine (33 isolates: 25.2%) followed by sputum and wound (27 isolates each: 20.6%) as shown in Table 3.1.

(Table 3.1): The percentage of *P. aeruginosa* isolates in different clinical samples.

Source	%	Source	%	Source	%	Source	%
Sputum	20.6%	Blood	3.82%	Pus	1.53%	Tracheostomy	0.76%
Urine	25.2%	Swab	1.53%	Drain	1.53%	Vagina	0.76%
Wound	20.6%	Cornea	0.76%	Tissue	3.82%	Eye	0.76%
Ear	5.34%	CVP Tip	1.53%	BAL	2.30%	Catheter	0.76%
Bed sore	0.76%	Body fluid	2.30%	Trap	4.58%	Stool	0.76%

Isolates were obtained both from outpatients (12 isolates: 9.16%) and patients admitted to the hospitals (119 isolates: 90.8%). Table 3.2 shows the distribution of *P. aeruginosa* isolates taken from inpatients in the different hospitals wards. Isolates collected from patients in the surgical ward were the majority (30 isolates: 22.9%) followed by those taken from the medical ward (20 isolates: 15.26%), ICU (19 isolates: 14.5%) and rehabilitation ward (12 isolates: 9.16%).

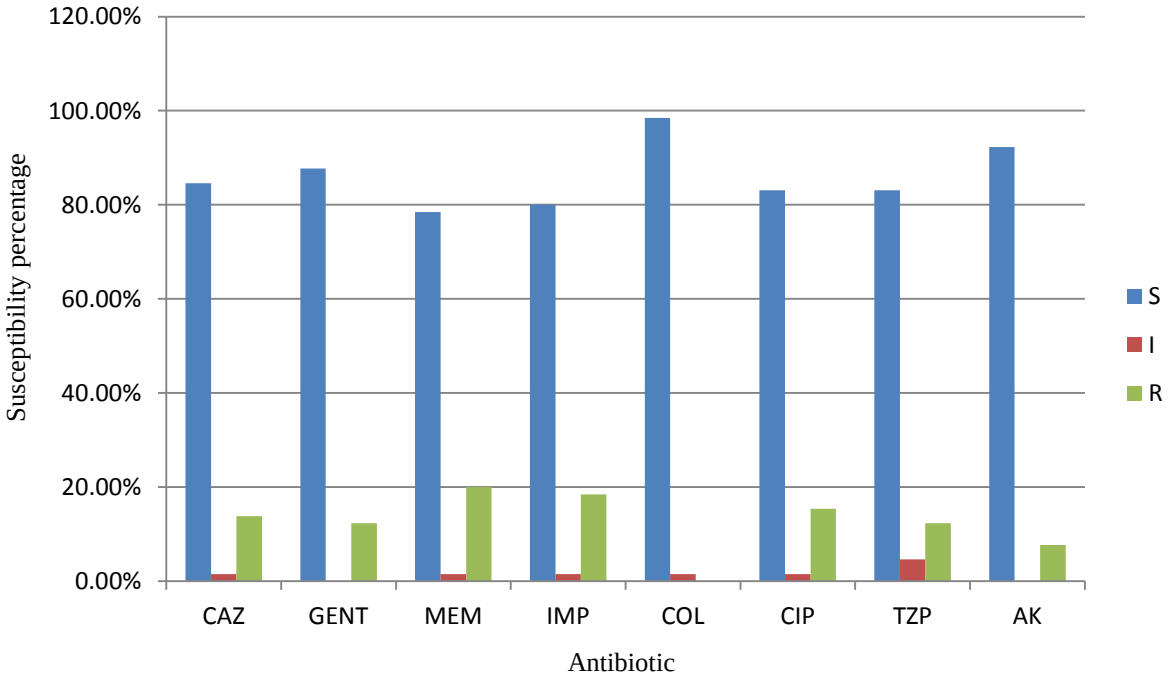
(Table 3.2): The percentage of *P. aeruginosa* isolates in the different hospitals wards.

Ward	%	Ward	%	Ward	%
Emergency	1.5 %	Oncology	2.29 %	Pediatric	3.8 %
ICU	14.5 %	Surgical	22.9 %	Neonate	2.29 %
Rehabilitation	9.16 %	Medical	15.26 %	Orthopedic	8.4 %
COVID-19	3.8 %	Admission	0.76 %	Day Care	0.76 %
Cardiac Care Unit (CCU)	3.8%	Pediatric cardiac	0.76 %	Obstetrics & Gynecology	0.76 %

3.3 Antimicrobial susceptibility testing of *P. aeruginosa* isolates.

Antimicrobial susceptibility information was available for 65 isolates out of the 131 for eight commonly used antibiotics i.e. Ceftazidime (CAZ), Gentamicin (GENT), Meropenem (MEM), Imipenem (IMP), Colistin-Sulphate (COL), Ciprofloxacin (CIP), Piperacillin/Tazobactam (TZP) and Amikacin (AK).

COL was the most effective antibiotic against *P. aeruginosa* isolates (98.46%). AK and GENT showed high susceptibility 92.3% and 87.7% respectively. Further, the susceptibility pattern against CAZ (84.61%), CIP (83.07%), TZP (83.07), IMP (80.0%) and MEM (78.46%) was also observed in this study as shown in Fig.3.3. Further, 44 out of 65 isolates (67.7%) were pigment producers while 21 isolates (32.3%) were non-pigment producers.

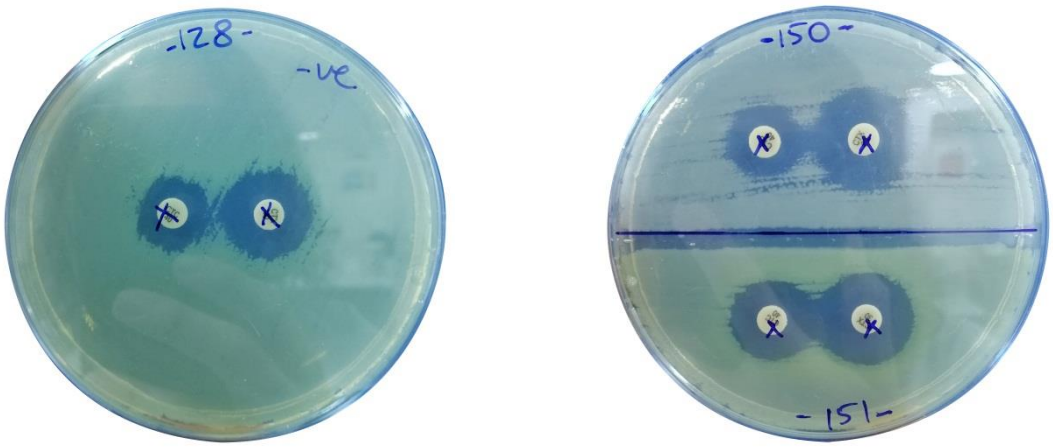


(Fig.3.3): Antimicrobial susceptibility of *P. aeruginosa* isolates.
S: Sensitive, I: Intermediate, R: Resistant

Eleven out of 65 (16.9%) *P. aeruginosa* isolates were found to be multi-drug resistant (MDR), i.e. showed intermediate or full resistance to at least three different classes of antimicrobial agents. The maximum number of MDR strains was isolated from wound samples compared to other samples. Further, both females and males had the highest MDR strains in the case of wound samples (n=3). Out of the 11 MDR isolates, however, pigment production was noticed in 4 isolates (36.36%) whereas no pigmentation was observed in the other 7 isolates (63.63%).

3.4 Phenotypic detection of ESBLs

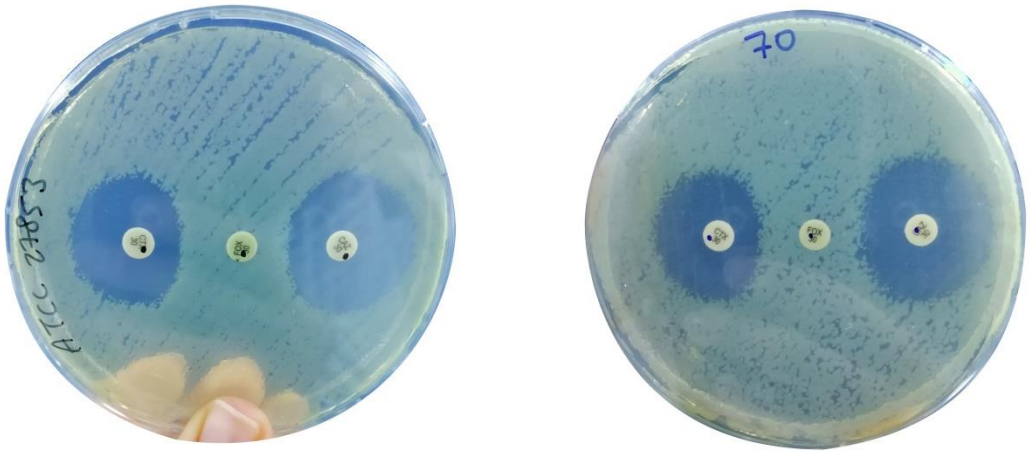
All isolates in addition to the control *P. aeruginosa* ATCC strains 27853 and 9027 were non-ESBL producers by the DDST phenotypic test as the zone of inhibition of the clavulanate containing disks was smaller compared with the zone of diameter of the disk containing cephalosporin (ceftazidime or cefotaxime) alone as shown in Fig.3.4.



(Fig.3.4): Phenotypic detection of ESBLs by DDST.

3.5 AmpC screening

Screening of AmpC using FOX-30, CAZ-30 and CTX-30 disks was positive for all isolates as well as for *P. aeruginosa* ATCC 27853 as shown in Fig.3.5.

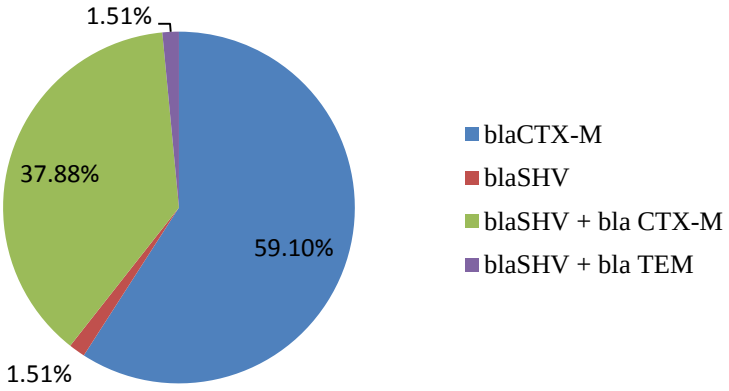


(Fig.3.5): AmpC positive *P. aeruginosa* isolates.

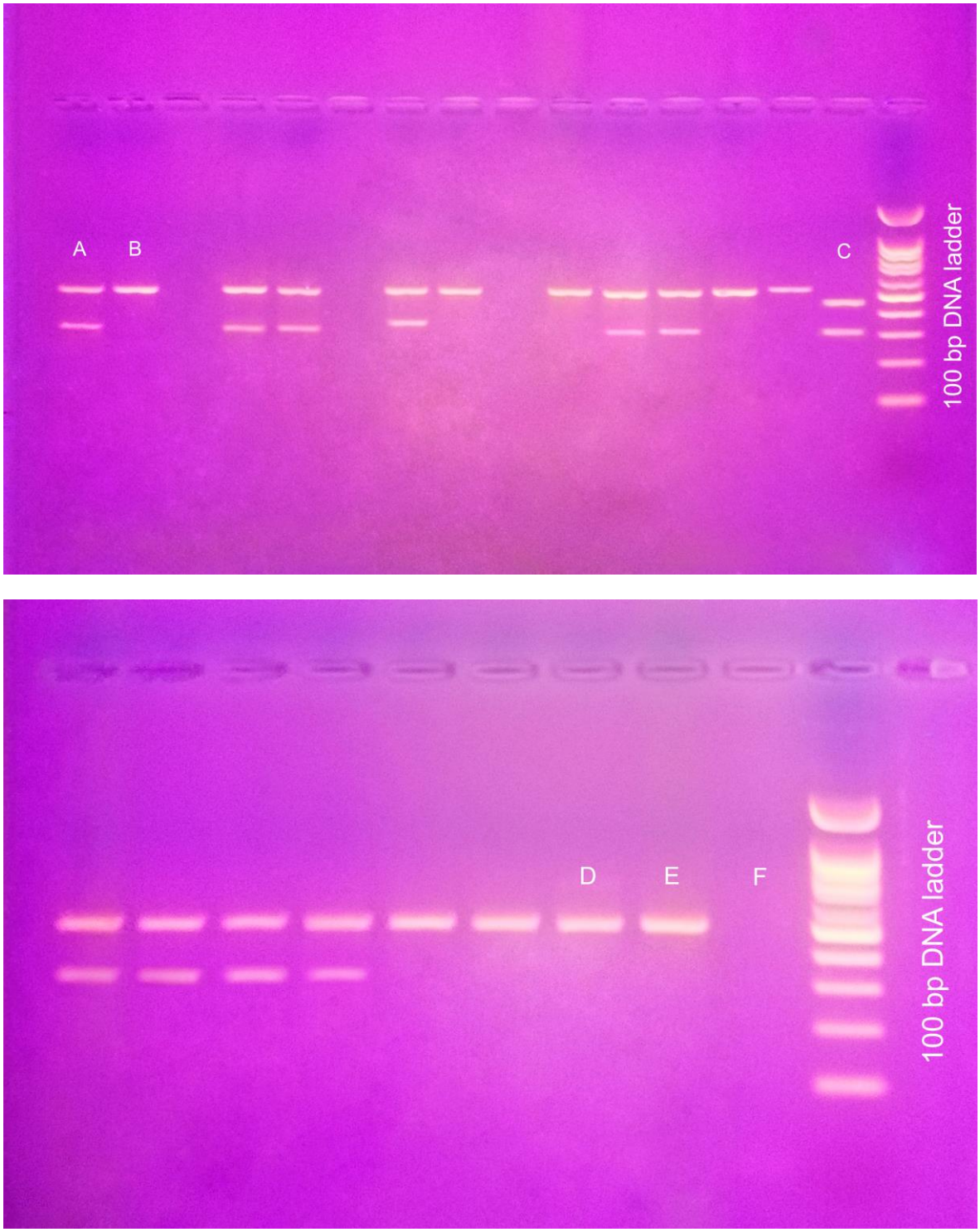
3.6 Genotypic detection of ESBLs by multiplex PCR

Sixty six *P. aeruginosa* isolates (50.38%) which were non-ESBL producers phenotypically were PCR positive for Ambler class A, whereas 65 isolates (49.6 %) were PCR negative for β- lactamase genes tested.

On the one hand, PCR results revealed amplification of 588 bp fragment corresponding to *bla*CTX-M in 39 isolates (59.1%) whereas, on the other hand, the 392 bp fragment corresponding to *bla*SHV was detected alone only in one isolate (1.51%). However, the 465 bp fragment corresponding to *bla*TEM was not detected alone in any of the isolates but in 1 isolate (1.51%) with the *bla*SHV gene. The co-occurrence of both *bla*SHV and *bla*CTX-M genes was detected in 25 isolates (37.88%) as shown in Fig.3.6. None of the ESBL positive isolates carried all the three genes together, also none carried *bla*CTX-M and *bla*TEM genes together. Fig.3.7 illustrates the occurrence of genes detected using agarose gel electrophoresis.



(Fig.3.6): Distribution of β- lactamase genes in ESBL positive isolates as detected by PCR.



(Fig.3.7): Ethidium bromide-stained agarose gel of a multiplex PCR assay of *P. aeruginosa* strains.

Lane A: *bla*CTX-M: 588 bp & *bla*SHV: 392 bp

Lane B: *bla*CTX-M

Lane C: *bla*TEM: 465 bp & *bla*SHV

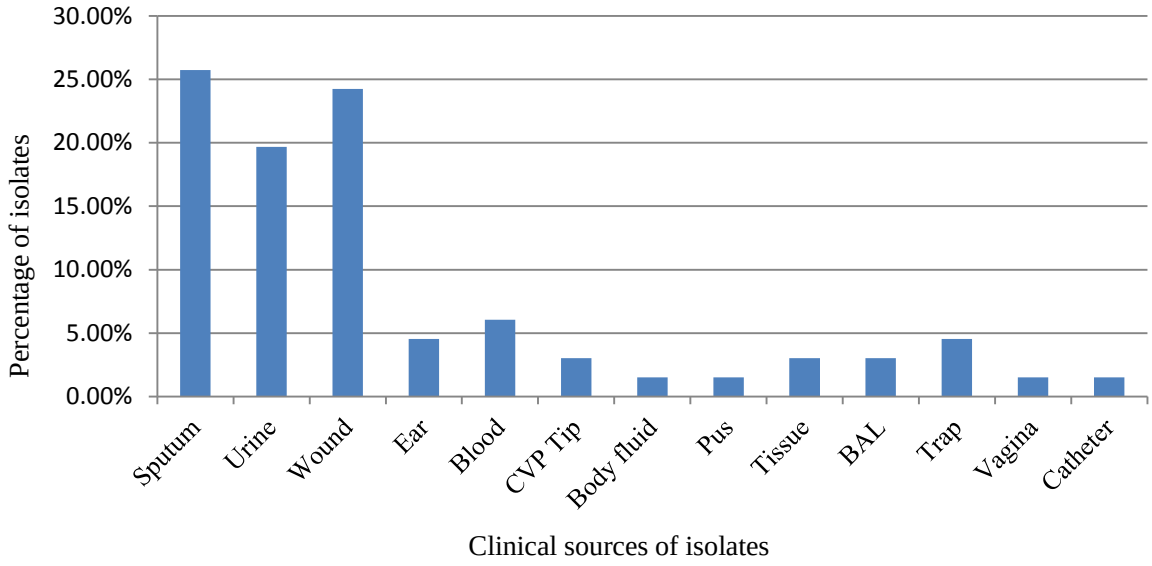
Lane D: *P. aeruginosa* ATCC 27853

Lane E: *P. aeruginosa* ATCC 9027

Lane F: Negative control

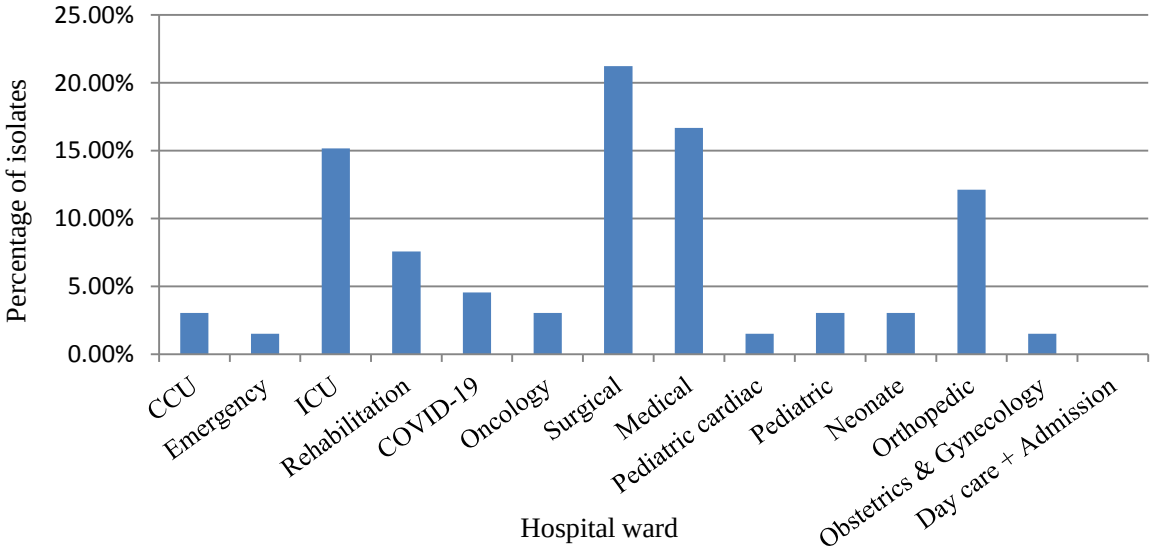
3.7 Distribution of *P. aeruginosa* ESBL-producing isolates according to clinical specimens

P. aeruginosa ESBL-producing isolates were mainly recovered from sputum (17 isolates out of 66: 25.75%) followed by wound (16 isolates out of 66: 24.24%) and urine (13 isolates out of 66: 19.69%) samples (Fig.3.8).



(Fig.3.8): Distribution of ESBL positive isolates obtained from various clinical samples

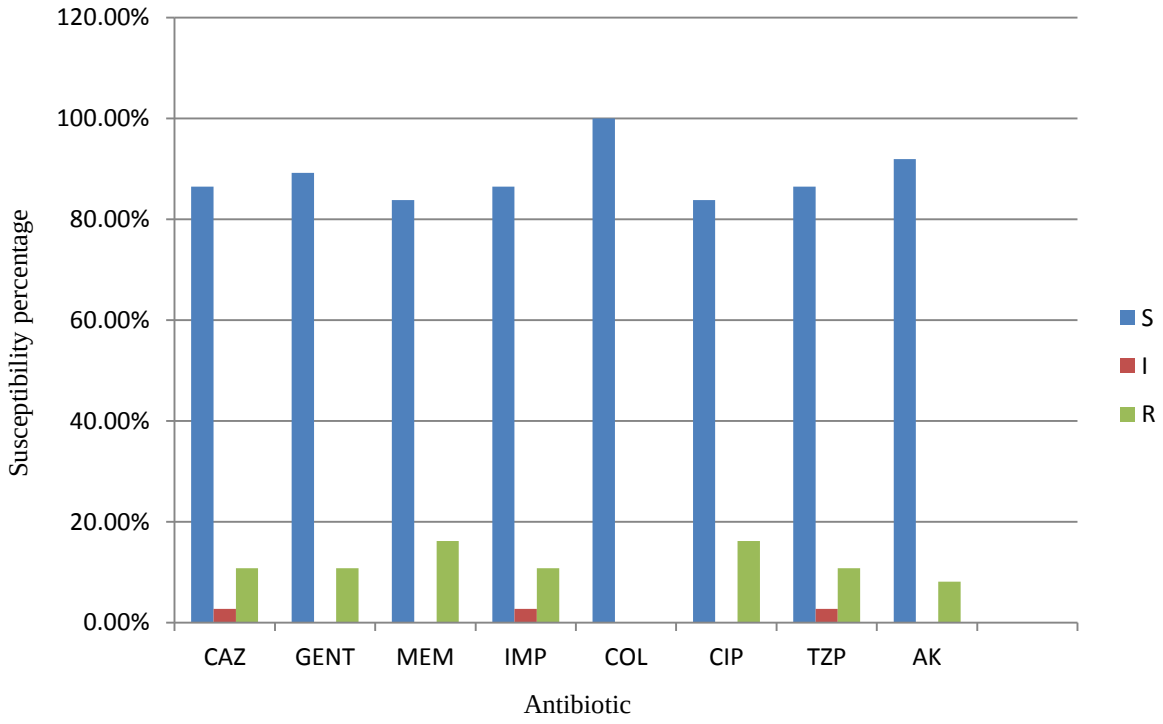
Higher number of ESBL-producing isolates was obtained from inpatients (62 out of 119 isolates: 52.1%) in comparison to outpatients (4 out of 12 isolates: 33.33%). The patients affected were mainly those admitted to the surgical ward (14 isolates out of 66: 21.21%), medical ward (11 isolates out of 66: 16.66%) and ICU wards (10 isolates out of 66: 15.15%) respectively (Fig.3.9).



(Fig.3.9): Distribution of ESBL positive isolates in different wards of hospitals

3.8 Antimicrobial susceptibility testing of ESBL-producing isolates

Out of the 65 isolates which antimicrobial susceptibility information was available, 37 isolates were ESBL positive. *P. aeruginosa* ESBL-producing isolates showed the highest susceptibility against COL (100%) followed by AK (91.9%) and GENT (89.18%). CAZ, IMP and TZP susceptibility was 86.48%. Further, susceptibility of both CIP and MEM was 83.78% as Fig.3.10 illustrates.



(Fig.3.10): Antimicrobial susceptibility of ESBL- producing *P. aeruginosa* isolates.

Chapter Four

4.1 Discussion

P. aeruginosa has been reported as a leading hospital acquired pathogen causing severe diseases (Rossolini & Mantengoli, 2005). Further ESBL enzymes are not uncommon in *P. aeruginosa* isolates (Lin et al., 2012). Although the property of resistance in ESBL-producing organisms has limited antibiotic options (Abbas et al., 2018), carbapenems are the treatment of choice for these infections caused by gram negative rods (Zafer et al., 2014).

There are few reports on the prevalence of ESBL in *Pseudomonas* isolates in Palestine and so this study was conducted to detect the prevalence of ESBL production of 131 isolates of *P. aeruginosa* from various clinical samples of different wards of different hospitals.

In the present study, it was observed that 62.6% of *P. aeruginosa* isolates were from males whereas 37.4% were from females which is in agreement to previous studies which reported that the incidence is higher in males than in females (Khan, Iqbal, Rahman, Farzana, & Khan, 2008; Kothari, Kumar, Omar, & Kiran, 2020). On the other hand, in this study, *P. aeruginosa* isolates were most commonly found in the age group of > 60 years which is in concordance with other studies which also reported that *P. aeurginosa* infection was more common in higher age groups (Kothari et al., 2020). It has been observed that age plays a major role in patient's susceptibility to infection by *Pseudomonas* since the highest number of patients was in the age group of above 60, followed by 46-60 then 0-15 years.

The highest percentage of *P. aeruginosa* isolates was observed in urine (25.2%), sputum and wound samples (20.6%). These results are different than the findings of others where the prevalence of *P. aeruginosa* was the highest in pus and urine (Khan et al., 2008; Syed, Thakur, Shafiq, & Sheikh, 2007) as well as pus and sputum (Kothari et al., 2020).

In the present study, samples obtained from in-patients were higher compared to those from out-patients which is similar to a previously published study (Negi, K, Bala, & Sharma, 2021) but unlike another where *Pseudomonas* isolation was more common in outpatients rather than in-patients samples (Kothari et al., 2020). *P. aeruginosa* isolates were commonly found in surgical wards (22.9%) whereas in the study conducted by Chen *et al*. in China, they depicted a much higher rate of *P. aeruginosa* isolates in the surgical ward (64.7%) (Chen et al., 2015). However, Khan *et al.* reported the highest rate of *P. aeruginosa* infection in orthopedic (24.61%) and OP Department (20%) (Khan et al., 2008).

The highest sensitivity (98.46%) was seen in COL which is in agreement with two previous studies where 0% resistance to this antibiotic was reported (Kothari et al., 2020; Negi et al., 2021). However, these results are in contrary to Anvarinejad *et al.* where CAZ was the most effective antibiotic and many isolates were resistant to all the other used antibiotics (Anvarinejad et al., 2014) and to another study where IMP and MEM were the most effective against *P. aeruginosa* and all isolates were resistant to many other antimicrobials (Mirsalehian et al., 2010).

P. aeruginosa isolates showed high susceptibility against AK (92.3%) which is in line with previous studies (Khan et al., 2008; Peymani, Naserpour-Farivar, Zare, & Azarhoosh,

2017), Unlike Khan *et al.* where resistance patterns against GENT, TZP, and CAZ were 67.8%, 35.1% and 30.2% respectively (Khan et al., 2008), in our study, antimicrobial tests showed that *P. aeruginosa* isolates were 87.7%, 83.07% and 84.61% susceptible to GENT, TZP, and CAZ respectively.

In a study carried out in Iran, it was revealed that all of the isolates were susceptible to CIP, MEM and IMP (Fazeli, Sadighian, Esfahani, & Pourmand, 2012). However, the present study results showed lower but still high susceptibility against CIP (83.07%), MEM (78.46%) and IMP (80.0%). On the other hand, the same study reported that CAZ resistance was 83.3% which is much higher than the present study where only 13.8% of *P. aeruginosa* isolates were CAZ resistant.

Around 16.9% of *P. aeruginosa* isolates were identified as MDR. These results are in a good agreement with a precedent Iranian study where 16.7% of isolates were MDR (Fazeli et al., 2012). Obviously, the results of these two studies are much lower than what was reported before about strains isolated from burn patients where 87.05% of the tested isolates were MDR (Mirsalehian et al., 2010). Further, in concordance to what was stated by Finalayson *et al.* our results showed that non-pigment-producing *P. aeruginosa* isolates were more MDR in comparison to pigment producers (Finlayson & Brown, 2011).

The present study showed that the phenotypic DDST method was unreliable compared to the molecular technique for ESBL detection in *P. aeruginosa* as all the isolates including the control *P. aeruginosa* ATCC 27853 strain showed AmpC production. The reduced level of susceptibility to the cephalosporin, cefotaxime, may be due to isolates containing AmpC β-lactamases since clavulanic acid ability to induce AmpC production may interfere with ESBL detection (Stürenburg & Mack, 2003). The finding of the present study is similar to a previous finding in which all isolates were having the AmpC gene. But in that study, molecular techniques were used for gene detection (Fazeli et al., 2012). However, recently, the co-existence of ESBLs and AmpC among species is obvious and, regarding this, it was explained that this could be due to the intense use of non-β-lactam antibiotics (Stürenburg & Mack, 2003).

The obtained result of ESBL production in this study was supported by a previous study where approximately 53% of phenotypically non-ESBL isolates were PCR positive for the targeted genes and 47% were PCR negative (Bajpai et al., 2017). Furthermore, a lower number of ESBL producers was reported phenotypically by an Egyptian study compared to the number reported using PCR (Zafer et al., 2014). Regarding these false negative results, researchers explained that this can be due to the presence of combined resistance mechanisms such as impermeability and efflux system (Lin et al., 2012; Peymani et al., 2017) as well as the overexpression of AmpC genes which may obstruct or even conceal ESBL detection (Laudy et al., 2017). Further, others added that there is a need for an improved method for the detection of ESBL-producing isolates in order to find those hidden genes (Bajpai et al., 2017).

The prevalence rate of ESBL in this study is much higher than the rate reported by two studies from burn centers in Iran (39.41% and 34%) (Mirsalehian et al., 2010; Shakibaie, Shahcheraghi, Hashemi, & Adeli, 2008) respectively. Further, studies from other countries reported a much lower prevalence rates (2.2%-7.4%) (Glupczynski et al., 2010; Woodford et al., 2008; Zafer et al., 2014). However, in comparison to this study, higher rates were reported by studies in Taiwan and China (61.4% and 87.5% respectively) (Chen et al.,

2015; Lin et al., 2012). This variability could be related to regional variations of the studies and the extent of strictness in implementing rules and regulation on the use of antibiotics.

In our study many isolates harbored more than one ESBL gene which is in line with an earlier study where more than one β-lactamase gene within the same isolate was observed in addition to the absence of the co-occurrence of the three genes together in one isolate (Bajpai et al., 2017). The occurrence of *bla*TEM with *bla*SHV gene was 1.51% but 0% alone in the current study just as a previous study carried out in Baghdad where no isolate had the *bla*TEM gene (Al Marjani et al., 2013). This, however, is unlike the study of *P. aeruginosa* isolates taken from burnt patients at Shafa-Hospital in Kerman, Iran (2.5%) (Shakibaie et al., 2008) and much lower than other previous studies in which the reported percentages were 48.7%, 92.3% and 39.2% (Bajpai et al., 2017; Lin et al., 2012; Rafiee et al., 2014) respectively.

*bla*SHV, *bla*CTX-M genes were not observed in a Brazilian study in 2013 (Zaranza et al., 2013) as well as in an Iranian study (Rafiee et al., 2014) and this was supported by the study of Weldhagen *et al.* where it was reported that these genes are rarely reported in *Pseudomonas* (Weldhagen, Poirel, & Nordmann, 2003). In the current study, however, *bla*CTX-M was the most prevalent gene (59.1%). In a study carried out in Iran on 75 ESBL-positive isolates of *P. aeruginosa*, the *bla*TEM (26.7%) was the most common gene, followed by *bla*CTX-M (17.3%), and *bla*SHV (6.7%), either alone or in combination (Peymani et al., 2017). Further, in the present study, *bla*CTX-M was found with the *bla*SHV gene in a percentage of 37.88% whereas in an Indian study the co-occurrence of these two genes together was not reported (Bajpai et al., 2017). The different prevalence rates of these genes among studies can be attributed to the geographical region variation as expressed by researchers previously (Bajpai et al., 2017; Weldhagen et al., 2003).

ESBL producers were more common in in-patients (52.1%) versus those isolated from out-patients (33.33%). This is in line with studies conducted by Kothari *et al.* (22% and 20% respectively) and Anupurba *et al.* in India (Anupurba, Bhattacharjee, Garg, & Sen, 2006; Kothari et al., 2020). This could be due to the fact that hospital environment harbor reservoir for infection and multidrug resistant pathogens.

The frequency of ESBL-producing isolates was highest in sputum samples followed by wound and urine samples and this is in harmony with earlier studies (Aggarwal, Chaudhary, & Bala, 2008; Shaikh et al., 2015; Shakibaie et al., 2008), but different than what was reported by Negi *et al.* where the distribution of ESBL-producing strains was the highest in pus samples followed by urine and catheter tip samples (Negi et al., 2021). Unlike this study, where ESBL producers were most commonly isolated from the surgical ward (21.21%) and those isolated from the ICU ranked the third position (15.15%), previous studies in Iran indicated that ESBL producers were most commonly isolated from the ICUs (Peymani et al., 2017; Ramazanzadeh, Chitsaz, & Bahmani, 2009). However, this study finding can be explained by the need for a long stay in hospitals post-operations thus colonization and subsequent infection occur.

In the current study, the highest rate of susceptibility in ESBL-producing isolates was for COL (100%) which is in concordance with what was reported by a study in Iran (Amirkamali, Naserpour-Farivar, Azarhoosh, & Peymani, 2017). However, although AK showed 100% susceptibility in ESBL-producing isolates of a former study in Nigeria (Okesola & Oni, 2012), in our study, a lower but still high susceptibility percentage was observed (91.9%). 89.18%, 86.48% and 83.78% of ESBL-producing *P. aeruginosa* isolates

were susceptible to GENT, CAZ and CIP respectively and these percentages are higher than what was reported previously (Okesola & Oni, 2012).

Former studies (Okesola & Oni, 2012; Shaikh et al., 2015) reported 100% susceptibility of ESBL-producing isolates to MEM and IMP. Meanwhile, in the present study, susceptibility to these both antibiotics was 83.78% and 86.48% respectively. Otherwise, a previous Palestinian study that took place in Nablus illustrated that 84% and 81% of MBL-producing isolates were resistant to IMP and MEM respectively (Adwan et al., 2016). The difference between all studies can be because vulnerabilities often differ between communities and even hospitals in the same community.

4.2 Conclusion and limitations

Our study showed high prevalence of ESBL-producing *P. aeruginosa* isolates carrying *bla*CTX-M followed by *bla*SHV in Palestine. The high prevalence of ESBL within hospitals in Palestine is considered a public health concern. DDST failed to detect ESBL producing *P. aeruginosa* isolates although it was inexpensive and easy to perform. The phenotypic methods recommended for enterobacteriacea are not always useful for *Pseudomonas* isolates. Multiplex PCR assay was very reliable and gave excellent results although it is expensive and not routinely performed in the clinical microbiology laboratories. COL, AK and GENT were proven to be the most effective treatments against infections caused by ESBL-producing *P. aeruginosa* isolates.

4.3 Recommendations

Modified phenotypic DDST method by incorporating other ESBL inhibitors such as tazobactum or boronic acid could be used. Timely detection of ESBLs is important to control their spread, prevent hospital outbreaks and optimize therapy. Constant monitoring of antimicrobial resistance and strict adherence towards infection control practices are the best protection against continuous spread of drug resistant *P. aeruginosa*.

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