Detection and molecular characterization of multi-drug resistant nonfermentative Gram-negative bacteria from clinical isolates

MD. NAZRUL ISLAM

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Department of Microbiology
University of Dhaka
Dhaka 1000, Bangladesh

Detection and molecular characterization of multi-drug resistant nonfermentative Gram-negative bacteria from clinical isolates

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Submitted by

Md. Nazrul Islam

Registration No. 195 (Session: 2014-2015)

Department of Microbiology
University of Dhaka
Dhaka 1000, Bangladesh

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CERTIFICATE

This is certified that the thesis entitled **"Detection and molecular characterization of multi-drug resistant nonfermentative Gram-negative bacteria from clinical isolates"** was carried out by Md. Nazrul Islam, Registration N0. 195 (Session 2014-2015) for the fulfillment of the degree of Doctor of Philosophy from the Department of Microbiology, University of Dhaka, Bangladesh.

This research work was carried out under our direct supervision and the style and contents of the thesis have been approved and recommended for the award of PhD degree.

Dr. Mahmuda Yasmin
Professor and Supervisor
Department of Microbiology
University of Dhaka, Bangladesh

Dr. Chowdhury Rafiqul Ahsan
Professor and Co-supervisor
Department of Microbiology
University of Dhaka, Bangladesh

Dr. Anowar Hossain
Senior Scientist and Co-supervisor
Laboratory Sciences Division
ICDDR,B; Mohakhali
Dhaka, Bangladesh

Dedicated

To my beloved parents

Ayesha Begum and *Osman Ali*

And

To my beloved children

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Introduction

Materials and Methods

Results

Discussion

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Appendixes

Abstract

The increasing rate of carbapenem resistant nonfermentative Gram-negative bacteria is a serious global health threat. Almost all studied isolates of *Pseudomonas aeruginosa* and *Acinetobacter baumannii* showed resistance to carbapenem. Carbapenem is the last option for treating Multi-drug resistant (MDR) *P. aeruginosa* and *A. Baumannii* but resistance against this drug has been emerging. Two types of β-lactamases differentiated by their mode of target hydrolysis, serine β-lactamase and metallo-β-lactamase (MBL). These are major foci of the present study in nonfermentative Gram-negative isolates of *P. aeruginosa* and *A. baumannii.* When studying various virulence properties, a majority of both *P. aeruginosa* and *A. baumannii* isolates showed hemolytic activity. However, formation of capsule, invasiveness and cytotoxic activity were observed only in the case of *A. baumannii*. Among the 100 *P. aeruginosa* and *A. baumannii* isolates tested, 92% and 35% showed MBL positive, respectively. Verona Integron-encoded metallo-β-lactamase (VIM) is an important MBL which confers resistance to regular using antimicrobials and now a days carbapenems became resistant due to this enzyme. We obtained a total of 16 VIM encoded MBLs from 100 *P. aeruginosa* isolates, but none from a similar number of *A. baumannii* strains. The sequences of VIM gene from 16 strains together with 22 other similar sequences, retrieved from the NCBI GenBank database were used to construct the phylogenetic tree to understand the nearest neighbour of the study sequences. Genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree. For VIM type of MBL, *P. aeruginosa* phylogenetic analysis showed similarities with the strains of India, Thailand, Nepal, Egypt, Turkey, UK, USA and Tunisia (93-100%). These findings suggest that the bla_{VIM} gene is similar to global circulating strains. From Bootstrap table it has been found that 90-100% nucleotide similarities exist among the 16 Bangladeshi strains. Comparable results of nucleotide and amino acid sequences of VIM showed differences with a reference strain retrieved from the NCBI for 4 Bangladeshi *P. aeruginosa* strains and among the changes, three were in important positions (72, 145 and 146) as these changes brought significant modification in amino acid sequences from polar/nonpolar to nonpolar/polar that might affect the enzymatic nature of VIM. We obtained 6 New Delhi Metallo-β-lactamase-1(NDM-1) positive isolates from 100 *A. baumannii* strains, but none from the same number of *P. aeruginosa* strains. Among these 6 $bla_{\text{NDM-1}}$ genes, 5 sequences together with 17 other similar sequences retrieved from the NCBI GenBank database were used to construct the phylogenetic tree to understand the nearest neighbor of the study sequences. Genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree. Amongst the DNA sequences obtained in our study, the isolates MN226842, MN226843, MN226844 and MN226846 formed distinct lineages with the strains from India, while only isolate MN226845 shared similarity and was found to be closely related to the sequences with Iran, Egypt and Korea. From these findings, we can predict that the NDM-1 may have been transmitted in Bangladesh from India. Bootstrap table shows 100% nucleotide similarities among the 5 Bangladeshi strains. On the other hand, 10 strains from China showed 52% similarity. Comparable results of NDM-1 of *A. baumannii* showed no differences in nucleotide and amino acid sequences with a reference strain retrieved from the NCBI which suggest that these are clonal. The study reports on the presence of bla_{VIM} and $bla_{\text{NDM-1}}$ gene in nonfermentative Gram-negative bacteria in Bangladesh for the first time.

Abbreviation

μL	Micro liter
AMR	Antimicrobial resistance
bp	Base pair
CNS	Central nervous system
DNA	Deoxyribo nucleic acid
EDDS	EDTA double disc synergy
ESBL	Extended spectrum beta-lactamase
GIT	Gastrointestinal tract
HGT	Horizontal gene transfer
hrs	Hours
icddr,b	International Centre for Diarroeal Disease Research, Bangladesh
ICU	Intensive care unit
IMP	Imipenemase
In	Intregon
IS	Insertion sequence
IS*CR*	Insertion sequence common region
KPC	*Klebshella pneumoniae* carbapinamae
LIMS	Laboratory Information Management System
LPS	Lipopolysaccharide
MBL	Metallo-beta-lactamase
MDR	Multi-drug resistant
MEM	Meropenem
MGE	Mobile genetic elements
MHT	Modified Hodge Test
MIC	Minimum inhibitory concentration
Min	Minutes
mL	Milliliter
MRSA	Methicillin resistant *Staphylococcus aureus*
NCBI	National Center for Biotechnology Information
NDM-1	New Delhi Metallo-β-lactamase-1
NFGNB	Nonfermentative Gram-negative bacteria
OM	Outer membrane
OMP	Outer membrane protein
OXA	Oxacillinase
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
QS	Quorum sensing
RNA	Ribonucleic acid
RND	Resistance-nodulation division
Tn	Transposon
UTI	Urinary tract infection
VAP	Ventilator-acquired pneumonia
VIM	Verona Integron-encoded metallo-β-lactamase
VRE	Vancomycin resistant enterococci
WHO	World Health Organization

1. Introduction

1.1 Nonfermentative Gram-negative bacteria

The term nonfermentative Gram-negative bacteria means all aerobic Gram-negative rods that can grow within 24 hrs on the surface of the non selective medium, but neither grow in nor acidify when inoculated below the surface of the medium (Koneman, *et. al.*, 1997). Nonfermentative Gram-negative bacteria can cause different type of infections and account for approximately 15% of all Gram-negative clinical isolates (Su *et al.*, 2009). Nonfermenters may differ in their pathogenic potential and transmissibility, and many are multi-drug resistant (MDR). For this reason, accurate identification of nonfermenters are important for appropriate patient management (Su *et al.*, 2009). Infections caused by nonfermenters may be endogenous or exogenous of origin, depending on several factors such as abuse of wide spectrum antimicrobial agents, use of immunosuppressant substance, prolong surgical wide spectrum antimicrobial agents, prolong surgical procedure and inadequate instruments (Frota and Moreira, 1998). In recent years due to liberal and empirical use of antimicrobial agents, nonfermentative Gram-negative bacteria are emerging as an important health care associated pathogen. They have been incriminated in infections such as septicemia, pneumonia, urinary tract infection (UTI) and surgical site infection.

Nonfermeters include different type of bacteria likely *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia*, *Moraxella catarrhalis*, *Sphingomonas paucimobilis*, *Achromobacter xylosoxidans* etc. Among them the most important and notorious clinical pathogens are *P. aeruginosa* and *A. baumannii* and they account for 90% of nonfermenters.

Broad-spectrum antibiotic resistance is worrisome in Gram-negative bacteria, and multi-drug resistant (MDR) bacteria are increasing day by day, particularly in nonfermenters, but there are no antimicrobial agents possessing suitable activity against *Pseudomonas* spp. or *Acinetobacter* spp. The increase in resistance has been further enhanced by the acquisition of metallo-β-lactamase (MBL) genes that can potentially confer broad-spectrum β-lactam resistance. These genes encode enzymes that can hydrolyse all classes of β-lactams and the activity of which cannot be neutralized by β-lactamase inhibitors such as sulbactam, clavulanate and tazobactam. MBL genes are often associated with aminoglycoside resistance genes and thus bacteria that possess MBL genes are often co-resistant to aminoglycosides, further compromising therapeutic regimes. These types of

genes can be found as gene cassettes carried by integrons that in turn are embedded within transposons/gene cassette providing a highly ambulatory genetic element. The dissemination of metallo-β-lactamase (MBL) genes is typified by the spread of bla_{VIM} believed to originate from a Portuguese patient in 1995, and is now present in over 20 counties.

Nonfermenting Gram-negative bacteria have emerged as important healthcare-associated pathogens. Accurate identification of all clinically significant nonfermenting Gram-negative bacteria is important to consider the intrinsic multi-drug resistance exhibited by these bacteria. Therefore, the present study was undertaken to identify *P. aeruginosa* and *A. baumannii* from patients attending at icddr,b diagnostic unit. The study was also done to assess their clinical significance, antimicrobial susceptibility pattern and molecular characterization.

1.2 The organism *P. aeruginosa* and *A. baumannii*

1.2.1 Characteristics of *P. aeruginosa*

P. aeruginosa is an aerobic Gram-negative rod with the remarkable adaptive capacity to stay alive and continue under a wide range of environmental situations. *P. aeruginosa* belongs to the *Pseudomonadaceae* family. Typical features of *P. aeruginosa* isolates include positive oxidase tests, the ability to be grown up to temperatures of 42°C and the ability to grow under anaerobic conditions by the presence of an alternative terminal electron acceptor such as nitrite or arginine, while utilizing more than eighty organic compounds as carbon and energy sources (Dworkin, 2006).

This is a glucose and lactose nonfermenting bacterium that commonly isolates from hospitalized patients and may be found in a variety of aqueous solutions, including disinfectants, soaps and eye drops. It can also be found in sinks, hot tubs, respiratory equipment, showerheads, and has the ability to produce the water-soluble pigments pyoverdine and pyocyanin, which together confer the bright green characteristic color of the organism when grown on *Pseudomonas* isolation agar (King, *et. al.*, 1954).

The bacterium can cause a variety of diseases and can be isolated from almost any type of specimen. Infections can be acquired from the community or during a stay in a healthcare facility. *P. aeruginosa* has been rare part of the microbial flora of healthy individuals, but may colonize the gastrointestinal tract of hospitalized patients, particularly those who have received previous antibiotic therapy (Dworkin, 2006). The colony morphology,

pigmentation of *P. aeruginosa* can be quite substantial heterogeneous. In a simple agar culture at 35-37°C, the morphological colony is smooth and large with an elevated center, giving it the appearance of a fried egg. As pointed out by Lau *et al*., 2004, pyocyanin is a compound with a redox-active effect and has multiple cytopathic influences on mammalian cells. *P. aeruginosa* has different forms of motility, which can be identified as swimming, twitching and swarming.

1.2.2 Characteristics of *A. baumannii*

The genus *Acinetobacter* underwent major taxonomic changes over the last 30 years. *A. baumannii* is the most significant species and becomes one of the most troublesome pathogens for health care institutions worldwide. Over the last 15 years, its clinical significance has been propelled by its notable ability to up-regulate or acquires resistance determinants. This characteristic has made it into one of the main organisms threatening the current antibiotic era (Peleg, *et. al.*, 2008).

Presumptively Acinetobacters can be identified as Gram-negative, catalase-positive, oxidase-negative, non-motile, nonfermenting, coccobacilli. *Acinetobacter* species of human origin grow well on solid media that are regularly used in clinical microbiology laboratories and hospitals, such as sheep blood agar or Tryptic Soy Agar, McConkey agar in 35-37°C.

Recently, reports of *A. baumannii*, which are resistant to all known antibiotics, have increased (Peleg *et. al.*, 2008). Moreover, its ability to survive for prolonged periods of time in hospital settings increases its capacity for nosocomial spread, in particular, to the immunocompromised patients. Hospital-acquired pneumonia is considered to be the most common infection caused by *A. baumannii*. However, more recently, infections involving the central nervous system, skin and soft tissue, and bone have emerged and becoming problematic for hospitals and health institutions (Peleg *et. al.*, 2008).

1.2.3 Infection caused by *P. aeruginosa*

P. aeruginosa can cause infection in almost any part of the body, although it does not typically cause infection in a healthy host. It is an opportunistic pathogen that causes respiratory illness, urinary tract infections, dermatitis, bacteremia, soft tissue infections and a variety of systemic infections. Outbreaks caused by this organism have been reported in diverse settings (Pirnay *et al*., 2003). It is the common cause of ear infection like external otitis and swimmer's ear, and also a cause of eye infections, including

keratitis and neonatal ophthalmia. *P. aeruginosa* can produce disease in any part of the gastrointestinal tract from the oropharynx to the rectum (Driscoll, *et al*., 2007). The majority of *P. aeruginosa* is acquired in hospitals and nursing homes, which accounts for 25% of all healthcare-associated Gram-negative bacteremia. Rarely, *P. aeruginosa* causes meningitis and brain abscesses due to invading to the central nervous system (CNS) from the paranasal sinus or inner ear.

P. aeruginosa colonization plays a main role within intensive care units (ICUs), and it has the capability to colonize inpatients, with ICUs clearly established as endemic settings (Erbay *et al*., 2003). Risk factors considerably related to the acquisition of this bacterium in ICUs include period of stay, mechanical ventilation (Talon *et al*., 1998), long-term use of antibiotics (Blanc, *et al*., 1998; Baddour *et al*., 1995; Carmeli, *et al*., 1999), alcoholism (Blanc *et al*., 1998; Talon *et al*., 1998) and the utilization of indwelling urinary catheters (Blanc *et al*., 1998). *P. aeruginosa* is infrequently found as a part of the human microflora of healthy individuals as this organism dies in the dry skin of healthy individuals (Bellais, *et al*., 1999). This organism is rarely responsible for community-acquired infections in healthy individuals. On the other hand, the incidence of *P. aeruginosa* associated infection is high in a hospital environment, particularly in immune compromised individuals, epithelium compromised cystic fibrosis patients, and individuals with severe burns, ulcerations and mechanical abrasions caused by catheterization. *P. aeruginosa* is the main cause of death in cystic fibrosis patients (Thuong *et al*., 2003). Infections caused by *P. aeruginosa* not only become increasingly resistant to one drug, as multi-drug resistance is also increasing and have been difficult to treat (Flamm *et al*., 2004). Due to increasing rates of antibiotic resistance in *P. aeruginosa*, the treatment of infections is still a serious medical challenge. Infection with MDR *P. aeruginosa* is associated with risk factors including severity of illness, invasive devices, a bedridden state, and in hospitalized patients, lead to increased length of stay and increased therapeutic costs as well as significant morbidity and mortality (Defez *et al*., 2004; Paramythiotou *et al*., 2004; Moore and Flaws, 2011b). *P. aeruginosa* has been a well known cause of infections for almost 130 years and will probably remain a leading cause of infections in humans for many years to come for various reasons (Moore and Flaws, 2011b). *P. aeruginosa* is ubiquitous in nature, increasing the probability of exposure, patients with chronic disease and immunosuppression for long periods are susceptible hosts for infection. In addition, *P.*

aeruginosa has many resistant mechanisms for antipseudomonal agents that are not always used judiciously and when overprescribed drive the development of resistance.

1.2.4 Infection caused by *A. baumannii*

A. baumannii infections prevalent in individuals with immunosuppression, prolonged hospitalization and critically ill patients in Intensive Care Units (ICUs) (Dijkshoorn, *et al*., 2007). Nosocomial infections caused by *A. baumannii* include pneumonia, bloodstream infections, skin and soft tissue infections, wound infections, urinary tract infections (UTI) and, rarely, gastrointestinal tract (GIT) infections (Zarrilli, *et al*., 2009).

Ventilator-acquired pneumonia (VAP) is the most commonly identified clinical manifestation of *A. baumannii* (Dijkshoorn *et al*., 2007; Peleg *et al*., 2008). This could be attributed to the colonization of airways by *A. baumannii* which soon develop into true pneumonia upon prolonged hospital admission and extensive administration of antibiotics. Bloodstream infections, particularly sepsis, are common in *A. baumannii* infections worldwide (Dijkshoorn *et al*., 2007; El-Mahallawy, *et al*., 2005; Munford, 2006). In the USA, *A. baumannii* was found as the 10th most common etiologic agent in nosocomial bloodstream infections (Peleg *et al*., 2008). *A. baumannii* bacteraemia can be secondary to pneumonia, and can also result from central venous line catheters, which act as a main route for dissemination of organisms into the blood stream (Simon *et al*., 2000).

Skin, soft-tissue and wound infections caused by *A. baumannii* is increasingly reported and highlight the importance of environmental contaminated infections. Most reports are from wounded military personnel, as well as in burn patients (Adams-Haduch *et al*., 2008; Hujer *et al*., 2006). *A. baumannii* infections have also been reported in causing urinary tract infections (UTI) associated with urinary catheters, as well as meningitis particularly with the presence of an external ventricular drain (Peleg *et al*., 2008).

There are certain risk factors that predispose patients to infection with *A. baumannii*. These risk factors can occur simultaneously or separately, but contribute significantly to the morbidity and mortality observed with *A. baumannii* infections. Those include surgery, trauma, underlying malignancy, previous administration of broad-spectrum antibiotics, previous admission to the ICU, exposure to contaminated equipment, mechanical ventilation, invasive procedures and presence of indwelling devices such as catheters (Falagas and Kopterides, 2006; Dijkshoorn *et al*., 2007; Giamarellou, *et al*., 2008).

1.2.5 Pathogenicity of *P. aeruginosa*

The pathogenicity of *P. aeruginosa* mainly depends on various virulence factors and genetic flexibility, enabling it to survive in various environments. Lung injury associated with *P. aeruginosa* infections result from both the direct destructive effects of the organism on the lung parenchyma, and the exuberant host immune responses. The factors that contribute to the pathogenesis of *P. aeruginosa* are based on the health status of the host. VAP patients are most at risk for pneumonia caused by *P. aeruginosa*. In order to cause any type of infection it must first enter the host and colonize. Entry is often through inhalation into the respiratory tract, but the organism is so ubiquitous that it is hard to tell exactly how the organism is acquired in all cases.

The virulence factors produced by *P. aeruginosa* are listed and summarized in Table 1.1 (Sadikot, *et al*., 2005; Driscoll *et al*., 2007). All of the virulence factors used by *P. aeruginosa* are also produced by other microorganisms except for pyocyanin, which is uniquely produced by *P. aeruginosa*. Many of these factors assist colonization, whereas others facilitate bacterial invasion. Several factors are required in bacterial colonization, including, pili or fimbriae, flagella and surface polysaccharides. Functionally, virulence factors are divided into two groups- factors that assist in the attachment of the organism to host cells, which are the fimbriae and flagella; and factors that support during the invasion of tissue and the inhibition of the immune response. These virulence factors include pili, and flagella, which play an initial role in motility and adhesion to the epithelium, as well as the lipopolysaccharide (LPS). These factors, then perform the main function in the irreversible adhesion to epithelial cells, which is the first significant step in colonization of the respiratory epithelium.

Table 1.1 Virulence factors of *P. aeruginosa* and their functions

Virulence Factor	Functions
Fimbriae	Attachment to host cells and activation of pro inflammatory gene expression
Polar flagella	Motility, attachment to host cells and activation of lnterleukin-8
Type III secretion system	Injects toxins (ExoS, ExoT, ExoU, ExoY) into host cells
ExoS	Stimulates tumor necrosis factor alpha production
ExoT	Activates GTPase
ExoU	Cytotoxin
ExoY	Adenylate cyclase activity
Quorum-sensing molecules	Coordinate expression of genes among other pseudomonal cells and promotes the formation of biofilms
Pyochelin and pyoverdin	Bind iron
Elastase, proteases, hemolysins and leukocidin	Aid in tissue invasion and lyse host cells
Pyocyanin	Inhibits lymphocyte proliferation and cilia function and produces reactive oxygen intermediates
Exotoxin A	Inhibits protein synthesis in host cells and helps organism disseminates
Lipopolysaccharide	Endotoxin
Alginate	Free radical scavenger; inhibits phagocytosis, neutrophils chemotaxis and activation of complement

P. aeruginosa produce pyocyanin (N-methyl1-hydroxyphenazine), the pigment that gives the blue-green color to the colonies. It has been found that pyocyanin may have various pathogenic effects such as increasing production IL-8, depressing host-response (Denning *et al*., 1998; Leidal, *et al*., 2001) and inducing apoptosis in neutrophils (Allen *et al*., 2005). In animal models of acute and chronic lung infection, pyocyanin was shown to be necessary for its virulence (Lau *et al*., 2004). In acute infections, invasion, dissemination and extensive tissue damage predominate. In chronic infections, primarily in cystic fibrosis patients, *P. aeruginosa* may also adjust, by losing its most immunogenic features such as flagella and pili to prevent clearance, and by isolating itself from host defenses and adhering to the respiratory epithelium by forming biofilms. A persistent inflammatory state is maintained by extracellular secreted virulence factors (Landsperger *et al*., 1994).

P. aeruginosa possesses a multiplicity of regulatory systems, allowing it to adapt to its environment and notably to host defenses (Mahenthiralingam, *et al*., 1994). Among these systems, quorum sensing (QS) displays *P. aeruginosa* adaptability. QS has been shown to be critical in maintaining airway inflammation through the production of virulence factor and to the formation of biofilm in chronic infections (Hentzer, *et al*., 2003).

1.2.6 Pathogenicity of *A. baumannii*

Limited information is available on the pathogenicity and virulence mechanisms of *A. baumannii*; however, colonization is important in *A. baumannii* than infection, which indicates a relatively low pathogenicity when compared with other Gram-negative organisms (Dijkshoorn *et al*., 2007). Sixteen genomic islands carrying putative virulence genes associated with cell-envelope and biogenesis, lipid metabolism, iron uptake and metabolism, quorum sensing and a type IV secretion system as have been identified (Dijkshoorn *et al*., 2007; Gordon and Wareham, 2010). The ability of *A. baumannii* to adhere to biotic and abiotic surfaces allows formation of biofilms, which are mediated by pili. Pili thereafter interacts with human epithelial cells and the production of lipopolysaccharide (LPS) promotes adherence to the host cells (Dijkshoorn *et al*., 2007; Gordon & Wareham, 2010). LPS also acts as an immunostimulatory factor leading to pro-inflammatory responses in *A. baumannii* infections (Dijkshoorn *et al*., 2007). However, the ability of *Acinetobacter* spp. to adhere to epithelial cells, to produce enzymes and toxins and to possess anti-phagocytic surface components is considered to be significant virulence mechanisms of this genus. Resistance to the bactericidal activity of the human serum is crucial for survival in the human host; in addition to quorum sensing and its role in biofilm formation. This consequently shows the ability of *A. baumannii* to survive and adapt to diverse environments, including the nutrient-limited conditions in a hospital setting and in the human body (Dijkshoorn *et al*., 2007). Iron uptake is an important mechanism in *A. baumannii* infections. The ability of *A. baumannii* to obtain and utilize iron has contributed to its ability to survive both in the host and in the environment, which is mediated by the secretion of a variety of molecules involved in iron acquisition, including the siderophore acinetobactin, and also the production of a haemin utilization system (Gordon and Wareham, 2010; Vallenet *et al*., 2008).

1.2.7 Treatment of diseases caused by *P. aeruginosa*

P. aeruginosa is often resistant to a wide range of antimicrobial agents, including fluoroquinolones, tetracycline, aminoglycoside, β-lactams, macrolides, rifampicin, cotrimoxazole (trimethoprim/sulfamethoxazole) and chloramphenicol. Several indigenous resistance mechanisms elucidate this pattern, such as active efflux pumps, low membrane permeability and production of β-lactamases (Hancock, 1998). *In vitro* susceptibility data are essential guides in the choice of a proper antibiotic for *P. aeruginosa* infections, due to the incidence and diverse acquired resistance found by clinical isolates. Antimicrobials that are typically considered effective toward *P. aeruginosa* can be generally classified into the following groups:

First are the penicillins, such as ticarcillin and piperacillin, β-lactam/β-lactamase inhibitor combinations including ticarcillin/clavulanic acid, and piperacillin/tazobactam (Lister, 2000).

Second are the cephalosporins, which include ceftazidime, cefoperazone and cefepime (Bush, 2002). From all third generation cephalosporins, ceftazidime is the only one active against *P. aeruginosa.* Among fourth generation cephalosporins, cefepime is the only one that is accepted in the United States for human use and has an extended range. Cefepime is active toward Gram-positive and Gram-negative bacteria, including *P. aeruginosa*. The fourth generation cephalosporins exhibit chemical properties that can result in increased resistance by Gram-negative microorganisms (Zuanazzi *et al*., 2010).

Third are the monobactams, this class of drugs is only represented by aztreonam (Jones, *et al*., 2002). Aztreonam is mainly used against Gram-negative aerobic microorganisms, as well as *P. aeruginosa* and *Klebsiella* spp. It is also recommended for use in urinary tract infections, soft-tissue infections, intra-abdominal and pelvic infections and pneumonia (Ennis and Cobbs, 1995).

Fourth are the carbapenems, which include ertapenem, doripenem, imipenem and meropenem. Meropenem is becoming more effective than imipenem with regard to resistance (Livermore, 2001). Extended infusions of meropenem (3 hrs) have been found in results in positive outcomes (Santos *et al.*, 2007).

Fifth are the fluoroquinolones, levofloxacin and ciprofloxacin. As judged by MIC, ciprofloxacin is more potent than levofloxacin. However, maximum doses of levofloxacin have superior pharmacokinetics, which lead to balance the decreased potency. Therefore,

these two fluoroquinolones are pharmacodynamically effective against *P. aeruginosa* (Tennenberg, *et al*., 2006).

Sixth are the aminoglycosides, which include amikacin, tobramycin, gentamicin, and netilmicin. In their *in vitro* study, found that aminoglycosides when combined with β-lactams has a synergistic role (Giamarellou *et al*., 1984).

Finally the polymyxin (colistin), which has lately re-appeared as an effective solution to treat MDR *P. aeruginosa*; (Giamarellou and Kanellakopoulou, 2008; Falagas, *et al*., 2005; Michalopoulos and Falagas, 2008; Kasiakou *et al*., 2005). The toxicity of this drug (nephrotoxicity, but also neuromuscular blockade and ototoxicity) has been much discussed, but has been reported to be controllable and lower than initially thought (Falagas *et al*., 2005). Primary experimental data recommends that colistin can be managed even in an aerosolized form to mechanically ventilated, non-cystic fibrosis patients with pseudomonal infections. Currently, colistin is becoming the foremost curative choice of strains with complicated MDR patterns (Falagas and Kasiakou, 2006; Kallel *et al*., 2007). Some confusion still exists concerning the different formulations used (Falagas and Kasiakou, 2006). Polymyxin B belongs to this group, while clinical knowledge of this drug is usually limited. It has been reported that Polymyxin B has been used as a rescue therapy in a series of patients with healthcare-associated pneumonia caused by MDR *P. aeruginosa* (Furtado *et al*., 2007). As a part of these antimicrobial classes, rifampicin has been suggested to be used in combination with this agent due to the potential synergistic results observed (Giamarellos-Bourboulis, *et al*., 2003).

1.2.8 Treatment of diseases caused by *A. baumannii*

The resistance patterns observed for *A. baumannii* in the clinical setting is leaving very few treatment options. Combination therapy is relied on in many centers to treat MDR strains of *A. baumannii*, where a significant synergy is observed *in vitro*. Combination therapy relies on an aminoglycoside with a 3rd generation cephalosporin, or colistin combined with rifampicin, ceftazidime or imipenem (Dijkshoorn *et al*., 2007; Gordon and Wareham, 2010). Combination therapy aims to prevent the emergence of resistance when using two different compounds, as well as provide coverage of a broad spectrum of pathogens in the case of mixed or unidentified infections (Legrand, *et al*., 2011; Peleg *et al*., 2008). Most importantly, antibiotic selection should rely on the susceptibility data of individual institutions (Peleg *et al*., 2008). Due to *A. baumannii* being inherently resistant

to many antibiotics, carbapenems are the ideal drugs in treating *A. baumannii* infections, but resistance is emerging very rapidly to carbapenem, leaving few available treatment options (Knapp and English, 2001). Fluoroquinolones were used to treat sporadic cases of *A. baumannii*, but resistance is now widespread in endemic strains (Dijkshoorn *et al*., 2007; Legrand *et al*., 2011). The use of polymyxins and tigecycline has emerged in recent years to overcome carbapenem and multi-drug resistance, and have proven successful in treating severe *A. baumannii* infections (Dijkshoorn *et al*., 2007; Gordon and Wareham, 2010). Resistance is still relatively rare for these compounds; however the use must be closely monitored in order to monitor the emergence of resistance. Colistin (polymyxin E) has proven effective in treating carbapenem and multi-drug resistant strains of *A. baumannii* (Legrand *et al*., 2011).

1.3 Antibiotics

1.3.1 Definition of antibiotic

The term antibiotic refers to a substance produced by microorganisms that inhibits or kills other microorganisms (Harremoës *et al*., 2001). Antibiotics are classified as bactericidal, exemplified by penicillin, or bacteriostatic. Bactericidal antibiotics cause bacterial cell death, whilst bacteriostatic antibiotics prevent the bacteria from growing (Walsh, 2010). Antibiotics can be classified according to their various mechanisms of action (Table 1.2) such as (1) interference with cell wall synthesis, (2) inhibition of protein synthesis, (3) interference with nucleic acid synthesis, (4) inhibition of metabolic pathways, and (5) disruption of bacterial membrane structure (Levy and Marshall, 2004; Tenover, 2006).

Table 1.2 Mechanisms of action of antibiotics

Mechanism of action	Antimicrobial agent(s)
1.Interference with cell wall synthesis	β- Lactams: penicillins, cephalosporins, carbapenems, monobactams. Glycopeptides: vancomycin, teicoplanin
2.Inhibition of protein synthesis	Macrolides, chloramphenicol, clindamycin, quinopristin-dalfopristin, linezolid, Aminoglycosides, tetracyclines
3.Interference with nucleic acid synthesis -Inhibition of DNA synthesis -Inhibition of RNA synthesis	Fluoroquinolones, rifampicin
4.Inhibition of a metabolic pathway	Sulfonamides, folic acid analogous
5.Disruption of membrane structure	Polymyxins, daptomycin

1.3.2 Penicillins

Penicillins have low toxicity and mostly show high activity against Gram-positive bacteria. Resistance to penicillin, such as that shown by *S. aureus* and *Enterobacteriaceae*, can occur via several mechanisms, including the production of the enzyme β-lactamase (Waksman, 1973; Walsh, 2000). Overall, the basic structure of penicillins includes a thiazolidine nucleus attached to the β-lactam ring and a side chain at the C6 position. The side chain represents different groups of penicillins- penicillin G (group 1), penicillin M (group 2), penicillin A (group 3), 6-α-substituted penicillins (group 4), α-carboxy- and α-sulfopenicillins (group 5), amidinipenicillins (group 6), and oxyimino penicillins (group 7) (Naga, 2012).

1.3.3 Cephalosporins

Cephalosporins are used to treat bacterial infections caused by both Gram-negative and Gram-positive bacteria. The cephalosporin's core structure has the β-lactam ring fused to a six-membered dihydrothiazine ring, with a sulphur atom at position 1 (Bryskier, 1997). The cephamycins are structurally similar to cephalosporins, but the cephamycin nucleus is fused with a 7-α-methoxyl group. The other group gives high-level resistance to class A β-lactamases (Essack, 2001). The first semi synthetic cephamycin was cefoxitin.

1.3.4 Carbapenems

The first carbapenem was thienamycin, isolated from a culture of *Streptomyces cattleya* (Weaver, *et al*., 1979). Carbapenems foster less resistance than other β-lactams because of their stability against hydrolysis by many β-lactamases, including AmpC enzymes and ESBLs (Bassetti, *et al*., 2013). Imipenem (N-formimidoyl thienamycin) is a chemically stable compound compared to thienamycin and was the first carbapenem approved for clinical use (Hellinger and Brewer, 1999). Meropenem has one of the largest ranges of antimicrobial activity available (Pfaller and Jones, 1997) and, due to its stability against the most β-lactamases, is often used against many MDR/XDR life threatening infections in infants and adults (Cohen-Wolkowiez *et al*., 2012). Other carbapenems approved for clinical use include ertapenem and doripenem (Nicolau, 2008; Paterson and DePestel, 2009).

1.3.5 Monobactams

Monobactams are β-lactam compounds where the β-lactam ring is a single cyclic structure and not fused to another ring. They are active only against Gram-negative bacteria. SQ-26180 was the first monocyclic β-lactam derived naturally from *Chromobacterium violaceum* (Sykes *et al.*, 1981) and was subsequently developed in 1985 after demethoxylation at the C3 position and substitution with a 2-amino-5 thiazolyloxime moiety into aztreonam (Sykes and Bonner, 1985). Aztreonam is the only monobactam in clinical use and displays high activity against *Enterobacteriaceae* and good efficacy against *P. aeruginosa* (Sykes, *et al*., 1988).

BAL30072 (SFM) is a new monocyclic β-lactam antibiotic with potent antimicrobial activity against a broad range of Gram-negative bacteria. It is a siderophore-monobactam with potent *in vitro* activity against MDR Gram-negative bacilli, representing a likely choice in treating carbapenem-resistant pathogens (Page, *et al*, 2010).

1.3.6 Uses of antibiotics

Antibiotics were introduced into medication well over 75 years ago, considerably changing life expectancy from certain diseases. Antibiotics have enabled the successful treatment of life-threatening contagions and enabled safe surgeries, previously thought to be untreatable due to the high hazards of infection. The first antibiotic, Salvarsan, was used to treat syphilis in 1910 (Harremoës *et al.*, 2001). The use of antibiotics expanded in the 1930's with the discovery of sulphonamide, a synthetic antibiotic with broad antibiotic

activity against Gram-positive bacteria, but with no effect on *Enterobacteriaceae*. It was not until the late 1990s and early 2000s that further new classes of antibiotic, such as daptomycin, tigecycline and linezolid, were discovered and made available for clinical use (Figure 1.1). With the unregulated availability of antibiotics, resistance was an inevitable outcome. Now, it is estimated that more than 70% of the bacteria that cause hospital-acquired infections are resistant to at least one of the antibiotics which are used to treat them. Antibiotic resistance continues to expand for many reasons, including extensive use of broad-spectrum antibiotics in hospitalized patients and in the community, use of antibiotics in animals as growth enhancers, increased international travel, poor hospital sanitation and abuse of antibiotics in both poor and wealthy countries (Kapil, 2005; Piéboji *et al*., 2004).

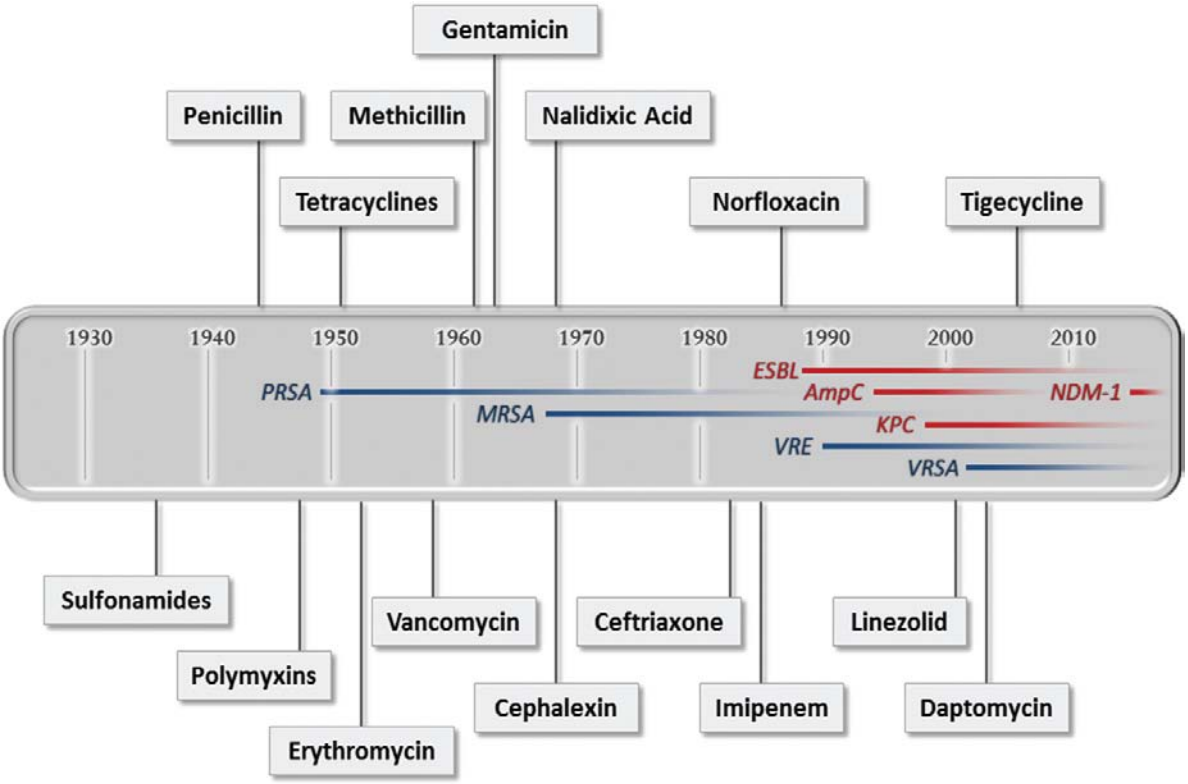


Figure 1.1 Chronology of discoveries of antibiotics (Molton, *et al*., 2013)

1.3.7 Emergence of antibiotic resistance

Antibiotic resistance is defined as "the ability of a microorganism to resist antibiotic stress and survive" (Walsh, 2000). In contrast, the susceptible bacteria would be eliminated. Over the last 75 years, the continued use of antibiotics has led to bacteria procuring and expressing various resistance mechanisms mediating resistance to one or more drugs: i.e.

multi-drug resistance (MDR) (Giedraitienė, *et al*., 2011) or, where only one or two classes are left, extensive drug resistance (XDR). MDR/XDR has appeared in *P. aeruginosa*, *A. baumannii*, *E. coli*, and *K. pneumoniae*, producing extended-spectrum β-lactamases (ESBL), vancomycin-resistant *Enterococci* (VRE), MRSA, vancomycin-resistant *Staphylococcus aureus* VRSA, XDR- *Mycobacterium tuberculosis* (Alekshun and Levy, 2007), *Salmonella enterica* serovar *typhimurium*, *Shigella dysenteriae*, *Stenotrophomonas*, and *Burkholderia* (Džidić, *et al*., 2008).

Antibiotic resistance mechanisms are either intrinsically mediated or acquired. The intrinsic resistance mechanism refers to the existence of chromosomal resistance genes or mutations in other genes/adjacent DNA altering their expression (Davies and Davies, 2010); for example, *Mycoplasma* intrinsically shows resistance to β-lactams and other cell-wall targeting antibiotics due to an alternate cell wall (Allen *et al*., 2010). In contrast, acquired resistance mechanisms are gained by bacteria through horizontal gene transfer (HGT) mechanisms (Sykes, 2010; Rowe-Magnus and Mazel, 1999); for example, many β-lactamase genes are acquired by bacteria through mobile genetic elements (MGEs-explained in later section) such as plasmids (Jeong *et al*., 2003; Toleman, *et al*., 2004), transposons (Toleman, *et al*., 2003) and insertion sequence common region (ISCR) elements (Toleman *et al*., 2006). In principle, a bacterial cell can become resistant to any antibiotic by three mechanisms: a) enzymatic inactivation or modification of the drug; b) reduced drug accumulation at the active site by decreased cell permeability and/or increased efflux from the cell surface; or c) alteration or modification of its targets (Figure 1.2) (Kunz and Brook, 2010). A brief overview of the different mechanisms will be given below to focus on resistance to β-lactams. A more detailed characterization of β-lactamases will be discussed later.

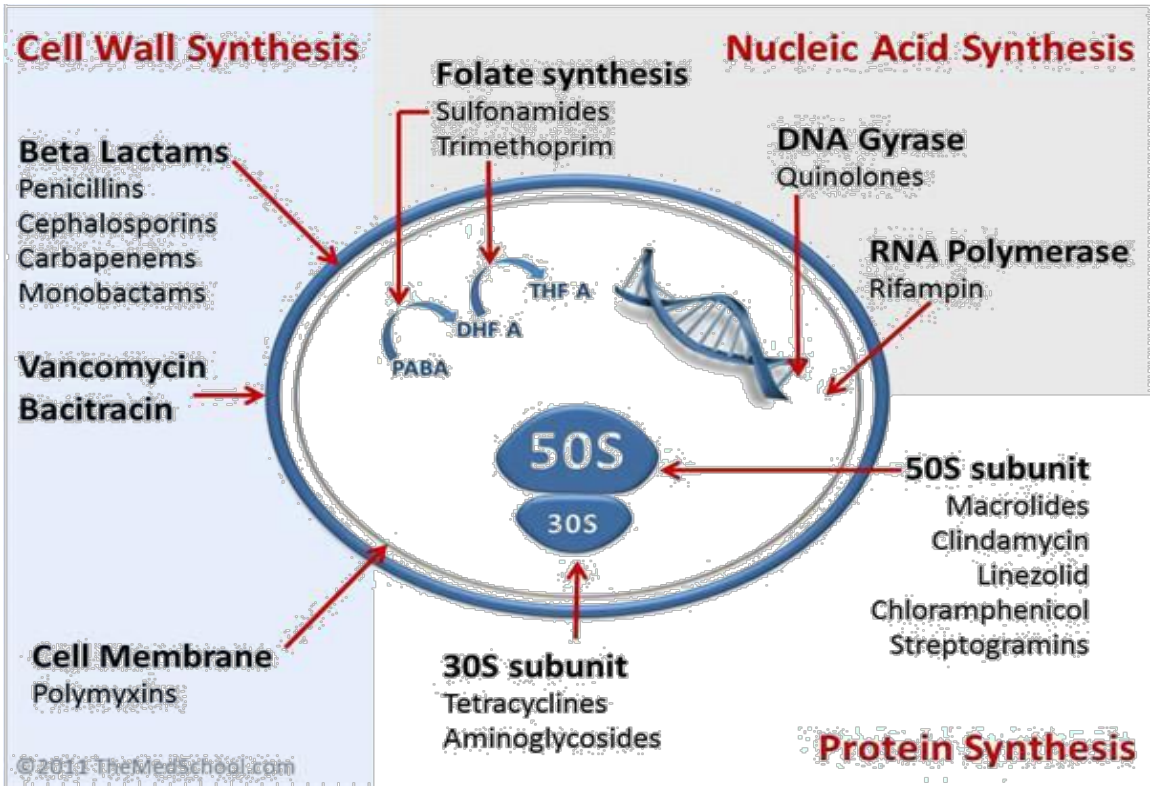


Figure 1.2 Target of action of different antibiotics in Gram-negative bacteria

1.4 Mechanisms of antibiotic resistance

1.4.1 Reduced permeability and active efflux

In many Gram-negative bacteria, outer membranes (OM) have protein channels, formed by porin proteins, to allow molecules to pass through the OM and enter the cell (Nikaido, 2003). These molecules include nutrients as well as antibiotics such as β-lactams or fluoroquinolones. These proteins form channels that traverse the OM and end in the periplasm. In order to prevent the entry of antibiotics, bacteria can reduce the access of antibiotics (also called flux) by changing the OM. Gram-negative bacteria like *K. pneumoniae, P. aeruginosa,* and *A. baumannii* shows resistance to antibiotics like β-lactams by altering the porins or by down regulating expression of the porins (Hancock, 1998). As an example, a combination of deletion of OM porins, with the increased expression of plasmid-mediated AmpC β-lactamases, in *K. pneumoniae* can confer resistance to imipenem (Cao *et al*., 2000). Many studies have shown that the combined presence of ESBLs and altered OM can mediate carbapenem resistance in *A. baumannii, P. aeruginosa* and other Gram-negative bacteria (Giani *et al*., 2009).

Efflux pump systems were discovered in the 1970s in *E. coli* and conferred resistance to tetracycline (Ball, *et al*., 1980). To date, five major efflux pump families have been

reported: ABC (ATP-binding cassette) superfamily, SMR (small multi-drug resistance), RND (resistance-nodulation division), MFS (major facilitator superfamily), and MATE (multidrug and toxin extrusion) superfamilies (Giani *et al*., 2009). *P. aeruginosa* harbours several efflux pumps, including MexAB-OprM, MexCD-OprJ and MexXY-OprM, with different spectra of substrate profiles that include various groups of antibiotics including β-lactams (Masuda *et al*., 2000). Exopolysaccharides (EPS) are responsible for expelling antibiotics from bacterial cells (Kumar and Schweizer, 2005). EPS play an important role in the physiology and homeostasis of bacteria (Mirelman, *et al*., 1974). Furthermore, EPS are useful tools for the cell to remove other molecules like heavy metals, dyes, or detergents (Livermore, 1995). While diverse mechanisms account for different resistance forms, the over-expression of efflux pumps extruding antibiotics is a major resistance mechanism for clinical isolates (Majiduddin, *et al*., 2002).

1.4.2 Enzymatic inactivation or modification of antibiotics

The enzymatic inactivation or modification of the drug is the most common mechanism of antibiotic resistance in many bacteria. The genes that encode these enzymes are often associated with MGEs such as plasmids, transposons or integrons (Azucena and Mobashery 2001). Most of the antibiotics are characterized by ester or amide bonds, which are hydrolytically susceptible, targeted by certain bacterial enzymes (e.g. β-lactamases) which make them inactive (Wright, 2005).

1.4.3 Target alteration

Bacteria can alter the targets of antibiotics, which structurally reduce the affinity for antibiotics; for instance, the modification of penicillin binding proteins (PBPs), the important targets for β-lactams, reduces their affinity for β-lactams (Macheboeuf, *et al*., 2006). Resistance to quinolones is caused by chromosomal mutation encoding substitutions in the DNA gyrase or topoisomerase IV (Patzer *et al*., 2004; Sharma & Mohan, 2006).

Since the discovery of penicillin, β-lactams have been the most significant antibiotic group for the past 75 years and are used to treat infections caused by both Gram-negative and Gram-positive bacteria (Siu, 2002). β-lactams can be classified into four main groups based on the structure and discovery; penicillins, cephalosporins, carbapenems, and monobactams. The four different classes of β-lactam antibiotics and their spectrums are described briefly in Table 1.3.

Table 1.3 Classes of β-lactams and the antibacterial spectrum

Chemical class	Examples	Spectrum of activity	
		Gram-negative	Gram-positive
Beta-lactams (Penicillin)	Penicillin-G, Penicillin-M	–	+
Semi-synthetic β-Lactams	Amoxicillin, Ampicillin	+	+
Beta-lactams (Cephalosporins)	1st generation Cephalothin, Cefazolin	±	+
	2nd generation Cefoxitin, Cefuroxime	+	±
	3rd generation Ceftazidime, Cefotaxime	+	±
	4th generation Cefepime, Cefpirome	+	+
Penems	Carbapenems Imipenem, Meropenem	+	+
Monobactams	Aztreonam	+	+

+: good activity; ±: reduced activity.

1.4.4 β-lactamases

Hydrolysis of β-lactam antibiotics by β-lactamases is the most common resistance mechanism in clinically important Gram-negative bacteria (Bush and Jacoby, 2010). The term β-lactamases refers to an enzyme inactivating the β-lactam by hydrolyzing the amide bond of it β-lactam ring (Siu, 2002). Now, there are more than 500 β-lactamases documented (www.lahey.org/studies.webt.htm). The substrate and inhibition profiles vary between different types of β-lactamases (Bush and Jacoby, 2010). The spread of β-lactamases is often associated with plasmid encoded ESBLs, mainly CTX–M, *K. pneumoniae* carbapenemase (KPC) and MBLs (VIM, IMP and NDM-1) (Pitout, 2010). In Gram-negative bacteria, β-lactamases usually accumulate in the periplasm where they hydrolyze the antibiotic. However, in Gram-positive bacteria β-lactamases are released into the surrounding environment as exo-enzymes to hydrolyze the drug outside the cell (Ghuysen, 1991).

1.4.5 Classification of β-lactamases

β-Lactamases have been classified either based on their functional characteristics (Bush, *et al*., 1995) or their primary structure based on amino acid similarities (Ambler, 1980). The

simplest classification is the Ambler molecular scheme where β-lactamases are divided into four molecular classes (A, B, C, and D) based on amino acid active motifs (Ambler *et al.*, 1991; Jaurin & Grundström, 1981). β-lactamases can be grouped structurally into two super families: serine β-lactamases (classes A, C, and D) and metallo-β-lactamases (class B). Classes A, C, and D include enzymes that hydrolyze their substrates by forming an acyl enzyme through an active site serine residue, whereas class B β-lactamases are metallo-enzymes that use at least one zinc ion in their active site to co-ordinate polarized water molecules responsible for the β-lactam hydrolysis (Bush & Jacoby, 2010).

1.4.5.1 Class A β-lactamases (Extended Spectrum β-lactamases (ESBLs)

Class A β-lactamases are the largest group of β-lactamases and are generally inhibited by serine β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam (Paterson, 2006). This class includes TEM, SHV and CTX-M-type groups and are mainly associated with ESBLs (Jacoby & Munoz-Price, 2005; Walther-Rasmussen and Høiby, 2007). The first CTX-M was discovered in 1989 and over 120 CTX-M types have been detected to date. CTX-M-14 and CTX-M-15 are the most important genotypes worldwide. Disseminating CTX-M types are often associated with insertion sequence common regions (IS*CR*s). CTX-M-15 has become the most widespread ESBL globally (Coque *et al*., 2008).

1.4.5.2 Class C β-lactamases

Class C β-lactamases can be encoded on chromosomes of many Gram-negative bacteria (Jacoby, 2009), the paradigm being AmpC. AmpC enzyme has been described in many Gram-negative including *C. freundii, Enterobacter* spp., *E. coli, M. morgannii, P. rettgeri, Providencia stuartii, P. aeruginosa,* and *S. marcescens* (Philippon, *et al*., 2002). They are more active against cephalosporins than penicillins and are usually resistant to β-lactamases inhibitor clavulanate and active against cephamycins such as cefoxitin (Bush and Jacoby, 2010). Over-expressed class C enzymes can decrease OM efflux and the resistance to carbapenems, especially ertapenem (Quale, *et al*., 2006). Plasmid-mediated class C enzymes include CMY, ACT, DHA, FOX, and MIR of which the most common is CMY-2 (Jacoby, 2009).

1.4.5.3 Class D β-lactamases

Class D β–lactamases are termed oxacillinase or OXA-type β-lactamases (OXAs) because of their ability to hydrolyze oxacillin (Poirel, *et al*., 2010). These enzymes also hydrolyze

cloxacillin, cephalosporins and some carbapenems. OXA enzymes are usually not affected by β-lactamase inhibitors, but may be inhibited *in vitro* by sodium chloride. OXA genes are embedded into class 1 integrons and recent studies have shown that other MGEs, including insertion sequences (IS) and transposons (Tn), are associated with OXA genes. The OXA group demonstrating carbapenemase activity is divided into five subfamilies: OXA-23, OXA-24/OXA40, OXA-48/181, OXA-58 and OXA-51. The first four groups are carried on transmissible plasmids, while the last group, OXA-51, is chromosomally located and intrinsic in *A. baumannii*.

1.4.5.4 Class B β-lactamases

Class B β-lactamases are either serine or metallo type. In serine β-lactamases amino acid serine is present on an active site, on the other hand all metallo-β-lactamases are Zn-dependent hydrolyses, and present either one or two metal ions in their active site. The clinically relevant and the more important MBLs are encoded in mobile genetic elements and include VIMs (Verona Integron-encoded Metallo-β-lactamase) (Lauretti *et al*., 1999), IMPs (Imipenemase) (Laraki *et al*., 1999), and the more recently emerged NDMs (New Delhi Metallo-β-lactamase) (Yong *et al*., 2009). Although there are different inhibitors have been tested in vitro, but there is no clinical drug able to inhibit any of the metallo-β-lactamases (King and Strynadka, 2013). These MBLs have an extended substrate spectrum, including not only carbapenems, but also penicillins and the last generation cephalosporins. MBLs are not able to hydrolyze aztreonam (Poeylaut-Palena *et al*., 2007), but they are usually co-expressed with serine β-lactamases and diminish the susceptibility of the bacteria towards this compound (Fisher, *et al*., 2005). MBLs are able to hydrolyze carbapenems and are not inhibited by the serine β-lactamase inhibitors like clavulanic acids, sulbactams and tazobactams (Crowder, *et al*., 2006).

The most common types of acquired MBLs identified in *Enterobacteriacea*e, *Pseudomonas*, *Acinetobacter* include the MBL subgroups IMP and VIM, together with the emerging NDM group, where the group KHM-1 is rare (Walsh, 2005). Generally, the level of carbapenem resistance observed for MBL-producing strains varies and attributed mortality, associated with MBL production, ranges from 18% to 67% (Daikos *et al*., 2009).

IMP Variants

The first MBL, IMP-1, was described in Japan in 1991 from an *S. marcescens* isolate (Watanabe, *et al*., 1991). Alleles from the IMP family were then found in *Enterobacteriaceae* and distributed among Asia and the rest of the world (Zhao and Hu, 2011). Overall, the IMP variants present 85%–99.6% of sequence similarity (80%–99.6% sequence identity), giving rise to a diffused dendogram that suggests a polyphyletic origin of IMPs (Pal and Tripathi, 2013), contrasting with the VIM group.

Two major groups can be distinguished among IMP variants, based on the identity of the second sphere residue at position 262, which has been considered as responsible for tuning the substrate preferences (Iyobe *et al*., 2000). IMP-1-like variants possess a Ser residue at position 262, while IMP-6-like variants present a Gly residue at the same position (IMP-6, IMP-3, IMP-12, IMP-25, IMP-27, and IMP-38). IMP-6 displays similar catalytic efficiencies to those shown by IMP-1 towards cefalothin, cefotaxime (Laraki, *et al*., 1999; Iyobe *et al*., 2000), cefoxitin, meropenem, and doripenem (Liu, *et al*., 2012). However, IMP-1 is more efficient than IMP-6 towards penicillins (in particular penicillin G and ampicillin), ceftazidime, cephaloridine, and imipenem (Oelschlaeger, *et al*., 2005). These different activities are also reflected on the respective MICs on *E. coli* cells (Iyobe *et al*., 2000). The first characteristic of IMP-6 (Yano *et al*., 2001) against meropenem showed a marked increase in the MIC and catalytic efficiency compared to imipenem. Thus, it was hypothesized that mutation S262G would be an adaptation towards newer carbapenems, in detriment of penicillinase activity (Yano *et al*., 2001). A more recent work on IMP-25 reported an increase in meropenem MIC for IMP-6 and IMP-25 (Liu *et al*., 2012), despite not as high as previously reported. In fact, the catalytic efficiencies did not show the dramatic increment observed by Yano *et al*. (Yano *et al*., 2001).

Two possible scenarios have been proposed. On one side, IMP-6 might be an ancestor of IMP-1, and in this context, the role of mutation G262S would have been to broaden the substrate profile. On the other side, if IMP-1 is an ancestor of IMP-6, the effect of mutation S262G would have been to improve the activity towards newer carbapenems at the expense of the penicillinase activity. These hypotheses heavily rely on assumptions of the evolutionary timeline of these enzymes that cannot be verified. However, it is evident that position 262 on IMP enzymes effectively affects their substrate preferences.

VIM Variants

VIM-1 was identified in 1999, in an Italian clinical isolate of *P. aeuruginosa* resistant to carbapenems (Lauretti *et al*., 1999). Soon after, the variant VIM-2 was reported, arised from an earlier clinical isolate in France (Poirel, *et al*., 2000). Since then, 40 different VIM variants emerged worldwide. Among them, VIM-2 is by far the most prevalent clinical variant as well as the one with the largest geographical spread (Bush and Fisher, 2011). VIM variants present sequence similarities ranging between 81% and 99.6% (72.9%–99.6% sequence identity, including VIM-7, the most divergent variant; 87.7%-99.6% identity without VIM-7), and show a directional and monophyletic mode of evolution with formation of two major clusters: VIM-1 and VIM-2 (Pal and Tripathi, 2013).

NDM Variants

Perhaps the most noteworthy is the New Delhi metallo-β-lactamase-1 (NDM-1), first identified from patients hospitalized in India, and now reported worldwide. The Indian subcontinent is clearly the main reservoir of NDM-1 producers (Khan and Nordmann, 2012). The $bla_{\text{NDM-1}}$ is not associated with a single species or with a specific plasmid backbone, but has been identified harbored on different plasmid types from unrelated Gram-negative bacteria (Poirel, *et al*., 2011), and inter-strain, interspecies, and inter-genus transmission of diverse plasmids containing this gene in more than 40 countries worldwide (Johnson and Woodford, 2013; Maya *et al*., 2013). The NDM-1 gene is located on a mobile genetic element and the pattern of spread appears to be more complex (Yong *et al*., 2009). Many studies have reported that $bla_{\text{NDM-1}}$ positive isolates are mostly nosocomial *K. pneumoniae* isolates, but the $bla_{\text{NDM-1}}$ gene has also been identified in community-acquired *E. coli* (Nordmann, *et al*., 2012). In addition, reports from different countries worldwide (Far and Middle East, USA and Canada and many countries in Europe) have documented the NDM-1 presence with links to India (Johnson and Woodford, 2013). However, these links were not always related to hospitalization, since some isolates were from community-associated infections, in patients that only had a history of travel to India. In addition, while numerous reports described the occurrence of NDM-1 positive bacteria in Indian hospitals (Johnson and Woodford, 2013), a measurement of the prevalence of the NDM-1 gene in drinking and sewerage water samples in New Delhi found that it was carried by various strains in a worrisome frequency (Walsh, *et al*., 2011). This indicates that NDM spread has passed the hospital walls and that environmental transmission is

occurring (McKenna, 2013; Johnson and Woodford, 2013). In addition, many patients colonized with NDM-1 producers originated from the Balkan states, pointing it as a second reservoir (Livermore, *et al*., 2011; Jovcic *et al*., 2011). Apart from that, many reports informed the presence of isolates from different countries with no history of travel to India, indicating the possibility of transmission by asymptomatic patients and local spread following importation (Johnson and Woodford, 2013). Currently, eight variants of NDM enzyme have been identified (Khajuria, *et al*., 2014; Jovcic *et al*., 2011). The NDM-2 variant has been identified in *A. baumannii* (Kaase *et al*., 2011), the NDM-3-producing *E. coli* strain is from Australia, the NDM-4-producing *E. coli* strain from India the NDM-5-producing *E. coli* strain from the United Kingdom, the NDM-6-producing *E. coli* strain from New Zealand, the NDM-7-producing *E. coli* strain from Canada and the NDM-8 producing *E. coli* strain from Nepal (Tada *et al*., 2013).

1.5 Epidemiology of NDM-1

NDM-1 is the most prevalent recently detected transferable class B β-lactamase. It was first described in 2008 in *K. pneumoniae* and *E. coli* isolated in Sweden from an Indian patient hospitalized in New Delhi, India (Yong *et al*., 2009). He was originally from India but, having lived in Sweden for many years, returned to India. The NDM-1 gene can hydrolyze all penicillins, cephalosporins and carbapenems, but not aztreonam (Kumarasamy *et al*., 2010). Most NDM-positive bacteria are resistant to other antibiotic classes and carry many other resistance mechanisms (e.g. to aminoglycosides and fluoroquinolones) (Muir and Weinbren, 2010). NDM-1 was found in many isolates, mostly *K. pneumoniae* and *E. coli* collected in the UK, India, Pakistan and Bangladesh in 2009, indicating that it had been well established before the first discovery (Kumarasamy *et al*., 2010).

Currently, results suggest that the Balkan states and the Middle East might act as secondary reservoirs for the spread of the $bla_{\text{NDM-1}}$ gene (Nordmann, *et al*., 2011). *Enterobacteriaceae*, harboring the NDM-1 gene, have been recovered from many clinical specimens, including UTI, septicemia, pulmonary infections, peritonitis, and soft tissue infections (Nordmann *et al*., 2011; Kumarasamy *et al*., 2010). NDM-1-producing bacteria have been recovered from the intestinal flora of travelers returning from India and undergoing microbiological examination for unrelated diarrheal symptoms (Leverstein-Van Hall *et al*., 2010). This is in keeping with observed environmental pollution by NDM-1-producing bacteria in New Delhi (Walsh *et al*., 2011). This distribution suggests the

association of the bla_{NDM-1} gene with promiscuous plasmids. NDM-1 was found in a widespread diversity of Gram-negative species, including *Enterobacteriaceae*, *Pseudomonas* spp., *Stenotrophomonas* spp., *Aeromonas* spp. and *V. cholerae*, isolated in New Delhi from sewage and tap water samples (Walsh *et al*., 2011). Worldwide distribution of MBLs is shown in Figure 1.3.

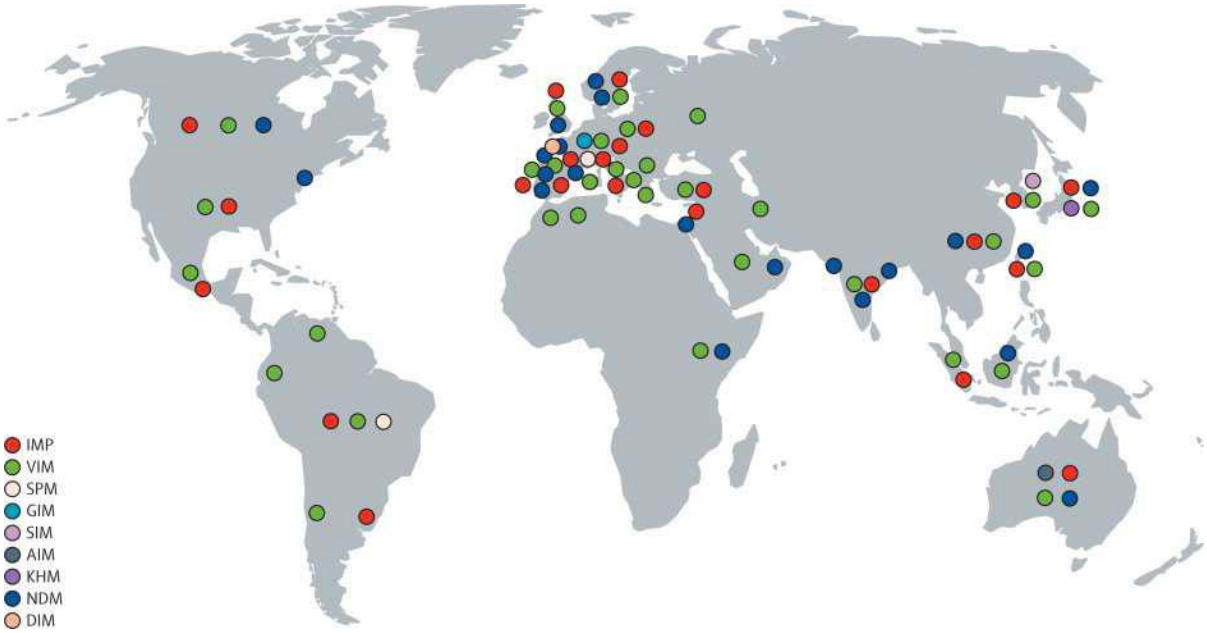


Figure 1.3 The worldwide distribution of metallo-β-lactamases (Cornaglia *et al.*, 2011): IMP, Imipenemase; VIM, Verona Integron-encoded Metallo-β-lactamase; SPM, Sao Paulo Metallo-β-lactamase; GIM, German Imipenemase; SIM, Seoul Imipenemase; AIM, Adelaide Imipenemase; KHM, Kyorin Health Metallo-β-lactamase; NDM, New Delhi Metallo-β-lactamase; DIM, Dutch Imipenemase.

1.6 Horizontal Gene Transfer (HGT)

Lateral gene transfer, recently, so called Lateral Gene Transfer (LGT) is also known as Horizontal Gene Transfer (HGT) (Frost, *et al*., 2005). The evolutionary importance of HGT in bacterial cells was seen in the spread of penicillin resistance through plasmid transfer across *Enterobacteriaceae* (Datta and Kontomichalou, 1965). HGT is an essential route in moving and rearranging DNA in prokaryotes and plays a key role in microbial evolution. The spread of resistance genes is a direct result of HGT and this has vast complications for human health (Gogarten, *et al*., 2002; Stokes and Gillings, 2011).

Stokes and Gilling have defined HGT as the process whereby DNA from one cell is physically transferred to another cell without an absolute need for cell division and the incorporation of that DNA into the recipient's DNA such that it can be stably inherited

(Stokes and Gillings, 2011). HGT requires three mechanisms to gain the foreign DNA in bacterial cell: 1) transformation, 2) transduction and 3) conjugation (Silva, *et al*., 2012).

1.7 Mobile Genetic Elements (MGEs)

Mobile genetic elements (MGEs) are a type of genetic materials that can move within a genome, or that can be transferred from one species to another. MGEs are found in all organisms. MGEs play a distinct role in evolution. Gene duplication events can also happen through the mechanism of MGEs. MGEs can also cause mutations in protein coding regions, which alter the protein functions. In addition, they can also rearrange genes in the host genome. Most importantly MGEs play a vital role in acquired resistance to antibiotics in microorganisms. Example of MGEs is plasmid, transposons, integron, insertion sequence etc.

1.8 Emergence of antibiotic resistance in *P. aeruginosa*

When a new antibiotic is established, a lot of bacteria are initially susceptible, however, resistance development is often observed after a short time (Hughes and Datta, 1983). In the 1960s, *P. aeruginosa* emerged as a main human pathogen due to wound and burn mediated infection, in addition to neutropenic, cystic fibrosis and surgical patients (Doggett and Aduan, 1979). In spite of the activity of anti-pseudomonal agents being one of the holy grails of pharmaceutical drug discovery for many decades, it remains one of the main recalcitrant and difficult to treat organisms. As therapeutic options run out, *P. aeruginosa* has attained “superbug” status. Antimicrobial resistance, which has become widespread today, seems to have been rare in the pre-antibiotic era (Moore and Flaws, 2011a). Antibiotic resistance among *P. aeruginosa* infection is an increasing global problem and a public health threat, posing many therapeutic challenges. Additionally, the susceptible strains of *P. aeruginosa* can acquire drug resistance during treatment, commonly with a relatively high frequency (Quinn, *et al*., 1986; Mesaros *et al*., 2007). The emergence of resistance in *P. aeruginosa* has been reported with all classes of antimicrobial agents including penicillins, cephalosporins, carbapenems, aminoglycosides and fluoroquinolones (Livermore, 2002; Lister, *et al*., 2009).

This emergence of resistance has limited the opportunity of therapeutic options and is leading to increased rates of morbidity and mortality and increased costs of treating patients with *P. aeruginosa* infection (Livermore, 2002; Moore and Flaws, 2011a). *P. aeruginosa* is considered the third most common healthcare-associated infections in our

society giving rise to a wide range of opportunistic infections. Its high intrinsic resistance to antibiotics and the ability to develop multi-drug resistance poses serious therapeutic problems (Zuanazzi *et al*., 2010; Cao, *et al*., 2004). The resistance to antimicrobial agents is constantly increasing with geographical differences frequently linked to local antibacterial strategies. (Jones *et al*., 2004). Generally, the fluoroquinolones are the antimicrobial class where the resistance rates are rising quickly (though, geographical difference subsists) with resistance to β-lactams and aminoglycosides. In Europe, this is more important, even though the emergences of colistin-resistant strains are being reported (Matthaiou *et al*., 2008; Walkty *et al*., 2008; Denton *et al*., 2002). A review (Bonomo and Szabo, 2006) described MDR as the decreased susceptibility to more than one of the following antimicrobial agents: quinolones, carbapenems, cephalosporins, β-lactam inhibitor combinations and aminoglycosides. Based on this description, a strain resistant to amikacin and ceftazidime will be considered as MDR, while there are sufficient treatment options. Multidrug-resistant strains were isolated more frequently from ICU and nursing home patients. Manian *et al*. (1996) analyzed resistance rates among *P. aeruginosa* isolated from an ICU and found that the rates of resistance to penicillins and cephalosporins was statistically related to prior treatment with third generation cephalosporins (Manian, *et al*., 1996). Overall, MDR rates in Europe are 5%, Asia and the US are 2%, while MDR rates for Latin America is more frequent at 8% of total isolates. Numerous reports have found that multidrug-resistant strains of *P. aeruginosa* usually arise after prolonged exposure to antipseudomonal therapies (Georges *et al*., 2006; Raja and Singh, 2007; Livermore, 2012) or after empirical treatment (Zavascki *et al*., 2006; Arancibia *et al*., 2002; Mesaros *et al*., 2007). A number of *P. aeruginosa* strains which showed resistance to β-lactam, reflect a previous treatment history of the patients with wide-range β-lactam. For example, a considerable association was observed in a single medical center between the antecedent use of ceftriaxone, piperacillin, ceftazidime and cefotaxime. A study of the occurrence of *P. aeruginosa* resistance to β-lactam antibiotics in ICU patients found a high risk emerging with imipenem, piperacillin/tazobactam and cefotaxime during treatment (Georges *et al*., 2006). A previous randomized assessment comparing imipenem to ciprofloxacin for the treatment of healthcare-associated pneumonia also illustrated a higher risk of emergence of resistance in the carbapenem arm (Aarts, *et al*., 2008). Several factors may lead to the increase in antibiotic resistance, such as hospital stay and period of hospitalization before ICU admission, surgical ICU stay, the type of operation, length of ICU stay, previous antibiotic use, and inappropriate use of

antipseudomonal drugs and inadequate adherence to infection control practices (Fridkin *et al*., 2002; Neuhauser *et al*., 2003; Siegel, *et al*., 2007). MDR has been found to be associated with a twofold increase in of duration of hospitalization, a ninefold elevation in secondary bacteraemia, a threefold raise in mortality and major increases in health care costs (Carmeli *et al*., 1999). Significant data from ICU isolates of *P. aeruginosa* resulting from Europe are even worse. From 1990 to 1999 resistance to ceftazidime reached 57%, resistance to aminoglycosides reached 37-70%, resistance to imipenem reached 52%, resistance to ciprofloxacin reached 56% and resistance to piperacillin/tazobactam reached 53% (Rossolini and Mantengoli, 2005).

1.9 Mechanisms of the antibiotic resistance in *P. aeruginosa*

P. aeruginosa is intrinsically resistant to certain antipseudomonal agents via three different mechanisms, including antibiotics cleavage by β-lactamase enzymes, antibiotic ejection by chromosomally encoded efflux mechanisms and decreased drug uptake due to loss of outer membrane porin protein (Poole, 2004; Pfeifer, *et al*., 2010). It has been observed that *P. aeruginosa* acquires resistance to antipseudomonal agents either by mutations to chromosomal genes or via the acquisition of plasmids encoding genes contributing resistance. Firstly, mutation of the *gyrA* gene alters the structure of DNA gyrase, which is the target of the quinolones (Lambert, 2002). When mutations occur in the respective genes, certain penicillin-binding proteins (PBPs) confer resistance to β-lactam (Lambert, 2002).

1.9.1 Enzymatic mediated mechanisms of resistance in *P. aeruginosa*

β-lactamase expression is the most common mechanisms of resistance to β-lactam antibiotics. β-lactamase are able to hydrolyze the four members of the β-lactam antibiotic family, including carbapenems, penicillins, cephalosporins and monobactams (Fisher *et al*., 2005; Henrichfreise, *et al*., 2007). The main cause of acquired resistance to β-lactam antibiotics in *P. aeruginosa* is an expression of the β-lactamase gene (Strateva and Yordanov, 2009). In a classification recognized as the Ambler scheme, β-lactamases can be classified depending on the amino acid and nucleotide sequences into 4 major classes: A through D (Ambler, 1980; Paterson and Bonomo, 2005). *P. aeruginosa* clinical isolates express all 4 Ambler classes, correlating with the functional classification defined by enzyme substrate and inhibitor profiles (Poole, 2004). Classes A, C and D act via a serine-based mechanism, while class B or metallo-β-lactamases (MBLs) require zinc for their

action. All of these molecular classes of β-lactamases were discovered in *P. aeruginosa*, including ESBLs of classes A.

MBLs or carbapenemases enzymes, mostly classified as Ambler class B MBLs, are zinc dependent and have a very wide substrate profile, such as carbapenems and expanded-spectrum cephalosporins. The three subclasses of clinically relevant mobile MBLs that have been identified thus far in *P. aeruginosa* are; *VIM*, *IMP* and *SPM*-1. A new fourth subclass of Ambler class B enzyme, *GIM*-1, was lately detected in isolates that originated from Germany (Castanheira, *et al*., 2004). Most recent resistances have emerged in Gram-negative bacteria (e.g., *NDM*-1) (Kumarasamy *et al*., 2010). In *P. aeruginosa*, acquired carbapenemases is an emerging cause of resistance and there has been an increase in the reports on carbapenemases over the last decade (Nordmann and Poirel, 2002).

Carbapenemase production establishes resistance to all β-lactams including the carbapenems (meropenem and imipenem). The hydrolytic features of MBLs do not affect the monobactam aztreonam (Bush and Mobashery, 1998). Metallo-β-lactamase (MBLs) production is a significant problem, especially in hospital isolates of *P. aeruginosa*. MBLs are spreading throughout various parts of the world. In 1988, it was found that the *IMP* type MBL emerged from a *P. aeruginosa* strain in Japan (Watanabe *et al*., 1991). However, other genes such as *SPM*, *VIM*, and *GIM* type MBLs were recognized worldwide (Navaneeth, *et al*., 2002; Gupta, *et al*., 2006). Carbapenem non-susceptible, *IMP*-1 producing bacteria were isolated in Europe, Korea and Singapore. In Malaysia, only *IMP-7* genes were found in *P. aeruginosa* (Lagatolla *et al*., 2004). Eleven percent of the *Pseudomonas* spp. carried *blaVIM* among imipenem-nonsusceptible isolates reported in Korea (K. Lee *et al.*, 2004). These *blaMBL* genes are located on class 1 integrons residing on mobile plasmids. The resistance cassettes carried on these MBL-containing integrons may vary, but this machinery enables resistance to spread horizontally between species in all cases (Rowe-Magnus and Mazel, 2002; Rice, 2002). The genes encoding *IMP*, *VIM* in addition to *GIM* were found as gene cassettes in class 1 integrons, although *IMP* MBL genes have also been found on class 3 integrons (Fluit and Schmitz, 1999). However, not all MBL genes are necessarily associated with integrons or transposons. The genetic context of *blaSPM*-1 is noticed in mobile genomic pathogenicity islet and is found on a plasmid of 180 kb (Shibata *et al*., 2003).

1.9.2 Non-enzymatic mediated mechanisms of resistance in *P. aeruginosa*

There are two types of non-enzymatic mediated mechanisms of resistance in *P. aeruginosa* and they are the efflux pump expression and outer membrane protein. Modified bacterial efflux systems are capable of extruding antibiotics and to date, five families of bacterial drug efflux pumps have been identified. They are the ATP-binding cassette (ABC) family, the major facilitator (MF) family, the small multidrug resistance (SMR) family, the resistance nodulation-division (RND) family, and the multidrug and toxic compound extrusion (MATE) family. The main clinically relevant efflux systems belong to the RND efflux systems because of their abundance and contribution to antibiotic resistance (Poole, 2004). These efflux pumps usually work as a tripartite system that consist of the cytoplasmic membrane protein that roles to efflux antibiotics through the cytoplasmic membrane, an outer membrane channel protein that supplies the means of access of drugs to the outer part of the cell and a linker protein that joins the two pumps to a membrane fusion protein (MFP) (Livermore, 2002). Together, these three proteins are capable of efficiently pumping a range of ions, dyes and antibiotics depending on the type of RND pump combined with the system.

Table 1.4 Substrate specificities of the efflux pump system in *P. aeruginosa*

Pump periplasmic linker	Cytoplasmic membrane	Outer membrane channel	Substrates
MexA	MexB	OprM	β-lactams except imipenem, macrolides, tetracycline, chloramphenicol, novobiocin, Quinolones, lincomycin
MexC	MexD	OprJ	meropenem, macrolides, novobiocin, Quinolones, lincomycin, tetracyclines, chloramphenicol, penicillins except sulbenicillin and carbenicillin, cefepime,cefpirome
MexE	MexF	OprN	Fluoroquinolones, carbapenems
MexX	MexY	OprM	Quinolones, cefepime, meropenem, cefpirome. aminoglycosides, macrolides, lincomycin, chloramphenicol, tetracycline's and, Penicillins except sulbenicillin and carbenicillin

The efflux pumps in *P. aeruginosa* are different from each other in various ways, which include the substrate antibiotics they extrude, and regulation of their operons. However, just the MexXY efflux system has been detected to supply both impermeability and adaptive resistance to aminoglycoside antibiotics in these bacteria (Hocquet *et al*., 2003; Sobel *et al*., 2003; Vogne *et al*., 2004). A dominant resistance mechanism in *P. aeruginosa* is the overexpression of efflux systems with wide substrate profiles. The effect of the overexpression of efflux systems one, the resistance to antipseudmonal antibiotics (aminoglycosides, β-lactams, fluoroquinolones and polymyxin B) are summarized in Table 1.3 (Livermore, 2002).

1.10 Mechanisms of antibiotic resistance in *A. baumannii*

A. baumannii is rapidly developing as a multi-drug resistance pathogen with the ability of acquiring and upregulating resistance mechanisms. All major resistance mechanisms reported in Gram-negative bacteria have been identified in *A. baumannii* such as modifications of target site, active efflux pumps, enzymatic deactivation of drugs and decreased influx (Gordon and Wareham, 2010; Hawkey, 1998; McGowan, 2006). This has rendered all current major antibacterial agents such as penicillins, cephalosporins, aminoglycosides and quinolones as inefficient treatment options for *A. baumannii* infections, which can also partially be attributed to the intrinsic resistance that the organism harbors towards these classes of antibiotics (Dijkshoorn *et al*., 2007). Carbapenems have been the drug of last resort for treating *A. baumannii* infections, but the rise in resistance is very worrying.

Recent research suggests that the observed increase in resistance can be attributed to the interplay of several resistance mechanisms: enzymatic and non-enzymatic, such as the decreased influx together with the expression of a resistance gene (Poirel and Nordmann, 2006b; Poirel, *et al*., 2012).

1.10.1 Enzymatic mechanisms of antibiotic resistance in *A. baumannii*

β-lactam antibiotics are widely used in the hospital setting and comprise the penicillins, cephalosporins, monobactams and carbapenems. Their high efficacy and safety in clinical use has increased the usage and consecutively resulted in emergence of resistance to β-lactam antibiotics. The main mechanism of resistance to β-lactam antibiotics is the production of β-lactamases. There are four classes of β-lactamases described in *A. baumannii* (Dijkshoorn *et al*., 2007). The spread of β-lactamases correlates with the usage

and development of β-lactam antibiotics. Carbapenems, the last development of β-lactam antibiotics are now the 'drug of choice' for many serious and multi-drug resistant infections, but we are entering an era of resistance to carbapenems, no newly developed compounds, which will ultimately result in untreatable infections.

1.10.2 Non-enzymatic Mechanisms of Antibiotic Resistance in *A. baumannii*

Resistance to carbapenems in *A. baumannii* may be enhanced by interactions between broad-spectrum β-lactamases and other resistance mechanisms, including porin(s) loss, active drug efflux, and (rarely) modification of penicillin-binding proteins (PBPs) (Poirel and Nordmann, 2006a).

Several reports have associated decreased expression of certain porins with antimicrobial resistance in *A. baumannii*, including several outer membrane proteins (OMPs) that have some homology with the monomeric OmpA porin found in *Enterobacteriaceae*. Porins of this family have been characterized in several species of *Acinetobacter*, including *A. radioresistens*, *A. junii* and *A. baumannii*, and are known as slow porins that allow the penetration of β-lactams (Gribun, *et al*., 2003). Three OMPs have been associated with resistance or decreased susceptibility to carbapenems, namely a 33–36 kDa protein, a 29kDa protein also known as CarO, and a 43 kDa protein, that show homologies with OprD from *P. aeruginosa*. An additional OMP (OmpW) has been identified in *A. baumannii* that shows significantly decreased expression in ceftriaxone-resistant clinical isolates. Efflux-mediated resistance is a common factor affecting antibiotic susceptibility in Gram negative bacteria, and several efflux pumps have been described in *A. baumannii*.

1.11 The role of penicillin-binding proteins (PBPs) and outer-membrane proteins (OMPs)

The role of PBPs in conferring antibiotic resistance in *A. baumannii* has been poorly investigated, but the reduced expression of PBPs has been reported to contribute to carbapenem resistance in isolates from Spain (Giamarellou *et al*., 2008). Another study found that the modification of PBPs was a source of imipenem resistance (Poirel and Nordmann, 2006a).

Porin loss has been found to significantly contribute to resistance to carbapenems (Dijkshoorn *et al*., 2007; Giamarellou *et al*., 2008). The loss of 22 and 33 kDa and OMPs together with the expression of an acquired β-lactamase resulted in

carbapenem resistance. CarO is an important OMP in the influx of carbapenems in *A. baumannii*, and studies have shown that the disruption of carO by insertion elements contributes to carbapenem resistance in clinical isolates (Poirel and Nordmann, 2006a).Tetracycline resistance is also mediated by transposon-mediated tetracycline-specific efflux pumps encoded by *tetA* and *tetB*, as well as ribosomal protection protein encoded by *tetM*, serving to protect the ribosome from the action of tetracycline (Giamarellou *et al*., 2008; Peleg *et al*., 2008; Perez *et al*., 2007).

1.12 The role of target site modifications

Target site modifications in *A. baumannii* seem to confer resistance to quinolones and aminoglycosides. Topoisomerase IV is the target of quinolones, and mutations at Ser80 and Glu84 of ParC subunit, combined with mutations at Ser83 of gyrA subunit contribute significantly to quinolone resistance (Dijkshoorn *et al.,* 2007; Vila, *et al*., 1997).

Aminoglycosides, on the other hand, bind to a highly conserved motif of 16s rRNA subunit, and methylation of *armA* prevents the antibiotic from binding to its target site, rendering the isolate resistant to aminoglycosides (Dijkshoorn *et al*., 2007; Lee *et al*., 2006; Peleg *et al*., 2008).

Table 1.5 Summary of β-lactamases in *A. baumannii*, their genetic environment and localization

Class of β-lactamase	Gene	Associated genetic structure	Localization
Class A β-lactamases	*bla*TEM-like	Transposons: Tn*1*, Tn*2*, Tn*3*	Plasmid
	*bla*SHV-like	Integrons, Insertion Sequence: IS*26*	Plasmid or chromosomal
	*bla*CTX-M-like	Insertion Sequences, IS*CR1*	Plasmid
	*bla*VEB-like	Integrons	Plasmid or chromosomal
	*bla*GES-like	Integrons	Plasmid or chromosomal
	*bla*PER-like	Integrons, IS*CR1*, Insertion Sequences: IS*4*	Plasmid or chromosomal
Class B β-lactamases	*bla*IMP-like	Integrons	Plasmid
	*bla*VIM-like	Integrons	Plasmid
	*bla*SIM-like	Integrons	Plasmid
	bla_{NDM}	Insertion Sequence: IS*Aba125*	Plasmid
Class C β-lactamases	*bla*AmpC	Insertion Sequence: IS*Aba1*	Chromosomal
Class D β-lactamases	*bla*OXA-23-like	Transposons: Tn*2006*, Tn*2007*, Tn*2008*. Insertion Sequence: IS*Aba1*	Plasmid
	*bla*OXA-40-like	XerC/XerD-like recombination sites	Plasmid or chromosomal
	*bla*OXA-51-like	Insertion Sequences: IS*Aba1*, IS*Aba2*, IS*Aba825*	Chromosomal
	*bla*OXA-58-like	Insertion Sequences: IS*Aba1*, IS*Aba2*, IS*Aba3*, IS*1006*, IS*1008*, IS*Aba825*	Plasmid

1.13 Current status of antibiotic uses

Over seventy five years, the antimicrobial era has been marked by successive discoveries of a wide range of antibiotics and the subsequent emergence of antibiotic resistance. Bacterial resistance continues to increase and, drug researchers and manufacturing industries are not producing new drugs to replace the existing antimicrobials against which resistance has developed. The effects on current infection rates cannot be simply estimated (Carlet *et al*., 2012).

Howard and Scott (2005) reported that the economic impact related to antimicrobial resistance was expected to cost over $105 billion annually worldwide (Howard and Scott, 2005). In recent times, development of antimicrobial resistance is rapidly changing, and the impending public health challenges these may cause in many health sectors need worldwide coordinated interventions. The Europe and the European Centre for Disease Prevention and Control (ECDC) had estimated that 25,000 people may die each year from infections related to antimicrobial resistance (Carlet *et al.*, 2012). The cost burden of

bacterial resistance worldwide may be difficult to quantify currently according to the World Health Organization (WHO) due to limited information on drug resistance investigations carried out between 2013 and 2014 among member states.

Only 17% of countries participated in Africa while in the other continents 62% to 100% responded (Organization, WHO 2015). This has prompted the WHO to adopt new strategies through education and to create awareness for its member states to know the extent of the problem of antimicrobial resistance at each country level. Indeed, the current emergence of carbapenem-resistant *P. aeruginosa* and *A. baumannii* and *Enterobacteriacea*e (PAE) are a source of worry for health care providers due to their limited treatment options, and has become a major public health challenge globally (Schwaber & Carmeli, 2008; Organization, WHO 2015).

1.14 Concern of antibiotic resistance in developing countries

Antibiotic resistance is a global problem, but unfortunately some countries lack the appropriate surveillance and are unaware of the problem. The decline in antibiotic development and the increasing reports of resistance has led public health sectors to release reports of action plans to combat antibiotic resistance as well as proposing incentives for pharmaceutical investments in antibiotic research in the US and Europe (Spellberg *et al*., 2008). This continuous surveillance of the epidemiology of infections and resistance rates, as well as the research undertaken in the developed world is not seen in the developing world. Limited data are available about the prevalence of hospital acquired infections, although the burden of infections is very high (Allegranzi *et al*., 2011). In a review article on prevalence of healthcare associated infections in developing countries by Allegranzi *et al.,* (2011) based on 271 relevant publications from 1995-2008 revealed that the prevalence of healthcare associated infections was much higher than Europe and the USA. Furthermore, Gram-negative pathogens represented the most common etiology of nosocomial infections, but with limited reports of the associated resistance patterns (Allegranzi *et al*., 2011). Interestingly, *Acinetobacter* spp. was the second most frequent pathogen identified in ventilator-associated pneumonia and bloodstream infections.

Recently, reports have been available concerning *A. baumannii* infections in the Middle East, and they have shown considerable heterogeneity in *A. baumannii* isolates from Bahrain, with all three CHDL enzymes present, resulting in the observed resistance to

carbapenems (Mugnier, *et al*., 2009). Similar heterogeneity is seen in *A. baumannii* isolates from a single centre in Cairo, in addition to the presence of several different $bla_{\text{OXA-51-like}}$ enzymes representing different clones. The first report of a plasmid-mediate $bla_{\text{PER-7}}$ was found in a clinical isolate of *A. baumannii* from the United Arab Emirates (Opazo *et al*., 2012). $bla_{\text{NDM-2}}$ has been reported in *A. baumannii* isolates in Egypt, Israel and the UAE (Espinal *et al*., 2011; Kaase *et al*., 2011; Ghazawi *et al*., 2012). American soldiers wounded in Iraq in 2003 were reported to have multi-drug resistant (MDR) *A. baumannii* (Hujer *et al*., 2006). These results indicate two important observations: first of all, there is an increased awareness of the epidemiology of nosocomial infections in the Middle East, and secondly the genome of isolates recovered from this region shows a large degree of diversity.

The lack of funding to undertake research in the developing world is one of the reasons behind the limited of reports available. Furthermore, there are no national surveillance systems, which in turn lead to poor and inaccurate data and un-standardized definitions. There is also limited communication between different healthcare facilities to alert of a certain outbreak. A large surveillance study was done in France and Belgium to contain the VEB-1 outbreak, where all hospitals were alerted managed to control the outbreak (Naas *et al*., 2006). If similar measures are taken in Asia and the Middle East, it would greatly decrease the incidence of nosocomial infections and spread of resistance. There is thus a need to create national and international surveillance systems across Asia and the Middle East.

1.15 Objectives of this study

The specific objectives of this study were:

- To determine the antimicrobial susceptibility profile for each isolate and prevalence of resistance.
- To determine the virulence factors of *P. aeruginosa* and *A. baumannii* to perceive the pathogenicity of these isolates.
- To determine the drug resistance genes bla_{VIM}, bla_{IMP} and $bla_{\text{NDM-1}}$ from these isolates according to the mechanisms of antibiotic resistance among these multi-drug resistant isolates.

- To elucidate whether the production of metallo-β-lactamase (MBL) contributes to imipenem/meropenem resistance in these isolates by sequencing the target gene mentioned.

- To compare the nucleotide and amino acid sequences of the bla_{VIM}, bla_{IMP} and $bla_{\text{NDM-1}}$ genes of *P. aeruginosa* and *A. baumannii* with that of the reference strain.

Hypothesis: The production of metallo-β-lactamase contributes to imipenem/meropenem resistance in the clinical isolates of *P. aeruginosa* and *A. baumannii*. Virulence factors of these strains might associate with the pathogenicity of these clinical isolates. Nucleotide and amino acid sequences changes of specific gene of *P. aeruginosa* and *A. baumannii* might impert with the diverse MDR patterns.

2.0 Materials and Methods

The current study aims to isolate and identify Multi-drug Resistant (MDR) nonfermentative Gram-negative bacteria from clinical sources, and 100 MDR *Pseudomonas aeruginosa* and 100 MDR *Acinetobacter baumannii* were selected to analyze. All the chemicals, reagents and media used in this investigation have been mentioned in Appendix I and II.

2.1 Specimen collection, transportation and storage

A total of 29,136 clinical specimens was collected and processed for culture between January 2012 and December 2015 over a period of three years from patients at the diagnostic unit of icddr,b Dhaka hospital, Bangladesh. These were routine samples received at the specimen reception unit (SRU) of icddr,b Dhaka hospital. As the icddr,b diagnostic laboratories are ISO:15189 accredited so, all the documents are well recorded like specimen collection time, laboratory receiving time, etc. After receiving through Laboratory Information Management System (LIMS) the specimens were processed as soon as possible. If required, the specimens were stored in refrigerated condition not more than 2 hrs for short term and at -80°C freezer for long term storage. Among the specimens, 14,323 were urine samples; 11,378 were blood; 535 were sputum, 413 were tracheal aspirate and 2,487 comprises other body fluids. Year wise sample distribution between male and female patients with age was described in Table 2.1.

Table 2.1 Year wise sample distribution of patient mean age between male and female

Year	Sample type	Number	Sex		Age (Mean±SD) in year	
			Male	Female	Male	Female
2012						
	Urine	3591	2298	1293	52±1.2	48±1.3
	Blood	2852	1796	1056	53±1.1	47±1.5
	Sputum	132	87	45	54±0.8	49±1.2
	Tracheal aspirate	105	68	37	51±1.3	48±1.1
	Otherbody fluid	631	403	228	52±0.9	47±1.3
	Year total	**7311**	**4652**	**2659**		
2013						
	Urine	3827	2411	1416	54±1.3	49±1.1
	Blood	2947	1886	1061	53±1.2	48±1.2
	Sputum	142	93	49	55±1.4	50±0.8
	Tracheal aspirate	108	69	39	52±1.5	47±1.6
	Otherbody fluid	652	410	242	53±1.2	47±1.2
	Year total	**7676**	**4869**	**2807**		
2014						
	Urine	4038	2584	1454	53±1.3	47±1.4
	Blood	3021	1903	1118	54±1.2	48±1.3
	Sputum	153	101	52	54±1.2	49±1.1
	Tracheal aspirate	121	75	46	52±1.6	47±1.7
	Otherbody fluid	701	448	253	55±1.3	48±1.4
	Year total	**8034**	**5111**	**2923**		
2015						
	Urine	2867	1806	1061	53±1.3	48±1.2
	Blood	2558	1586	972	52±1.4	47±1.3
	Sputum	108	70	38	55±1.1	50±0.9
	Tracheal aspirate	79	51	28	51±1.6	46±1.8
	Other body fluid	503	327	176	54±1.5	48±1.6
	Year total	**6115**	**3840**	**2275**		
	Grand total	**29136**	**18472**	**10664**		

SD-Standard Deviation

2.1.1 Isolation and identification of bacterial isolates

All the specimens were processed for culture following standard bacteriological procedure. The biochemical identification was made accordingly as per the demand of the concern physicians or the hospital authority. Briefly, urine specimens were cultured on to Blood and MacConkey's agar plates (BA and MC), sputum, blood, tracheal aspirate, pus and other body fluids were cultured on to Blood agar, Chocolate and MacConkey's agar plates and incubated overnight (Murray *et al*., 2015). Colonies with typical cultural properties were selected and characterized on the basis of Gram staining and biochemical tests. The isolates were tested for the production of oxidase, then subject to biochemical reactions on Kligler Iron Agar (KIA), Motility Indole Urea (MIU) and citrate agar media

(McFaddin 2000). Among the identified isolates, 100 *Pseudomonas* spp. and 100 *Acinetobacter* spp. with MDR patterns were randomly selected for this study. All the selected *Pseudomonas* spp. and *Acinetobacter* spp. were identified elaborately following defined standard biochemical procedures and confirmed by a secondary biochemical battery of API 20NE (bioMeriux).

2.1.2 Analytical Profile Index (API)-20 NE nonfermentating identification systems

The API kit for the identification of nonfermenting bacteria provides an easy way to inoculate and to read tests relevant to members of the family *Pseudomonadaceae* and associated organisms. A plastic strip holding twenty mini-test tubes were inoculated with a saline suspension of 0.5 McFarland standard pure cultures of bacteria (as per manufacturer's directions). A few tubes were completely filled (NO3, TRP etc. figure not shown) and some tubes were overlaid with mineral oil such that anaerobic reactions could be carried out (GLU, ADH, URE). Tubes and cupules are filled for GLU, ARA, MNE, MAN, NAG, MAL, GNT, CAP, ADI, MLT, CIT, and PAC. After incubation in a humid chamber for 18-24 hrs at 35-37°C, the color of reactions were read (some with the aid of added reagents). The oxidase test was done separately. The profile was matched to the profile index of API 20NE software confirming our specific targeted species as *P. aeruginosa* and *A. baumannii* respectively.

2.2 Antimicrobial susceptibility test

Antimicrobial susceptibility of the pathogens was determined using a modified disc diffusion method onto Mueller-Hinton agar. Inoculum was prepared by growing the bacteria for 4-6 hrs in Mueller Hinton broth so that they are in log phase of growth and then adjusted to 0.5 McFarland standards. The tests were performed following Clinical and Laboratory Standard Institute (CLSI 2012) guideline. There was a committee for the selection of antimicrobial drugs to be used for the patients, including hospital physicians and laboratory personnels. As per the committee guideline antibiotics discs (Oxoid Ltd. Basingstock Hamshire, England) used were amikacin 30µg, ceftazidime 30µg, cefixime 5µg, ciprofloxacin 5µg, trimethoprim/sulfamethoxazole 1.25µg, ceftriaxone 30µg, gentamicin 10µg, imipenem 10µg, meropenem 10µg, netilmicin 30µg, nitrofurantoin 300µg, polymyxin-B 300µg and colistin 10µg. The diameter of the zone of inhibition was measured and the isolates were classified as ‘resistant’, ‘intermediate’ and ‘sensitive’ based on CLSI guideline (CLSI 2012).

2.3 Determination of Minimum Inhibitory Concentration (MIC) of Imipenem and Meropenem

Minimum Inhibitory Concentration (MIC) of Imipenem (IMP) and Meropenem (MEM) were determined by the microdilution method according to CLSI guideline. (CLSI 2012).

2.3.1 Preparation of IMP and MEM stock solution

IPM and MEM stock solution were prepared by dissolving 5 mg of IMP and MEM powder (Titan Biotech Ltd., India) in 5 mL (1 mg/mL) 0.01M phosphate buffer having a pH value of 7.2 (Appendix II) to obtain a concentration of 1mg/mL respectively. The solutions were sterilized by membrane filtration using 0.22 µm filter. Aliquots of stock solution were prepared in 1.5 mL eppendorf tubes and stored at -80°C immediately after preparation for further use.

2.3.2 Microdilution methods

To determine the level of MIC of IMP and MEM for selected isolates, each well of 96 well microtiter plates were filled with 90 µl of Mueller-Hinton broth and supplemented with different concentration of IMP and MEM (2,4,8,16,32,64,128,256,512) from stock solution. Each isolate was grown in 5 mL of Mueller-Hinton broth without IMP or MEM for 3-6 hrs at 35-37°C on a rotary shaker (120 rpm) to obtain 0.5 McFarland standard. Ten microlitter of bacterial inoculums were placed in each respective well, so that the final inoculums were $5x10^5$CFU/mL. One well of each row was set as a negative control (respective medium only) and an MDR *K. pneumoniae* (Islam *et al*., 2012) as a positive control. Microtiter plates were incubated at 35-37°C for 18-24 hrs. Bacterial growth and density were measured using EZ Read 400 Microplate Reader (Poweam) at 600nm. The experiment was done in duplicate (Wiegand *et al*., 2008).

2.4 Selection of Multi-drug Resistant (MDR) strains

The strains of *P. aeruginosa* and *A. baumannii* showing resistant to at least one agent in three or more antimicrobial categories were included in the study as MDR strains for our investigation (Magiorakos *et al*., 2012).

2.5 Data collection and strain preservation

Retrospective analysis of data was carried out from laboratory records at the Clinical Microbiology and Immunology Laboratory, from January 2012 to December 2015. Age, sex and antimicrobial susceptibility pattern of 13 drugs were recorded for all of 100 *P.*

aeruginosa and 100 *A. baumannii* MDR strains isolated during the study period, according to icddr,b hospital guideline.

2.6 Phenotypic detections of carbapenemase

Modified Hodge test (MHT) was used to determine carbapenemase production. The presence of a 'cloverleaf shaped' zone of inhibition due to carbapenemase production by the test strain will be considered as positive (Noyal *et al*., 2009). An inoculum of *E. coli* ATCC 25922 was prepared and incubated for 2 hrs and adjusted to 0.5 McFarland standard and was inoculated on an MHA plate. After drying, 10 µg meropenem disk was placed at the centre of the plate and the test strain was streaked from the edge of the disk to the periphery of the plate in four different directions. The plate was incubated overnight at 35-37°C. The presence of a 'clover leaf' zone of inhibition due to carbapenamase production by the test strain was considered as positive. MDR *K. pneumoniae* (Islam *et al*., 2012) was used as a positive control.

2.7 Phenotypic detections of metallo-β-lactamase (MBLs)

Mettalo-β-lactamase (MBLs) was determined by EDTA double disk synergy (EDDS) test. The presence of an expanded growth inhibition zone between the two disks were interpreted as positive for MBL production (Noyal *et al*., 2009). EDDS test was done with two different β-lactam (meropenem and ceftazidime) and EDTA disk. A 0.5 M EDTA solution was prepared by dissolving 1.86 g of disodium EDTA in 10 mL distilled water. The pH was adjusted at 8.0 and was sterilized by autoclaving. An inoculum of test strain was prepared and incubated for 2 hrs and ajusted to 0.5 McFarland standard and was inoculated on a MHA plate. After drying, a 10 µg meropenem or 30 µg ceftazidime disk was placed on the agar. A blank disk (6 mm in diameter) was impregnated with 10 µL of 0.5 M EDTA. This disk was placed on previously inoculated plate and was kept 10 mm edge-to-edge apart from the meropenem or ceftazidime disk. After incubating overnight at 35-37°C, the presence of an expanded growth inhibition zone between the two disks was interpreted as positive for MBL production. MDR *K. pneumoniae* (Islam *et al*., 2012) was used as a positive control and *P. aeruginosa* ATCC 27853 was used as a negative control.

2.8 Phenotypic detections of AmpC MBL by AmpC disk test

An inoculum of *E. coli* ATCC 25922 was prepared as before and after drying, a 30 µg cefoxitin disk was placed on to MHA plate and a blank disk (6 mm in diameter) was moitened with sterile saline and inoculated with a few colonies of test strain. This disk was

placed beside the cefoxitin disk almost touching it. A flattening or indentation of the cefoxitin inhibition zone in the vicinity of the disk with test strain were interpreted as positive for the production of AmpC β-lactamase (Noyal *et al*., 2009). An undistorted zone were considered as negative. MDR *K. pneumoniae* (Islam *et al*., 2012) was used as a positive control and *P. aeruginosa* ATCC 27853 was used as negative control.

2.9 Examination of virulence factors of *P. aeruginosa* and *A. baumannii*

2.9.1 Detection of hemolytic activity

Hemolytic activity of the isolates of *P. aeruginosa* and *A. baumannii* was performed by streaking the isolates on 5% sheep blood agar plate (Cota-Gomez, *et al*. 1997). The plates were incubated overnight at 35°C, and the diameter of the zones of hemolysis was compared, to detect whether hemolytic activity was present or not. *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 was used as a positive and negative control respectively.

2.9.2 Detection of capsule production

Clean and greeze free three glass slides were taken. Using sterile technique a heavy smear was prepared on each slide with different test organisms after growing in brain heart infusion broth for 72 hrs. The smears were allowed to air dry. The smears were flooded with crystal violet and let stand for 5 min. The smears were washed with 20% copper sulphate solution (Schooley *et. al*., 2017). Gently blot dried and examined under oil immersion magnification.

2.9.3 Detection of invasiveness (keratoconjunctivitis assay) by Sereny test

The Sereny test was performed according to the procedure described elsewhere (Sasakawa,*et al.*, 1986), briefly, overnight culture of bacteria, suspended to a density of approximately 10^{10} viable cells in 20 µL of phosphate-buffered saline (PBS), was dropped into the conjunctival sac of the guinea pig. The other eye served as the control and was dropped only PBS. The guinea pigs were observed daily for 72 hrs and their inflammatory responses were graded. This experiment was carried out in the animal resources facility lab of icddr,b obliging the ethical issues set by this organization.

2.9.4 Detection of cytotoxicity (rabbit Ileal loop assay)

The cell pellet of *P. aeruginosa* and *A. baumannii* were washed with PBS (pellet was suspended in 5 mL normal saline and centrifuged at 12,000 X g for 10 min) and repeated

for three times. The washed pellet was then re-suspended in 5 mL of PBS. The cells were placed in an ice cool chamber and sonicated by ultrasonic vibration (30 sec x 15 min). Sonicated samples were then centrifuged at 12,000 X g for 20 min at 10°C and supernatant was then filtered through the 0.22 µm millipore membrane filter and was stored at -20°C aliquoting in eppendrof tubes.

The rabbit ileal loop assay was performed according to the method described earlier (Singh and Sanayl, 1978). Briefly, adult albino rabbit (New Zealand) of about 9-10 weeks age of nearly 2 kg of body weight were starved for 48 hrs allowing water only. After proper anesthesia with lower doses of pentobarbital sodium (0.5 mL/kg body weight, intravenous), the intestine was exposed and loops of 5-7 cm in length with 3-5 cm intervals between each were tied beginning near the ileocaecal junction. One hundred µL supernatant of each strain was inoculated in each loop and usually 5 loops were made in one rabbit. The animals were sacrificed after 18 hrs with excess of pentobarbital sodium. The length of each loop and the volume of fluid accumulation were measured to determine the amount of fluid accumulated per unit length of the gut. Each test was done in two rabbits. The results were considered valid only if the positive and negative controls gave appropriate responses in each animal. Animal experiments were carried out in the animal resources facility lab of icddr,b obliging the ethical issues set by this organization.

2.10 Extraction of DNA from the subculture

P. aeruginosa and *A. baumannii* strains from the stocks were subjected to subculture on Tryptic Soya Agar (TSA) plates and the plates were allowed to incubate 24 hrs at 35-37°C for vigorous growth. The sterile 1.5 mL eppendorf tubes were taken and each tube was filled with 1 mL (1000 µL) USP water. A loopful of *P. aeruginosa* and *A. baumannii* culture were suspended in water inside the tube respectively and homogeneous mixer was prepared by vortexing spending at least 2 min. Then the uniform mixer was boiled for 10 min at 100°C in a water bath and subsequently the tubes were kept at -20°C for about 10 min for an abrupt and explosive heat shock. After this operation the tubes were centrifuged at 2500 rpm for 10 min at 25°C.

After centrifugation, the tubes were kept in an ice box to protect DNA from breaking down. 200 µL of supernatant from each eppendorf tube was transferred into a new sterile tube and the extracted DNAs were preserved at -80°C. A $bla_{\text{NDM-1}}$ positive strain

(*Klebsiella pneumoniae*) (obtained from Islam *et al*., 2012) was used as a positive control in our study and *P. aeruginosa* ATCC 27853 was used as a negative control.

2.11 MBL primer

The MBL primers NDM-1, IMP, and VIM used in this study were described in Table 2.2.

Table 2.2 MBL primers used in this study with product size

Primers	Nature	Primer sequence (5′→3′)	Bases	Tm value (°C)	PCR Product (bp)	Source of reference
NDM-1	forward	CTTCCAACGGTTTGATCGTC	20	51.8	465	Islam *et al*., 2012
	reverse	TAGTGCTCAGTGTCGGCATC	20	53.8		
IMP	forward	GTTTATGTTCATACWTCG	18	41.2	432	Amudhan *et al*.,2012
	reverse	GGTTTAAYAAAACAACCAC	19	43.5		
VIM	forward	TTTGGTCGCATATCGCAACG	20	51.8	500	Amudhan *et al*.,2012
	reverse	CCATTCAGCCAGATCGGCAT	20	53.8		

Tm-Melting temperature

2.12 Polymerase Chain Reaction (PCR) of *bla*$_{\text{NDM-1}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ genes

All the IMP and/or MEM resistant isolates were subjected to amplify the *bla*$_{\text{NDM-1}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ genes by PCR. Positive samples were then purified and sequenced. The sequence alignment and phylogenetic analysis were done for further analysis. To find out the presence of *bla*$_{\text{NDM-1}}$, *bla*$_{\text{VIM}}$ and *bla*$_{\text{IMP}}$ genes, PCR was carried out in a thermocycler (Eppendorf, Germany). Besides a standard PCR setting include 5 µL of 5 X PCR reaction buffer with $MgCl_2$-15mM (Biolabs, New England); 10mM deoxynucleotide triphosphates (dNTP) (Fermentas) and Taq DNA polymerase (3 U/mL) (Biolabs, New England). Our standardized PCR conditions described below. The master mix for the monoplex PCR was prepared following Table 2.3 and mixed well by vortexing. Then 20 µL of master mix was transferred to each tube. Finally, 5 µL of DNA template of each clinical isolate was added and mixed by artful repeated pippeting. The PCR tubes were transferred to Mastercycler gradient (Eppendorf, Germany) to run PCR following our standardized conditions. The cycling parameter of PCR program used for amplification of target DNA. Amplification reactions were carried out under the following conditions: initial denaturation at 94°C for 5 min and 36 cycles of 1 min at 94°C, 30 sec at 55°C and 45 sec at 72°C, followed by 7

min at 72°C for *bla*$_{NDM-1}$ gene and initial denaturation of 5 min at 94°C and 36 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 7 min at 72°C for *bla*$_{VIM}$ and initial denaturation of 5 min at 94°C and 36 cycles of 1 min at 94°C, 1 min at 45°C, and 1 min at 72°C, followed by 7 min at 72°C for *bla*$_{IMP}$ gene respectively.

Table 2.3 Reaction mixture of the monoplex PCR for the detection of *bla*$_{NDM-1}$/ *bla*$_{IPM}$/ *bla*$_{VIM}$ gene

Reagents	For each sample
5X Buffer ($MgCl_2$ 15mM)	5.0µl
PCR grade water	13.55µl
dNTP (10mM)	0.5µl
Primers (10µM/µl)	
NDM-1-forward	0.4µl
NDM-1-reverse	0.4µl
Taq Polymerase	0.15µl
DNA template	5.0µl
Total volume	25.0µl

2.13 Post-PCR detection of amplified DNA by electrophoretic analysis

The successful amplifications of *bla*$_{NDM-1}$, *bla*$_{IMP}$ and *bla*$_{VIM}$ genes were done by resolving the PCR product on 1% agarose gel. The 1 X TBE buffer was used to dissolve agarose (Invitrogen USA) to make 1% gel (W/V) through boiling at 100°C for 10 min. Then 3µL Ethidium Bromide (EtBr) was added before pouring on a gel tray. Two combs were set and the gel was poured on gel tray and allowed to solidify. After solidification of the gel, combs were removed and the gel was dipped into the 1X TBE buffers of the electrophoretic apparatus. Ten microlitter of PCR product was mixed with 1µL of sample loading buffer 5X (Bio-RAD) and finally 10 µL mixer was taken to load the each gel slot. 100mA was applied to move the DNA through the gel and the EtBr stained DNA bands were observed on a UV transilluminator (Gel Doc, Bio-Rad, USA). The photographs of gels were captured by Gel Doc, Bio-Rad (USA) machine attached to a computer and the product sizes were estimated using 1 kb (50 µg/mL) DNA molecular ladder (Bio-RAD).

2.14 Sequence analysis

2.14.1 Sequencing of amplified product

Sequencing was done using dideoxy nucleotide chain termination method with the ABI PRISM BigDye terminator cycle sequencing reaction kit (Perkin-Elmer Applied Biosystem, Foster City, Calif) on an automated sequencer (ABI PRISM 310). The consensus forward and/or reverse primers 5'TTTGGTCGCATATCGCAACG3' for VIM and 5'CTTCCAACGGTTTGATCGTC3' for NDM-1 were used to sequence the specific gene.

2.14.2 Cycle sequence

Cycle sequence was performed according to the following Table 2.4.

Table 2.4 Reaction mixture composition for cycle sequencing

Reagents	Quantity (μL)
Termination ready reaction mixes (BigDye)	4.0
Template	1.0
Primer (forward or reverse 5 pmol)	1.0
Deionized water	4.0
Total volume	10

2.15 Bioinformatics tools for sequence analysis

Available sequences were analyzed using Chromas, SeqMan, ClustalW, BioEdit Sequence Alignment Editor and MEGA Bioinformatics tools.

2.15.1 Sequence editing by Chromas tool

The chromatogram sequences were inspected with Chromas 2.3 (Technelysium, Australia), and Multiple sequence alignment was performed using ClustalW Multiple Alignment in Bioedit (Hall, 1999), version 7.1.3, and manually edited there.

2.15.2 Consensus preparations by SeqMan tool

After editing by Chromas tool both the forward and reverse sequences were aligned by SeqMan tool and saved the file as fasta format.

2.15.3 Sequence checking by ExPASy for appropriate protein expression

The fasta format file containing both forward and reverse sequences were checked by ExPASy web for the appropriate protein sequences.

2.15.4 BLAST search and collect similar sequences from global database and alignment preparation

To identify the similarity of sequences all the sequences were submitted to online BLAST (basic local alignment search tool) program at the National Center for Biotechnology Information website (available at: *http://www.ncbi.nlm.gov/BLAST/*). Sequences were edited using the BioEdit (Hall, 1999). Then the sequences were aligned with reference sequences using ClustalW Multiple Alignment in Bioedit software, version 7.1.3 (Hall, 1999).

The chromatogram sequencing files were inspected using Chromas 2.3 (Technelysium, Queensland, Australia). Nucleotide and amino acid sequence similarity searches were performed using the National Center for Biotechnology Information (NCBI; National Institute of Health, Bethesda, MD) BLAST (basic local alignment search tool) server on the GeneBank database, release 145.0 (Altschul *et al*., 1990). Sequence data were edited by SeqMan II (DNASTAR, Madison). Multiple sequence alignments were calculated using CLUSTALW and CLUSTALX (Thompson *et al*., 2002). The flowchart for sequence analysis is given below in Figure 2.1:

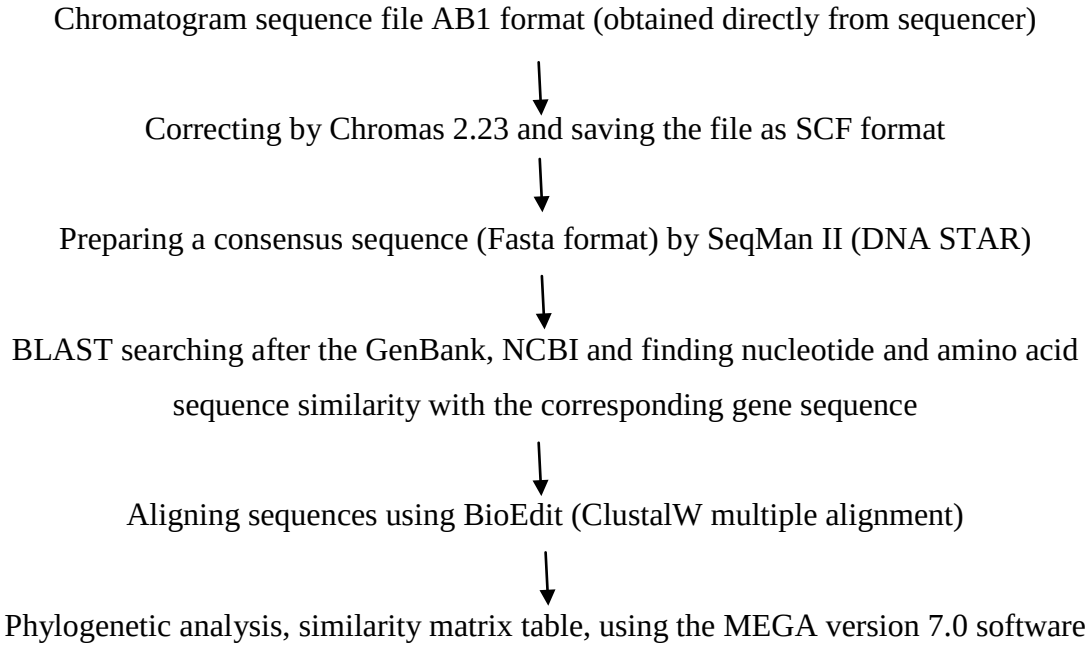


Figure 2.1 Flowchart for sequence analysis

2.16 Sequence alignment and identification

Sequences, obtained by using forward and reverse primers, were combined to full length sequences via the SeqMan Genome Assembler (DNA star, USA) and were compared to the GenBank database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/GenBank) by means of the basic local alignment search tool (BLAST) to identify close phylogentic relatives.

2.17 Construction of similarity matrix

The similarity percentages between nucleotide sequences were calculated by the p-distance model using MEGA software version 7.0 (Kumar, *et al.*, 2016)

2.18 Construction of phylogenetic tree

Phylogenetic and molecular evolutionary analyses of the $bla_{\text{NDM-1}}$ and bla_{VIM} genes were constructed using the following bioinformatics software:

Reference sequences were downloaded from

a. -NCBI: http://www.ncbi.nlm.nih.gov
   -EMBL: http://www.ebi.ac.uk
   -DDBJ: http://ddbj.nig.ac.jp

b. Acquired sequences were aligned by using
   -ClustalW
   -MEGA 7.0

c. Phylogenetic tree was constructed by
   -MEGA 7.0

Briefly, the multiple sequence alignment of the retrieved reference sequences from the NCBI and representative isolates sequences were performed by ClustalX software. Aligned sequences were exported to the Molecular Evolutionary Genetics Analysis (MEGA7) (Kumar *et al*., 2016) software for phylogenetic tree construction using the Neighbor-joining method. Further analyses of the genes were carried out using the Distance and Pattern analysis tool in the MEGA software. A Bootstrap consensus tree was inferred from 1000 replicates (Felsenstein 1985).

2.19 DNA Sequences and amino acid sequences comparison

Sequences obtained in this study were compared with reference sequences using BioEdit program after clustalW multiple alignment. Nucleotide sequences were converted into

amino acid sequences by using an ExPaSy translating tool. From that sequence provided amino acid without interval were selected as a corresponding amino acid sequence and these sequences were further checked by protein BLAST (BLASTp). Finally, all amino acid sequences obtained in this study were compared with reference sequences using BioEdit program after clustalW multiple alignment.

2.20 Nucleotide sequence accession number

The nucleotide sequences reported in this study were submitted to GenBank, the NCBI by using Sequin, Version 6.50 under accession number of *A. baumannii* MN226842 to MN226846 (a total of 5 sequences) and for *P. aeruginosa* MN256618 to MN 256633 (a total of 16 sequences). In this thesis these accession numbers were used in tables and figures in the results section and the detailed was given in Appendix II.

2.21 Statistical data analysis

Analyses of the data were performed using the Statistical Package for Social Sciences (SPSS, version 20.0 for Windows, SPSS Inc. Chicago, IL, USA). Student's t-test was used to test the null hypothesis that there is no significant difference between each individual parameter measured in the control and treatment groups over time. Difference was considered to be significant if $p \leq 0.05$.

3. Results

A total of 29,136 clinical samples was collected, processed and cultivated in bacteriological media. The samples were taken from various clinical sites of the body depending upon the type of infection (detail is given in the previous section). All the samples were processed and pathogens were identified as per hospital's recommendation and need. Among the identified isolates, only *Pseudomonas* spp. and *Acinetobacter* spp. were considered for this study.

3.1 Isolation and identification of *Pseudomonas* spp. and *Acinetobacter* spp.

Among 29,136 processed specimens 2,340 (8%) were identified as *Pseudomonas* spp. and 1,073 (4%) were identified as *Acinetobacter* spp. Primarily, the *Pseudomonas* and *Acinetobacter* spp. were screened by selecting non-lactose fermenting (NLF) colonies (Figure 3.1) and their morphologies on MacConkey agar plates.

The isolates were confirmed as *Pseudomonas* spp. and *Acinetobacter* spp. by conventional biochemical tests using KIA, MIU and Simmon citrate agar and later confirmed by API 20NE (data not shown).

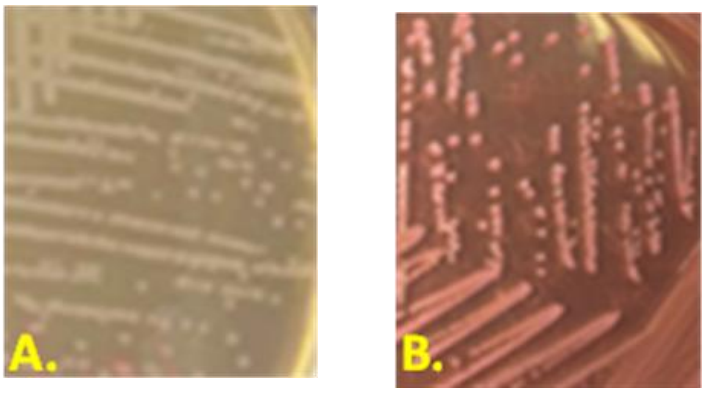


Figure 3.1 Characteristic colony morphology of *Pseudomonas* spp. and *Acinetobacter* spp. on MacConkey agar plate. A. *Pseudomonas* spp. and B. *Acinetobacter* spp.

Off the 2,340 isolates of *Pseudomonas* spp., the highest percentage was identified from different types of body fluids, accounting nearly 40% of total pseudomonads isolates. Next highest percentages of pseudomonads were obtained from urine and blood samples, which accounts for more than 22 and 18%, respectively. Sputum yielded the least number of *Pseudomonas* spp. (9.2%). A total of 1,073 isolates (4% of all samples) was identified as *Acinetobacter* spp. Here also the highest number of isolates was obtained from body fluids and the sputum being the lowest. The details are given in the Table 3.1.

Table 3.1 Specimen specific distribution of *Pseudomonas* spp. and *Acinetobacter* spp. among male and female

Specimen	Female		Male		Total	
	***Pseudomonas* spp.**	***Acinetobacter* spp.**	***Pseudomonas* spp.**	***Acinetobacter* spp.**	***Pseudomonas* spp. (%)**	***Acinetobacter* spp. (%)**
Tracheal aspirate	83	79	155	182	238(10.2)	261(24.3)
Urine	183	64	338	144	521(22.3)	208(19.4)
Sputum	76	38	140	84	216(9.2)	122(11.3)
Blood	153	39	283	84	436(18.6)	123(11.5)
Other body fluid	330	110	599	249	929(39.7)	359(33.4)
Total	825	330	1515	743	2340(100)	1073(100)

There were 20 wells in API 20NE and these contained different biochemical reagents. After overnight incubation with the inoculums, wells were read with/without reagents and every three wells produced a numeric number and ultimately species identification was done by a seven numbered containing the profile. We performed API 20NE for 100 MDR *Pseudomonas* spp. and 100 *Acinetobacter* spp. previously confirmed by conventional biochemical tests and among 100 *Pseudomonas* spp. all were confirmed as *Pseudomonas aeruginosa* and 100 *Acinetobacter* spp. were confirmed as *Acinetobacter baumannii*.

3.2 Antimicrobial susceptibility patterns of *Pseudomonas* spp. and *Acinetobacter* spp.

All the isolates of pesudomonads and *Acinetobater* spp. were subjected to antimicrobial susceptibility test to a number of antibiotics using modified Kirby-Baur method. Antimicrobial resistance irrespective of specimen sources showed that 71% of the *Pseudomonas* spp. were resistance to cefixime, 70% to ceftriaxone, 60% to gentamicin, 56% to piperecillin+tazobactam, 55% to cotrimoxazole, 50% to ciprofloxacin, 49% to amikacin, 46% to netilmicin, 45% to ceftazidime, 30% to meropenem, 26% to imipenem and 19% to polymyxin B. However, the *Pseudomonas* spp. isolated from urine samples showed higher resistance by 86% to nitrofurantoin (Figure 3.2).

On the other hand, 82% of *Acinetobacter* spp. showed resistance to ceftriaxone, 78% to cefixime, 65% to ciprofloxacin, 64% to ceftazidime, 64% to cotrimoxazole, 62% to

gentamicin, 58% to amikacin, 48% to meropenem, 47% to imipenem, 37% to netilmicin, 13% to colistin and 1% to polymyxin B. However, the *Acinetobacter* spp. isolated from urine samples showed greater resistance, by 84% to nitrofurantoin (Figure 3.2).

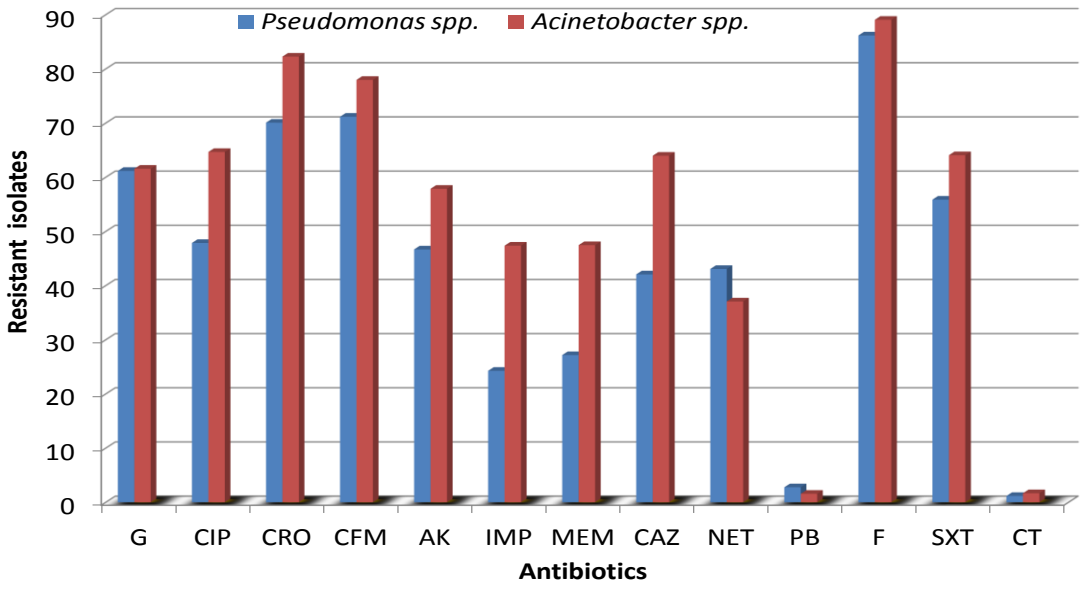


Figure 3.2 Antibiogram of *Pseudomonas* spp. and *Acinetobacter* spp.: G-Gentamicin, CIP-Ciprofloxacin, CRO-Ceftriaxone, CFM-Cefixime, AK-Amikacin, IMP-Imipenem, MEM-Meropenem, CAZ-Ceftazidime, NET-Netilmicin, PB-Polymyxin B, F-Nitrofurantoin, SXT-Cotrimoxazole, CT-Colistin.

3.3 MDR selection criteria

The bacterial isolates which showed resistance to at least one agent in three or more antimicrobial categories were considered as MDR. The sample size of these selected clinical isolates was 100 for each of the genera which comprised *P. aeruginosa* and *A. baumannii*.

The isolates of *P. aeruginosa* and *A. baumannii* which showed resistance to at least one main classes of antibiotics, namely carbapenem, cephalosporine, aminoglycoside, and fluoroquinolone were considered. For each type of bacteria 100 MDR isolates were included in this study and performed further characterization. Distribution of 100 *P. aeruginosa* and 100 *A. baumannii* among specimen and gender were shown in Table 3.2.

The selected isolates of *P. aeruginosa* and *A. baumannii* showed high MIC values. Selected 16 *P. aeruginosa* showed 256 µg/L against imipenem as well as meropenem and 6 *A. baumannii* showed 256 µg/L against imipenem and 128 µg/L against meropenem.

Table 3.2 Distribution of 100 *P. aeruginosa* and 100 *A. baumannii* isolated from various clinical specimens of male and female

Specimen	Female		Male		Total	
	P. aeruginosa	***A. baumannii***	***P. aeruginosa***	***A. baumannii***	***P. aeruginosa***	***A. baumannii***
Tracheal aspirate	25	15	37	26	62	41
Urine	2	6	6	13	8	19
Sputum	2	1	4	3	6	4
Blood	2	3	2	2	4	5
CSF	1	-	-	-	1	-
Other body fluid	3	5	16	26	19	31
Total	35	30	65	70	100	100

3.4 Antimicrobial resistance patterns of 100 MDR *P. aeruginosa* and 100 MDR *A. baumannii*

When the antimicrobial resistance patterns were analyzed for the selected 100 MDR strains of *P. aeruginosa*, a different picture was revealed. It was found that among 100 MDR *P. aeruginosa*, 2% and 6% of the isolates were resistant to 12 and 11 antibiotics respectively, whereas 46% of the isolates showed resistance to 10 antibiotics (Table 3.3). Only 6% of the isolates showed resistance against seven antibiotics namely gentamicin, cotrimoxazole, ciprofloxacin, ceftriaxone, cefixime, amikacin and imipenem.

Table 3.3 Antimicrobial resistance profile of 100 MDR *P. aeruginosa* isolated from different sample sources

Resistance or susceptibility profile	% of isolates in sample from				
	Urine	Blood	Sputum	Tracheal aspirate	Other body fluid
	n=8	n=4	n=6	n=62	n=20
G-CO-CI-CR-CF-A-I-M-CZ-N-P-CT	-	-	-	-	2
G-CO-CI-CR-CF-A-I-M-CZ-N-P	2	-	-	3	1
G-CO-CI-CR-CF-A-I-M-CZ-N	2	2	1	27	14
G-CO-CI-CR-CF-A-I-M-CZ-N	1	1	1	12	2
G-CO-CI-CR-CF-A-I-M-CZ	1	-	3	9	1
G-CO-CI-CR-CF-A-I-M	1	1	1	6	-
G-CO-CI-CR-CF-A-I	1	-	-	5	-

n, number; %, percentage; G, Gentamicin; CO, Cotrimoxazole; CI, Ciprofloxacin; CR, Ceftriaxone; CF, Cefixime; A, Amikacin; I, Imipenem; M, Meropenem; CZ, Ceftazidime; N, Netilmicin; P, Polymyxin B; CT, Colistin.

On the other hand, among 100 MDR strains of *A. baumannii*, 2% and 3% of the isolates showed resistant to 12 and 11 antibiotics, respectively, whereas 60% isolates showed resistance to ten antibiotics (Table 3.4). Only 2% isolates showed resistance to 7 antibiotics, namely gentamicin, cotrimoxazole, ciprofloxacin, ceftriaxone, cefixime, amikacin and imipenem.

Table 3.4 Antimicrobial resistance profile of 100 MDR *A. baumannii* isolated from different sample sources

Resistance or susceptibility profile	% of isolates in sample from				
	Urine	Blood	Sputum	Tracheal aspirate	Other body fluid
	n=19	n=5	n=4	n=41	n=31
G-CO-CI-CR-CF-A-I-M-CZ-N-P-CT	-	-	-	1	1
G-CO-CI-CR-CF-A-I-M-CZ-N-P	-	-	-	1	2
G-CO-CI-CR-CF-A-I-M-CZ-N	6	2	1	25	26
G-CO-CI-CR-CF-A-I-M-CZ	8	3	-	12	1
G-CO-CI-CR-CF-A-I-M	5	-	2	1	1
G-CO-CI-CR-CF-A-I	-	-	1	1	-

n, number; %, percentage; G, Gentamicin; CO, Cotrimoxazole; CI, Ciprofloxacin; CR, Ceftriaxone; CF, Cefixime; A, Amikacin; I, Imipenem; M, Meropenem; CZ, Ceftazidime; N, Netilmicin; P, Polymyxin B; CT, Colistin.

3.5 Carbapenemase positivity of *P. aeruginosa* and *A. baumannii* by Modified Hodge Test (MHT)

Carbapenems are the last resort for treating MDR *P. aeruginosa* and *A. baumannii* but resistance against carbapenems emerged producing novel enzyme carbapenamases. So, we did the Modified Hodge test to determine the carbapenamase. Among the one hundred tested *P. aeruginosa*, 48% showed carbapenemase positive. Strains isolated from tracheal aspirate (TA) showed highest (24%) positivity. Of the 100 *A. baumannii*, 32% was positive and tracheal aspirate showed maximum (22%) positivity (Figure 3.3).

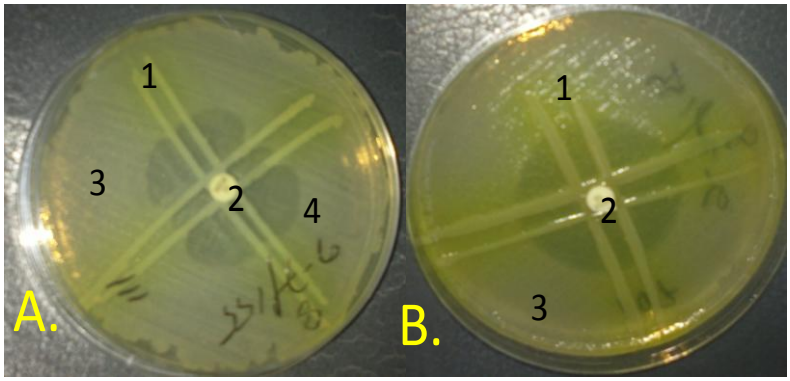


Figure 3.3 Determination of MHT: 1-Test strain, 2-Meropenem disk, 3-*E. coli* ATCC 25922, 4-Clover leaf shaped inhibition. A. MHT positive and B. MHT negative.

3.6 AmpC β-lactamase positivity of *P. aeruginosa* and *A. baumannii*

Sometimes resistance is due to inducible AmpC β-lactamase. This is chromosome mediated β-lactamase. Off 100 studied *P. aeruginosa*, 34% of the strains gave positive reaction for AmpC β-lactamase, whereas, 25% of MDR *A. baumannii* showed a positive reaction (Figure 3.4).

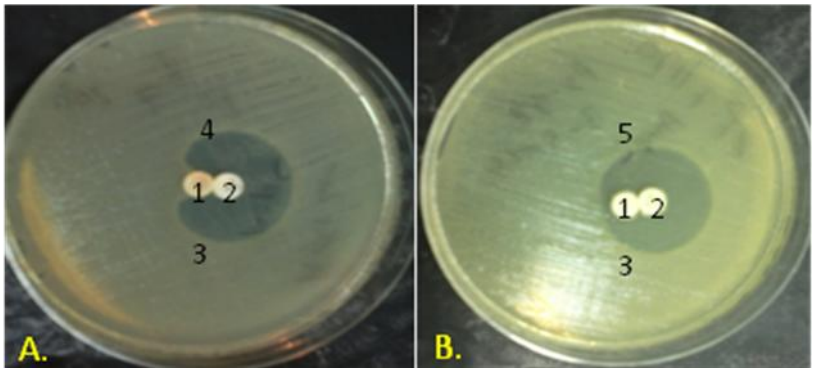


Figure 3.4 Determination of AmpC β-lactamase : 1-Disk with test strain, 2-Cefoxitin disk, 3-*E. coli* ATCC 25922, 4-Indentation of Cefoxitin zone, 5-Undistorted zone. A. AmpC β-lactamase positive and B. AmpC β-lactamase negative.

3.7 Metallo-β-lactamase positivity of *P. aeruginosa* and *A. baumannii*

Among the one hundred tested *P. aeruginosa*, 92% showed metallo-β-lactamase positive results of EDTA Double Disc Synergy (EDDS) test (Figure 3.5). The highest rate of metallo-β-lactamase producer (38%) was found from tracheal aspirate followed by strains from other body fluids (30%) and urine (17%), respectively (Table 3.5).

The positivity of EDDS test was revealed in 35% of *A. baumannii* and the highest rate was found to be from the isolates of tracheal aspirate (21%).

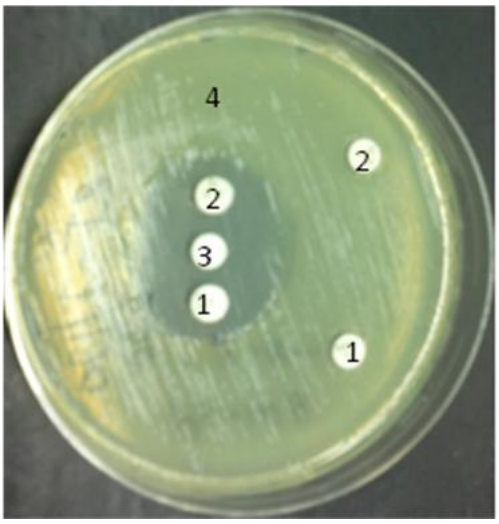


Figure 3.5 Determination of EDTA Double Disc Synergy (EDDS) test: 1-Meropenem disk, 2-Ceftazidime disk, 3-EDTA disk, 4-Test strain. Meropenem and Ceftazidime alone cannot produce any zone of inhibition, but in combination with EDTA they produce a zone of inhibition.

Table 3.5 Summary of EDDS test for 100 *P. aeruginosa* and 100 *A. baumannii*

Types of specimen	Type of EDTA Double Disc Synergy (EDDS) test							
	CAZ (%)		CAZ+MEM (%)		MEM (%)			
	PA	AB	PA	AB	PA	AB	PA	AB
Tracheal aspirate	2	5	3	5	33	11	38	21
Urine	1	-	7	-	9	1	17	1
Sputum	-	1	2	1	2	-	4	2
Blood	1	-	1	-	1	3	3	3
CSF	-	-	-	-	-	1	-	1
Other body fluid	4	1	9	1	17	5	30	7
Total	8	7	22	7	62	21	92	35

CAZ-Ceftazidime; MEM-Meropenem; PA- *P. aeruginosa*; AB- *A. baumannii*

3.8 Virulence properties of selected *P. aeruginosa* and *A. baumannii*

In our study, we observed the various virulence properties including hemolysin production, formation of capsule, invasiveness, cytotoxic activity of our studied organisms *P. aeruginosa* and *A. baumannii* isolates.

Hemolytic activity

The obtained results showed the strong hemolytic activity possessed by the test strains of both *P. aeruginosa* 94% (15/16) and *A. baumannii* 100% (6/6) (Figure 3.6) in compared with positive control strain *S. aureus* ATCC 25923.

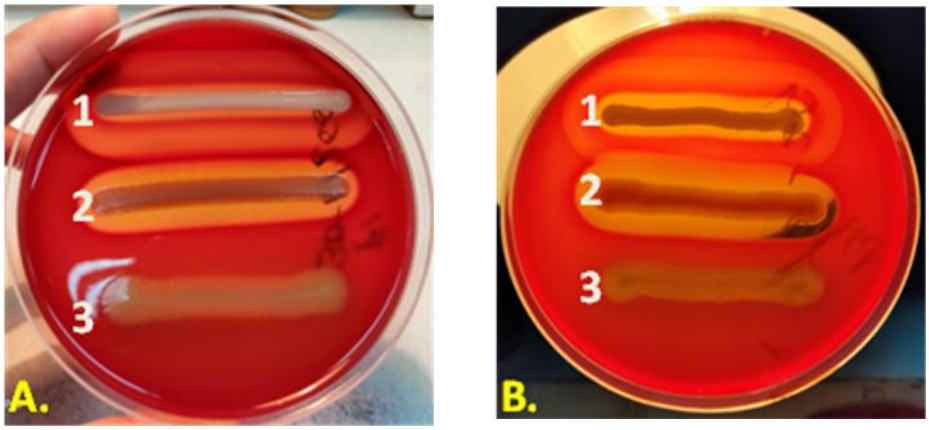


Figure 3.6 Hemolytic activity of *P. aeruginosa* (A) 1-positive control; 2-test strain MN256619 *P. aeruginosa*; 3-negative control; and *A. baumannii* (B) 1-positive control; 2-test strain MN226843 *A. baumannii*; 3-negative control.

Capsule formation

In this study, we observed for both *P. aeruginosa* and *A. baumannii* can produce capsule during their growth. Only *A. baumanni* showed the formation of a capsule (Figure3.7). When observed the cells under the microscope and we found that not all the cells equally producing the capsule. This might be due to the dissimilarity of the growth rate of individual cells.

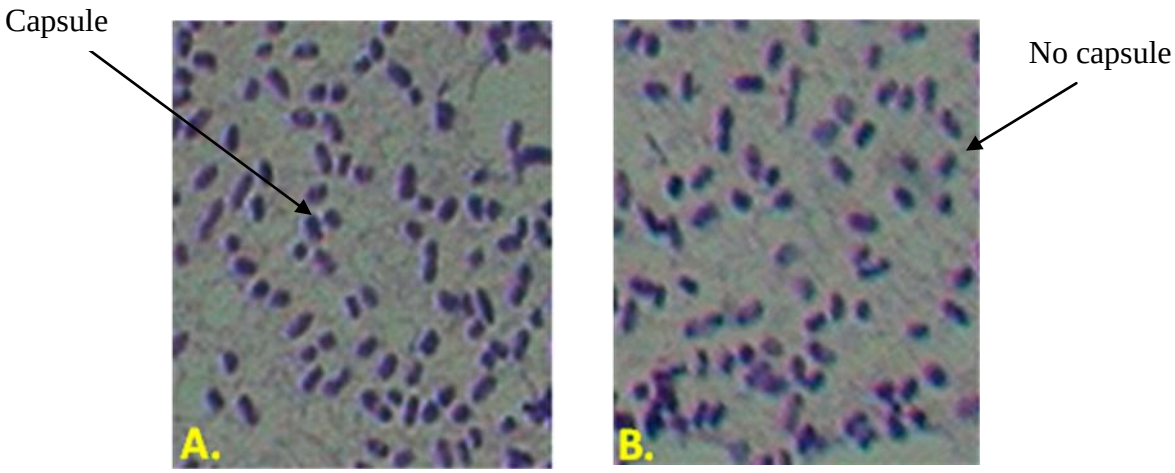


Figure 3.7 Capsule staining of strain MN226843 *A. baumannii* showed capsule production (A) and no capsule production of strain MN256619 *P. aeruginosa* (B).

Invasiveness

Strains producing hemolysin and capsule were tested for invasiveness. The invasiveness test was performed in guinea pig eyes (Figure 3.8) described in materials and methods. We checked the invasiveness of both *P. aeruginosa* and *A. baumannii*, however, only *A.*

baumannii showed the keratoconjunctivitis in the guinea pig eyes after 48 hrs of inoculation (Figure 3.8 B). This result indicated the invasive nature of this strain.

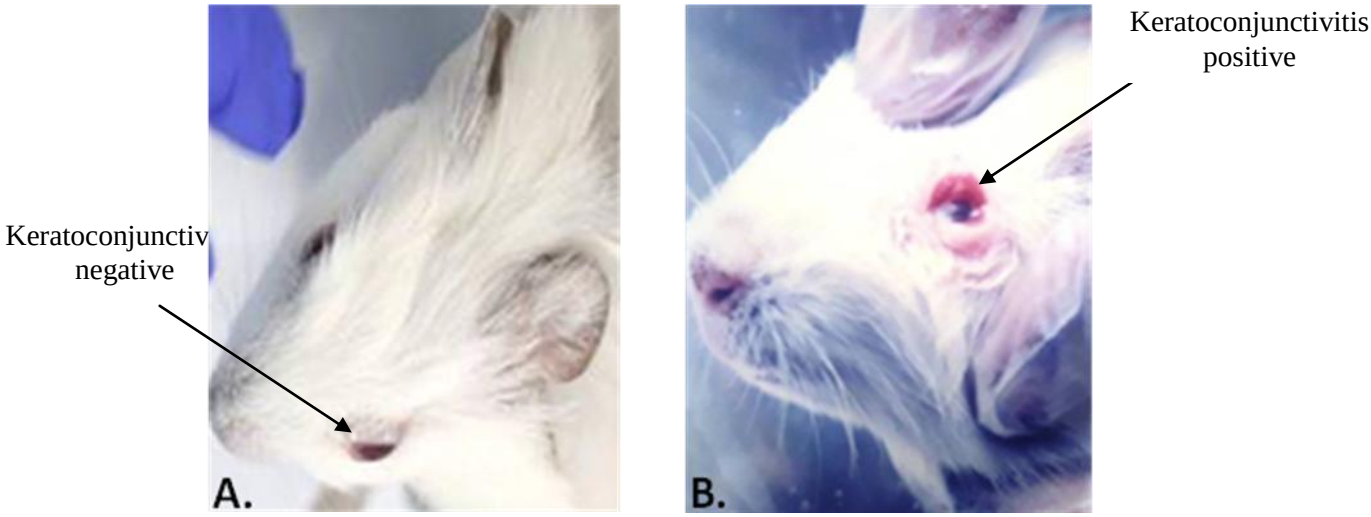


Figure 3.8 Guinea pig eyes showing the characteristics of keratoconjunctivitis. A. Only 20 µL of phosphate-buffered saline inoculated as negative control and B. Test strain MN226843 *A. baumannii* (positive).

Cytotoxic test (fluid accumulation)

For assaying the cytotoxic property of the tested organisms, we performed rabbit ileal loop assay, where we inoculated the cell lysates. After 24 hrs of incubation, we observed the fluid accumulation in the ligated ileal loop. Fluid accumulation was observed only in the case of *A. baumannii* but not in the case of *P. aeruginosa* (Figure 3.9). Mean fluid accumulation mL/cm was shown in figure 3.10.

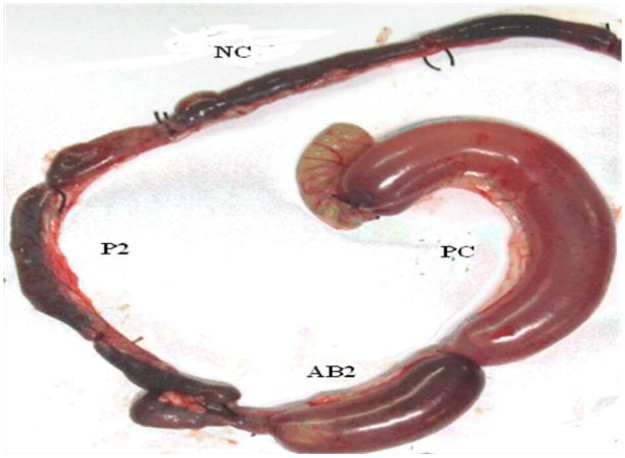


Figure 3.9 *A. baumannii* cell lysate induced fluid accumulation in rabbit ileal loop. PC-positive control-*Vibrio cholerae* strain No. 569B; AB2 test strain MN226843 *A. baumannii*; P2- test strain MN256619 *P. aeruginosa*; NC-negative control *E. coli* ATCC 25922 (no visible fluid accumulation).

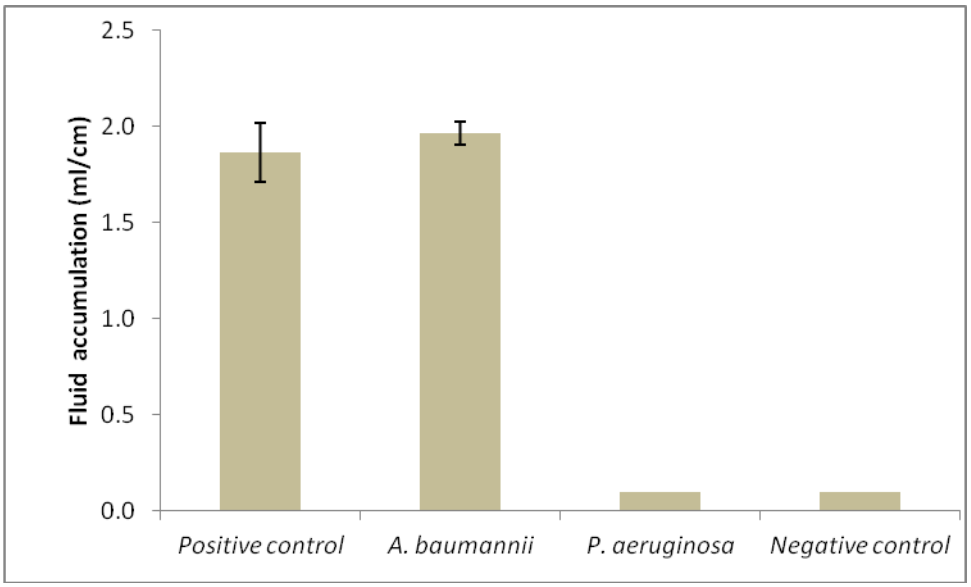


Figure 3.10 Mean fluid accumulation (mL/cm) in rabbit ileal loop after inoculation of cell lysate. Each experiment was performed in triplicate. Data are presented as meam ± SME.

3.9 High frequency of Verona integron-encoded metallo-β-lactamase gene in *P. aeruginosa*

Verona integron-encoded metallo-β-lactamase (VIM) is an important MBL which confers resistance to regular using antimicrobials and now-a-days carbapenems became resistant due to VIM. Therefore, we performed the amplification of VIM containing *P. aeruginosa.* Only β-lactamase producing gene Verona integron-encoded metallo-β-lactamase (VIM) was detected in 16% *P. aeruginosa* but no IMP or NDM-1 was detected (Figure 3.11). Specimen wise distribution shown in Table 3.6 where it was found that tracheal aspirate showed maximum (50%) isolates followed by urine isolates.

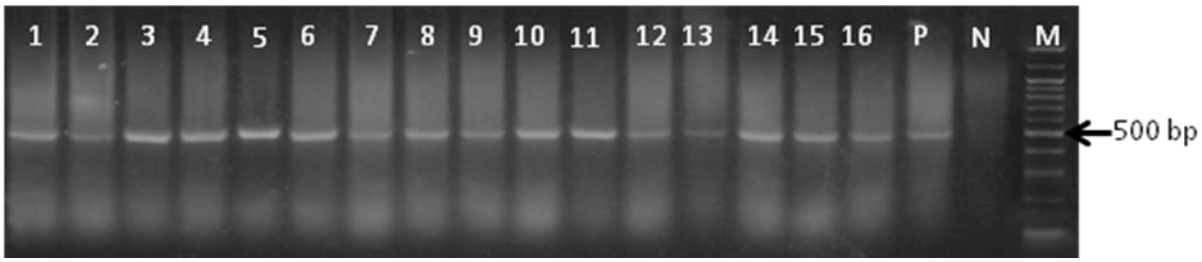


Figure 3.11 PCR products of VIM MBL from *P. aeruginosa*: Lane 1-16 representative test strains, M-100bp Marker, P-Positive control, N-Negative control.

Table 3.6 Specimen wise distribution of VIM in 100 *P. aeruginosa*

Specimen	Total
Tracheal aspirate	8
Urine	5
Sputum	1
Blood	1
Others	1
Total	16

3.10 New Delhi metallo-β-lactamase-1 gene in *A. baumannii*

Only New Delhi metallo-β-lactamase-1 (NDM-1) gene was detected in 6% *A. baumannii* but no IMP or VIM (Figure 3.12). Specimen wise distribution was shown in Table 3.7, where it was found that maximum isolates were from tracheal aspirates (83%) followed by urine isolates.

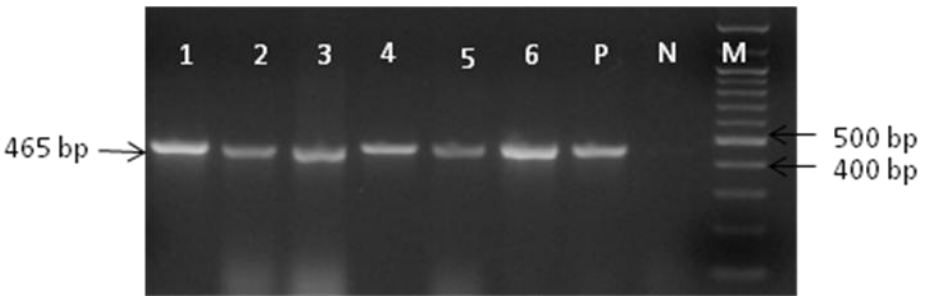


Figure 3.12 PCR products of NDM-1 MBL from *A. baumannii*: Lane 1-6 representative test strains, M-100bp Marker, P-Positive control (*K. pneumoniae*), N-Negative control

Table 3.7 Specimen wise distribution of NDM-1 in 100 *A. baumannii*

Specimen	Total
Tracheal aspirate	5
Urine	1
Total	6

3.11 Sequence analysis results of *P. aeruginosa* and *A. baumannii*

We obtained a total of sixteen VIM producers from one hundred *P. aeruginosa* isolates, but none from a hundred of *A. baumannii* strains. These sixteen DNA sequences of *P. aeruginosa* together with twenty two other similar sequences, retrieved from the NCBI GenBank database were used to construct the phylogenetic tree to understand the nearest neighbour of the study sequences. The genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree (Figure 3.13). For VIM type of MBL, *P.*

aeruginosa phylogenetic tree showed similarities with those of isolates from India, Thailand, Nepal, Egypt, Turkey, UK, USA, and Tunisia (93-100%). These findings suggest that the VIM gene is similar to global circulating strains. From Bootstrap table it was found that 90-100% nucleotide similarities exist among the sixteen Bangladeshi strains (Figure 3.14). The scenarios are similar to global circulating strains. The age, sex and sample type of VIM containing *P. aeruginosa* were described in Table 3.8.

Table 3.8 Age, sex and sample distribution of VIM containing 16 strains of *P. aeruginosa*

Strain	Age of patient (Yr)	Sex	Sample source
MN256618	50	M	Tracheal aspirate
MN256619	75	M	Tracheal aspirate
MN256620	20	M	Urine
MN256621	52	M	Urine
MN256622	12	M	Tracheal aspirate
MN256623	55	M	Urine
MN256624	55	M	Blood
MN256625	46	M	Tracheal aspirate
MN256626	70	M	Urine
MN256627	54	M	Tracheal aspirate
MN256628	65	M	Tracheal aspirate
MN256629	78	F	Urine
p13-MN256630	60	M	Tracheal aspirate
MN256631	5	M	Wound swab
MN256632	24	M	Endo tracheal tube
MN256633	55	M	Sputum

F-Female and M-Male

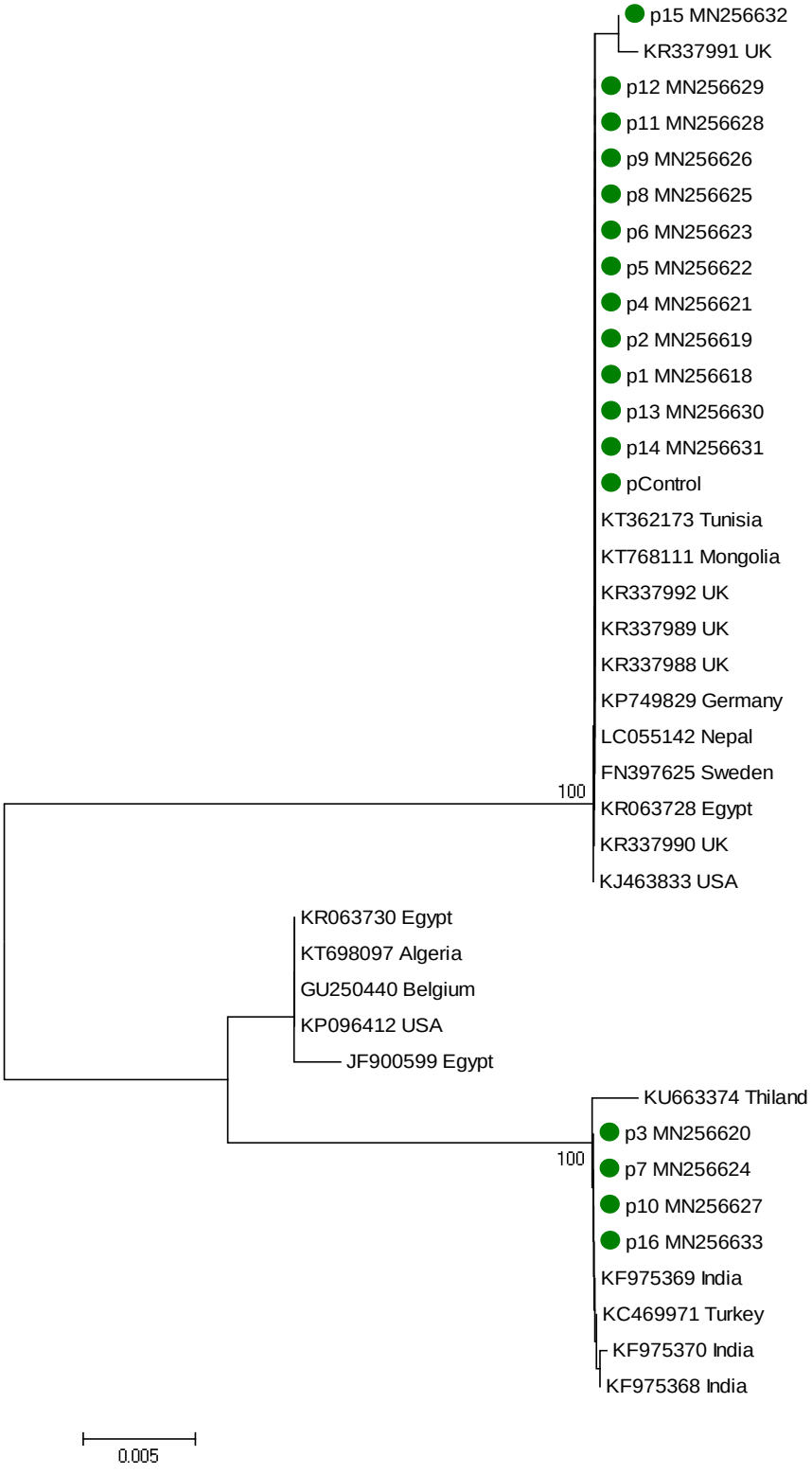
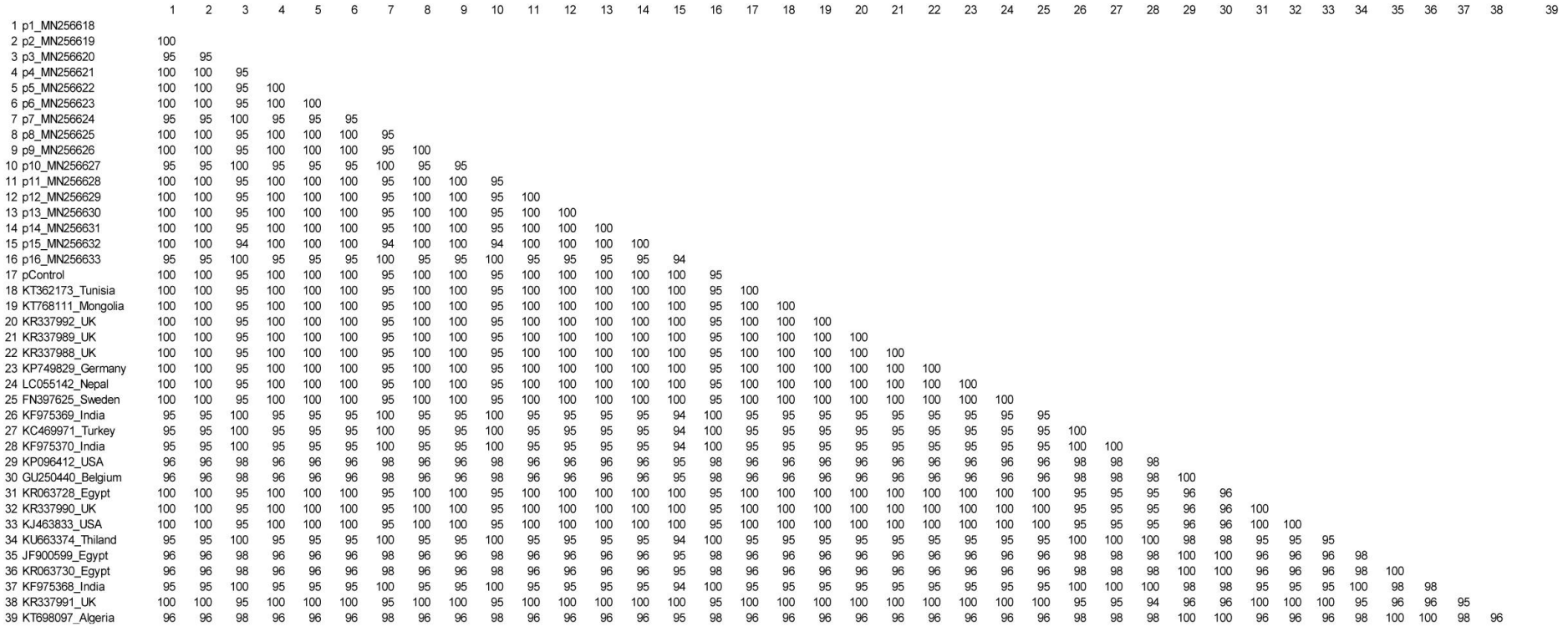


Figure 3.13 Neighbour-Joining Phylogenetic tree of Verona integron-encoded metallo-β-lactamase (VIM) producing MDR *P. aruginosa* of Bangladeshi strains. The numbers adjacent to the nodes represent the value of Bootstrap support (100 replicates) for the clusters to the right of the node. Bootstrap values lower than 99% were not shown; 0.005, Substitution/site (5 nucleotide changes from 1000). Green color solid circle indicates Bangladeshi strains.



		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39
1	p1_MN256618																																							
2	p2_MN256619	100																																						
3	p3_MN256620	95	95																																					
4	p4_MN256621	100	100	95																																				
5	p5_MN256622	100	100	95	100																																			
6	p6_MN256623	100	100	95	100	100																																		
7	p7_MN256624	95	95	100	95	95	95																																	
8	p8_MN256625	100	100	95	100	100	100	95																																
9	p9_MN256626	100	100	95	100	100	100	95	100																															
10	p10_MN256627	95	95	100	95	95	95	100	95	95																														
11	p11_MN256628	100	100	95	100	100	100	95	100	100	95																													
12	p12_MN256629	100	100	95	100	100	100	95	100	100	95	100																												
13	p13_MN256630	100	100	95	100	100	100	95	100	100	95	100	100																											
14	p14_MN256631	100	100	95	100	100	100	95	100	100	95	100	100	100																										
15	p15_MN256632	100	100	94	100	100	100	94	100	100	94	100	100	100	100																									
16	p16_MN256633	95	95	100	95	95	95	100	95	95	100	95	95	95	95	94																								
17	pControl	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95																							
18	KT362173_Tunisia	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100																						
19	KT768111_Mongolia	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100																					
20	KR337992_UK	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100																				
21	KR337989_UK	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100																			
22	KR337988_UK	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100	100																		
23	KP749829_Germany	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100	100	100																	
24	LC055142_Nepal	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100	100	100	100																
25	FN397625_Sweden	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100	100	100	100	100															
26	KF975369_India	95	95	100	95	95	95	100	95	95	100	95	95	95	95	94	100	95	95	95	95	95	95	95	95	95														
27	KC469971_Turkey	95	95	100	95	95	95	100	95	95	100	95	95	95	95	94	100	95	95	95	95	95	95	95	95	95	100													
28	KF975370_India	95	95	100	95	95	95	100	95	95	100	95	95	95	95	94	100	95	95	95	95	95	95	95	95	95	100	100												
29	KP096412_USA	96	96	98	96	96	96	98	96	96	98	96	96	96	96	95	98	96	96	96	96	96	96	96	96	96	98	98	98											
30	GU250440_Belgium	96	96	98	96	96	96	98	96	96	98	96	96	96	96	95	98	96	96	96	96	96	96	96	96	96	98	98	98	100										
31	KR063728_Egypt	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100	100	100	100	100	100	95	95	95	96	96									
32	KR337990_UK	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100	100	100	100	100	100	95	95	95	96	96	100								
33	KJ463833_USA	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100	100	100	100	100	100	95	95	95	96	96	100	100							
34	KU663374_Thiland	95	95	100	95	95	95	100	95	95	100	95	95	95	95	94	100	95	95	95	95	95	95	95	95	95	100	100	100	98	98	95	95	95						
35	JF900599_Egypt	96	96	98	96	96	96	98	96	96	98	96	96	96	96	95	98	96	96	96	96	96	96	96	96	96	98	98	98	100	100	96	96	96	98					
36	KR063730_Egypt	96	96	98	96	96	96	98	96	96	98	96	96	96	96	95	98	96	96	96	96	96	96	96	96	96	98	98	98	100	100	96	96	96	98	100				
37	KF975368_India	95	95	100	95	95	95	100	95	95	100	95	95	95	95	94	100	95	95	95	95	95	95	95	95	95	100	100	100	98	98	95	95	95	100	98	98			
38	KR337991_UK	100	100	95	100	100	100	95	100	100	95	100	100	100	100	100	95	100	100	100	100	100	100	100	100	100	95	95	94	96	96	100	100	100	95	96	96	95		
39	KT698097_Algeria	96	96	98	96	96	96	98	96	96	98	96	96	96	96	95	98	96	96	96	96	96	96	96	96	96	98	98	98	100	100	96	96	96	98	100	100	98	96	

Figure 3.14 Nucleotide sequence similarity percentage of VIM producing *P. aeruginosa* of Bangladeshi strains with that of strains from other countries

The nucleotide similarity was compared with the reference strain LC055142 retrieved from the NCBI GenBank database and it was found that there were nucleotide differences in several positions for four Bangladeshi strains of *P. aeruginosa* (Figure 3.15 and detail of 477 bp was given in Appendix III). Detailed nucleotide differences were described in Table 3.9.

Figure 3.15 Nucleotide similarity among 16 *P. aeruginosa* strains compared with the reference strain retrieved from the NCBI GenBank database

Table 3.9 Nucleotide changes of strains MN256620, MN256624, MN256627 and MN256633 of *P. aeruginosa* with reference strain LC055142.

Nucleotide changes	Changes in positions
A to G	17, 345, 384, 430
A to C	114,437
A to T	434
G to A	36, 214, 222, 390, 407
G to C	429
C to A	215, 414
T to C	150, 266, 357, 381, 382, 408, 417, 433
T to A	402

We obtained a total of six NDM-1 positive isolates from one hundred *A. baumannii* but none from one hundred *P. aeruginosa* strains. Among these six $bla_{\text{NDM-1}}$ gene positive isolates, five were sequenced. These five DNA sequences together with seventeen other similar sequences retrieved from the NCBI GenBank database were used to construct the phylogenetic tree to understand the nearest neighbour of the study sequences. The genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree (Figure 3.16). Amongst the DNA sequences obtained from our study, those from isolates AB1 MN226842, AB2 MN226843, AB4 MN226844 and AB6 MN226846 were found to form distinct lineages with India, while only AB5 MN226845 shared similarity and was found to be closely related to the sequences with Iran, Egypt, Korea. From these findings, we can predict that the NDM-1 has been transmitted in Bangladesh from India. From Bootstrap table it was found that hundred percent nucleotide similarities exist among the five Bangladeshi strains. On the other hand, ten strains from China showed 52% similarity (Figure 3.17). The age, sex and sample type of NDM-1 containing *A. baumannii* were described in Table 3.10.

Table 3.10 Age, sex and sample distribution of NDM-1 containing 5 Bangladeshi strains of *A. baumanni*

Strain	Age of patient (Yr)	Sex	Sample source
MN226842	22	M	Tracheal aspirate
MN226843	60	F	Tracheal aspirate
MN226844	75	M	Tracheal aspirate
MN226845	40	M	Tracheal aspirate
MN226846	1	F	Urine

F-Female and M-Male

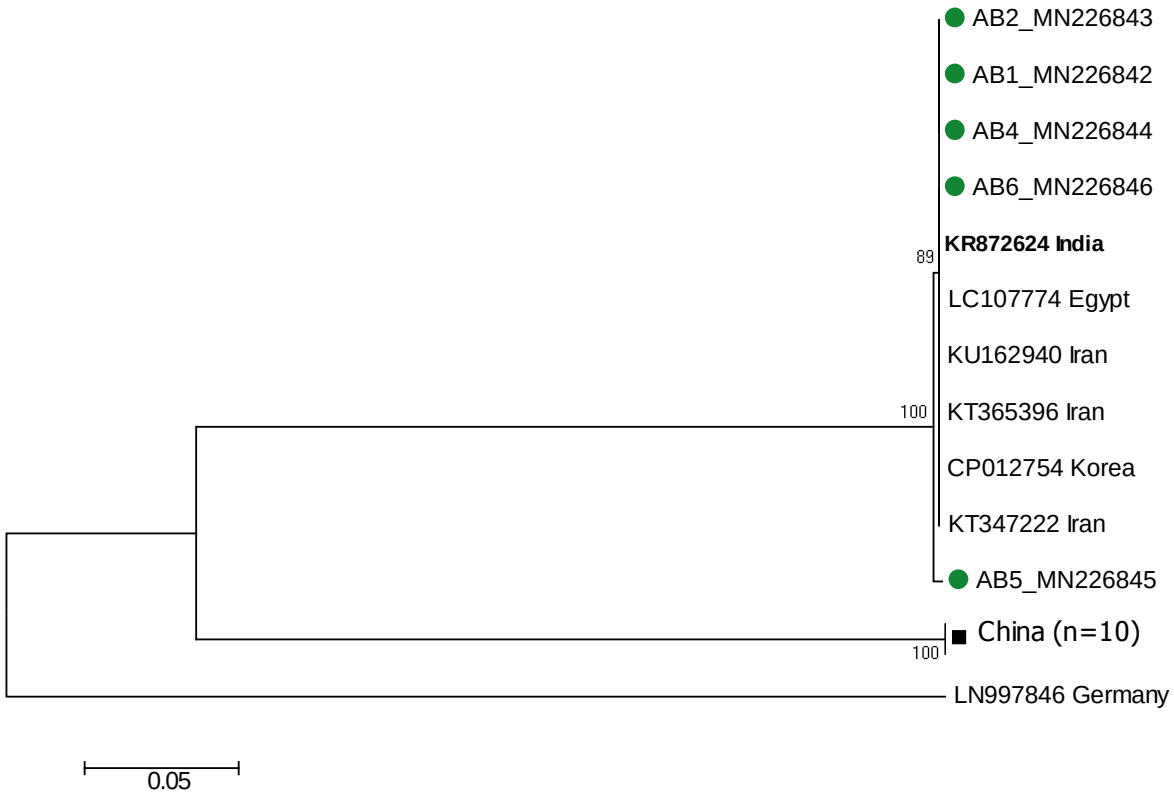


Figure 3.16 Neighbour-Joining Phylogenetic tree of New Delhi metallo-β-lactamase-1 producing MDR *A. baumannii* Bangladeshi strains. The numbers adjacent to the nodes represent the value of Bootstrap support (100 replicates) for the clusters to the right of the node; 0.05, Substitution/site (5 nucleotide changes from 100). Green color solid circle indicates Bangladeshi strain.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1 AB1_MN226842	100																					
2 AB2_MN226843	100	100																				
3 AB4_MN226844	100	100	100																			
4 AB5_MN226845	99.6	99.6	99.6	100																		
5 AB6_MN226846	100	100	100	99.6	100																	
6 KR872624_India	100	100	100	99.6	100	100																
7 KP772191_China	51.6	51.6	51.6	51.6	51.6	51.6	100															
8 KP772189_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100														
9 KP772149_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100													
10 KP772146_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100												
11 KP772195_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100	100											
12 KP772181_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100	100	100										
13 KP772164_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100	100	100	100									
14 KP772187_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100	100	100	100	100								
15 KP772185_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100	100	100	100	100	100							
16 KP772180_China	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100	100	100	100	100	100	100						
17 LC107774_Egypt	100	100	100	99.6	100	100	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	100					
18 KU162940_Iran	100	100	100	99.6	100	100	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	100	100				
19 KT365396_Iran	100	100	100	99.6	100	100	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100			
20 KT347222_Iran	100	100	100	99.6	100	100	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100		
21 CP012754_Korea	100	100	100	99.6	100	100	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	51.6	100	100	100	100	100	
22 LN997846 Germany	39.3	39.3	39.3	39.1	39.3	39.3	39.1	39.1	39.1	39.1	39.1	39.1	39.1	39.1	39.1	39.1	39.3	39.3	39.3	39.3	39.3	100

Figure 3.17 Nucleotide sequence similarity percentage of NDM-1 producing *A. baumannii* of Bangladeshi strains with that of strains from other countries

The nucleotide similarity of *A. baumannii* was compared with a reference starin KR872624 retrieved from the NCBI GenBank database and it was found that there were no nucleotide differences of 5 *A. baumannii* strains of Bangladesh origin (Figure 3.18) with the reference strain KR872624.

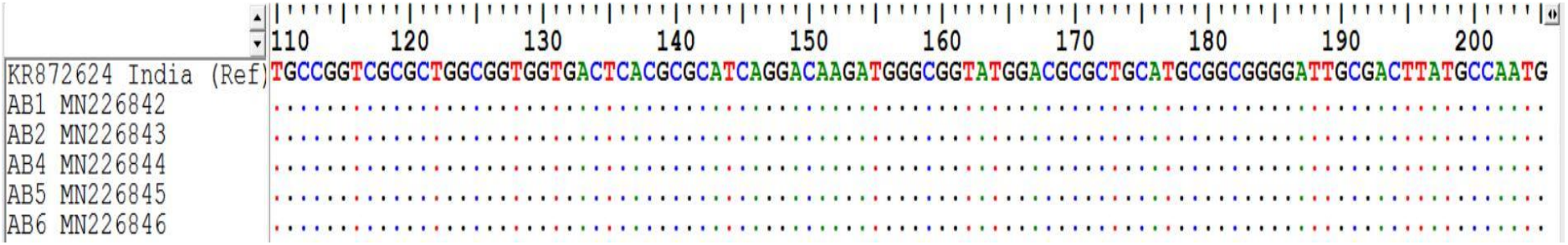


Figure 3.18 Nucleotide similarity among 5 *A. baumannii* strains compared with the reference strain KR872624 retrieved from the NCBI GenBank database

Strains MN256620, MN256624, MN256627 and MN256633 of *P. aeruginosa* had amino acid position changed at 72, changed from alanine (A) to lysine (K). Alanine is non polar amino acid whereas lysine is a polar (basic) one. In the position 145, tyrosine (Y) has been changed to leucine (L); here tyrosine is polar (acidic) and leucine is non polar (Figure 3.19). On the other hand, in position 146, glutamic acid (E) (polar acidic) amino acid has been changed to alanine (A) which is non polar. In conclusion, we can say that in strain number MN256620, MN256624, MN256627 and MN256633 of *P. aeruginosa* there were three positions changed where the first one was from non polar to polar (basic), secondly from polar (acidic) to non polar and thirdly also from polar (acidic) to non polar. Whether these changes have any effect on enzyme function/structre can only be conjuncted.

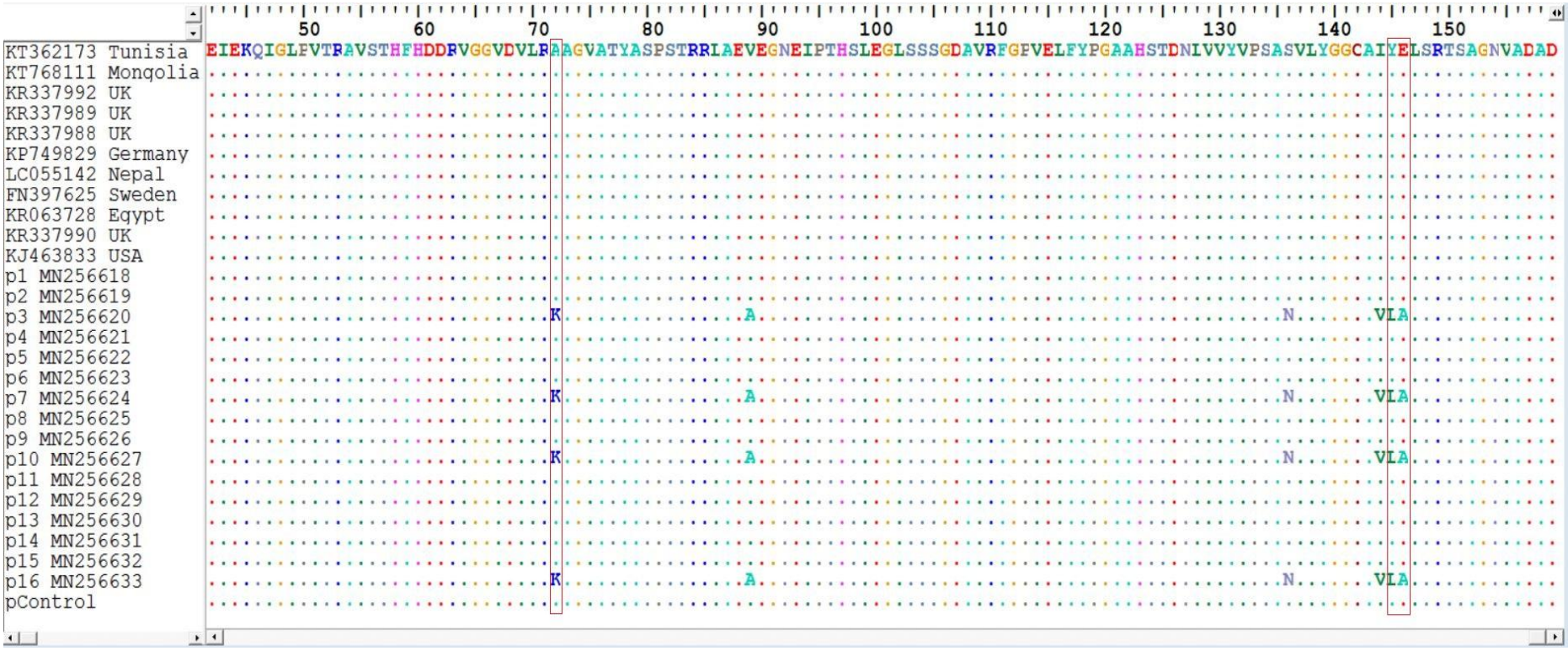


Figure 3.19 Amino acid sequence changes in 16 Bangladeshi strains of *P. aeruginosa*. Marked lines are important as these changes brought from nonpolar/polar amino acids to polar/nonpolar amino acids.

Nucleotide positional changes found in four Banhladeshi strains of *P. aeruginosa* among 16 strains. This changes bring the composition of amino acid sequence changes of VIM type MBL that may have changed the protein/enzyme nature. Among the identified 6 amino acid changes 3 were vital as these 3 changed from nonpolar/polar amino acid to polar/nonpolar. On the otherhand in NDM-1 gene, there was no significant changes in nucleotide and amino acid sequences as well.

4. Discussion

Antimicrobial resistance is a worldwide burning problem with severe implications for developing countries, where highly infectious disease burden coexists with rapid emergence and spread. The increasing rate of carbapenem resistance in nonfermentative Gram-negative bacteria (NFGNB) is a serious global health threat. Historically, metallo-β-lactamases (MBLs) were widespread in *P. aeruginosa* and *A. baumannii* but more recently have emerged in *Enterobacteriaceae.* The carbapenem-resistant in *Enterobacteriaceae* (CRE) have been focused due to a number of studies, guidelines, and infection control efforts and their increasing prevalence, their association with carbapenemase production, and multiple reports of nosocomial outbreaks with high rates of morbidity and mortality. However, nonfermentative Gram-negative bacteria have not received much attention (Aktas and Keyacan, 2008). Clinically, NFGNB can also cause difficult-to-treat life-threatening infections, often in patients with significant morbidities. As intrinsic antibiotic resistance to multiple antibiotic classes is common with NFGNB, carbapenems are frequently used for treatment. If carbapenem resistance becomes more widespread, therapeutic options may become tragically few or none.

The multi-drug resistant (MDR) nonfermentative Gram-negative bacteria *P. aeruginosa* and *A. baumannii* cause serious nosocomial infections that are more difficult to treat and are increasingly acquiring carbapenem resistance. Moreover, resistance gene transfer can occur between Gram-negative species, regardless of their ability to ferment glucose. Thus, the acquisition of carbapenemase genes by these organisms increases the risk of inter-genus carbapenemase spread worldwide. Ultimately, infection control practitioners and clinical microbiologists need to work together to determine the risk carried by carbapenem-resistant NFGNB in their institution and what methods should be considered for surveillance and detection.

The current study aimed to apply various phenotypic and genotypic techniques to identify and characterize of MDR nonfermentative Gram-negative bacterial isolates in Bangladesh. A number of carbapenemase resistance genes have been reported worldwide, including many Asian and Southeast Asian countries. But in Bangladesh, no studies have been published on carbapenemase producing genes using clinical isolates.

In our investigation, MDR strains of *P. aeruginosa* showed high resistance 99% to ceftriaxone and cefixime followed by ciprofloxacin, amikacin and netilmicin 94%, imipenem 93%, cotrimoxazole 92%, meropenem 91% and ceftazidime 86%. Only polymyxin B 4% and colistin showed 2% resistance which was reported by Deplano, *et al*., (2005), they showed 100% resistance to cefepime, meropenem, imipenem, gentamicin, tobramycin, and ciprofloxacin; 94% to piperacillin, piperacillin/tazobactam, ceftazidime, aztreonam and 83% to ticarcillin/clavulanic acid.

This broad-spectrum resistance drastically restricted the choice of antimicrobial therapy, leaving polymyxin B and colistin as the drug of last resort. This high resistance can be explained by the accumulation of multiple resistance mechanisms, including gene mutation, overexpression of efflux pumps, loss or modification of porins, and acquired extended spectrum β-lactamases.

One hundred MDR strains of *A. baumannii* all were resistance to ceftriaxone, co-trimoxazole, cefixime and meropenem followed by imipenem 99%, ciprofloxacin 97%, amikacin 95% and ceftazidime 94%. Only Polymyxin B and colistin showed lower frequency, 2% and 3% resistance respectively. From this data it has been found that *A. baumannii* is more resistant than pseudomonads. Nhu *et al.* (2014) also showed that *A. baumannii* even more resistance than *P. aeruginosa*. They also showed that VAP was the main clinical event for *A. baumannii* infection, which is proven by our study because 62% of *A. baumannii* was isolated from tracheal aspirate in our study.

NFGNB are intrinsically resistant to many of the antimicrobial agents and acquired resistance through production of carbapenamases. By producing carbapenamase NFGNB became MDR phenotype. In our study *P. aeruginosa* showed 48% carbapenemase positive and tracheal aspirate showed the highest (24%) positivity whereas; *A. baumannii* showed 32% positive and tracheal aspirate showed maximum (22%) positivity. Yum *et al.,* (2002) described in their study that 50% of MDR *A. baumannii* produced carbapenamase which is compareable with our study.

AmpC cephalosporinase is characteristically chromosomal or plasmid encoded in *P. aeruginosa*. Some antibiotics, such as the carbapenems, are strong inducers of this β-lactamase but not in *A. baumannii*. Interestingly, clavulanate can induce expression of the AmpC β-lactamase, resulting in antagonism of the bactericidal activity of ticarcillin (Lister *et al*., 1999, Tausk *et al*., 1986). Therefore, it has been suggested that ticarcillin-

clavulanate should be avoided when selecting an antipseudomonal β-lactam antibiotic (Lister *et al*., 1999). Tazobactam does not induce hyperproduction of the AmpC β-lactamase (Lister *et al*., 1999). Most importantly, antibiotic therapy selects derepressed mutants that permanently hyperproduce AmpC β-lactamase. Stably derepressed mutants that hyperproduce the AmpC β-lactamase may lead to resistance to ticarcillin, piperacillin, and third-generation cephalosporins (Livermore and Yang, 1987).

Although the current CLSI guidelines do not describe any method for the detection of isolates producing AmpC β-lactamases. We followed simple and easily applicable method and found 34% of *P. aeruginosa* and 25% of *A. baumannii* produce AmpC β-lactamases. From our findings, it could be concluded that more than one third of the NFGNB isolates were AmpC producer.

The MBLs hydrolyze all β-lactam groups of antibiotics except aztreonam *in vitro*. The detection of MBL production in *P. aeruginosa* and in *A. baumannii* is essential for the optimal treatment of patients and to control the spread of resistance. We found 92% of clinical isolates of *P. aeruginosa* and 35% of *A. baumannii* were MBL producers detected by EDTA Double Disc Synergy (EDDS) test and this was high when compared with a study from Karachi, Pakistan (Irfan *et al*., 2008). Our study was comparable to the study conducted by Aktaş, *et al.*, (2008), they found 78.5% *P. aeruginosa* and 63.6% *A. baumanni* isolates were positive in the EDDS test with imipenem and 0.5 M EDTA disks. Another study conducted by Siarkou *et al.* (2009) described 50% MBL production by *P. aeruginosa*.

We showed that both the experimental organisms possessed different genes to show resistance against various antibiotics. Besides these resistance characteristics, these organisms also possess various virulence properties to show their pathogenicity. In our study, we investigated some of the virulence properties of *P. aeruginosa* and *A. baumannii*, like production of hemolysin, formation of capsule, invasiveness and cytotoxicity. Both the test organisms showed strong hemolytic activity, however, only *A. baumannii* showed the formation of capsule (Figure-3.7 at page 58), invasiveness in the guinea pig's eye and accumulation of fluid in the rabbit ileal loop (Mendez *et. al*., 2012). All these results indicated that *A. baumannii* is likely more virulent as compared with *P. aeruginosa*.

In this study, we investigated the molecular mechanism of resistance to carbapenems in *P. aeruginosa* and *A. baumannii*. All MBL positive *P. aeruginosa* possessed bla_{VIM}. These data confirm previous studies performed in the Mediterranean basin, which concluded that the main MBL produced by *P. aeruginosa* was VIM-2 type (Sefraoui *et al*., 2014). Recently, Al Bayssari *et al*. (2014) reported the emergence of VIM-2 in a series of clinical isolates of carbapenem resistant *P. aeruginosa*. Additionally, Hammami *et al*. (2010) demonstrated the incidence of the MBL VIM-2 as a gene cassette in class 1 integron in *P. aeruginosa* collected from different wards at a hospital. These findings may reflect the current spread of MBLs in clinically relevant Gram-negative nonfermentative strains throughout the world isolates and share the same genetic pool.

β-lactamase producing gene Verona integron-encoded metallo-β-lactamase (VIM) was detected in 16% *P. aeruginosa* but no genes for IMP or NDM-1 was detected from our study. None of one hundred *A. baumannii* strains contained the gene for VIM. The amplified DNA from sixteen VIM were sequenced and compared together with twenty two other similar sequences, retrieved from the NCBI GenBank database. Close relative of the sequence was found from the phylogenetic tree with India, Thailand, Nepal, Egypt, Turkey, UK, USA, and Tunisia (93-100%). The genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree (Figure 3.13). These findings suggest that the VIM gene is similar to global circulating strains.

The nucleotide sequence of VIM positive 16 Bangladeshi *P. aeruginosa* strains were compared with a reference strain retrieved from the NCBI GeneBank database and it was found that twelve strains were similar to reference strain, but four strains showed differences in several nucleotide positions and the amino acid sequence comparison with a reference strain also showed differences in several amino acid sequence positions. Among the amino acid sequence changes three were vital as these changes bring from nonpolar/polar amino acid to polar/nonpolar amino acids, which may have been affected the enzymatic property of the protein. On the other hand, in NDM-1 gene, there was no significant changes in nucleotide sequences and as well as in amino acid sequences.

Carbapenemase-producing bacteria currently constitute a dramatic problem worldwide, because most of the carbapenemase-encoding genes are located on transferable genetic elements that are most often associated with other antibiotic resistance genes, leading to their rapid transfer and to the spread of uncontrollable superbugs (Rolain *et al*., 2012). Additionally, new antibiotic resistance genes may emerge from different sources, as

recently exemplified with NDM-1, and their diffusion is unpredictable in a complex bacterial community (Rolain *et al*., 2012). The problem is heightened by the absence of development of new antibiotic compounds, and has led to renewed interest in old antibiotics, such as colistin (Biswas *et al*., 2012). Unfortunately, the current increase in the use of colistin as the last-resort treatment in these situations has led to the emergence of resistance to colistin in these bacteria (Biswas *et al*., 2012), which we believe will be the next major public health challenge in the context of antibiotic resistance in the coming years (Diene *et al*., 2014; Rolain *et al*., 2012)

Molecular epidemiology is useful in hospital epidemiology as the molecular epidemiological techniques can aid a hospital in its infection control measures highlighting areas of weakness and identifying the source of hospital-acquired infections. On a global scale, use of molecular epidemiological techniques can aid the authorized persons and communities in developing control strategies to prevent the spread of drug-resistant pathogens by recognizing successful, epidemic, international clones or easy spread mobile genetic elements. Both of antimicrobial susceptibility testing, and molecular epidemiology is essential in the development of new drugs and treatment options as well.

The emergence and rapid spread of carbapenemases in *Enterobacteriaceae*, *Pseudomonas* and *Acinetobacter* (EPA) species is becoming a major public health crisis worldwide, and is responsible for a large number of hospital-acquired and nosocomial infections. The epidemiology and prevalence of these carbapenem-resistant Gram-negative bacteria among countries in the world are high and the rates of resistance are some of the highest in South and Southeast Asia. These countries house more than a third of the world's population, and several are also major medical tourism destinations. There are significant data gaps, and the almost universal lack of comprehensive surveillance programs that include molecular epidemiologic testing has made it difficult to understand the origins and extent of the problem in depth. A complex combination of factors such as inappropriate prescription of antibiotics, overstretched health systems, and international travel (including the phenomenon of medical tourism) probably led to the rapid rise and spread of these bacteria in hospitals in South and Southeast Asia.

South Asia is probably the source of the NDM-1 gene, which encodes one of the most widely, distributed carbapenemases in terms of geographic spread and bacterial species (Kumarasamy *et al*., 2010). This metallo-β-lactamase has received wide attention

because of its association with extreme resistance, presence in many common pathogens, and rapid spread. Microbiological surveys in India and Pakistan suggest that $bla_{\text{NDM-1}}$-carrying bacteria are now extremely common, at least in tertiary care hospitals. A study of 310 carbapenem-resistant Gram-negative bacteria from a tertiary care hospital in Mumbai, India collected between September 2009 and May 2010 found that 57 were *Enterobacteriaceae*, 173 *Pseudomonas* species, and 71 *Acinetobacter* species. Among the 57 *Enterobacteriaceae*, 49 were $bla_{\text{NDM-1}}$ positive, with *Klebsiella* (28) and *E. coli* (13) accounting for the majority. The plasmid on which it is carried is mobile and typically encodes other resistance determinants, so the bacteria are usually resistant to multiple classes of antibiotics (Wilson *et al*., 2012). The spread of NDM-1 has a complex epidemiology involving the spread of a variety of species of NDM-1 positive bacteria and the inter-strain, interspecies and inter-genus transmission of diverse plasmids containing $bla_{\text{NDM-1}}$, with the latter mechanism having played a more prominent role to date.

Acinetobacter species may play a pivotal role in spreading $bla_{\text{NDM-1}}$ genes for its natural reservoir to other members of *Enterobacteriaceae*. The emergence of the New Delhi metallo-β-lactamase (NDM-1) constitutes a critical medical issue. Indeed, this enzyme compromises the efficacy of almost all β-lactams (except aztreonam), including the last resort carbapenems. The Indian subcontinent, the Balkans regions, and the Middle East is considered to be the main reservoirs of NDM-1 producers. Since therapeutical options are limited to very few antibiotics such as colistin, tigecycline, and fosfomycin, hospital and community acquired infections caused by NDM-1 producers are difficult to eradicate. Isolation of infected patients and carriers and rapid diagnostic techniques are the key factors that contribute to contain this outbreak that threatens the efficacy of the modern medicine.

These findings highlight that even though *A. baumannii* is usually recognized as a final acceptor for resistance genes, it may acquire several resistance determinants and then transfer them to *Enterobacteriaceae* and *Pseudomonas* species.

β-lactamase producing gene New Delhi metallo-β-lactamase-1 (NDM-1) gene was detected in 6% *A. baumannii* but no genes for IMP or VIM was detected in our study. None of one hundred *P. aeruginosa* strains contained the gene for NDM-1. Among these six NDM-1, five PCR products were sequenced. These five DNA sequences together with seventeen other similar sequences, retrieved from the NCBI GenBank database

were used to construct the phylogenetic tree to understand the relativity of the study isolates sequences. The genetic divergence and homogeneity of the sequences were apparent in the phylogenetic tree (Figure 3.16). Close relative of the sequences NDM-1 obtained in our study MN226842, MN226843, MN226844 and MN226846 were also reported from India (Accession nos. KR872426 India). Homology was also evident with the sequences deposited from other countries, Egypt, Iran and Korea (Accession nos. LC107774 Egypt, KU162940 Iran, CP012754 Korea respectively) with strain MN226845.

From these findings, we can predict that the NDM-1 may have been transmitted in Bangladesh from India. Bootstrap table showed 100% nucleotide similarities among the five Bangladeshi strains. On the other hand, ten strains from China showed 52% similarity. The nucleotide sequences of NDM-1 positive 5 Bangladeshi *A. baumannii* strains were compared with a reference strain (KR872624) retrieved from the NCBI GeneBank database and it was found that all strains were similar to the reference strain hence there was no significant change in nucleotide sequence and amino acid sequence as well which suggest that these were clonal.

A number of studies have already reported the carriage of $bla_{\text{NDM-1}}$ in *A. baumannii* in different parts of the world (Chen, *et al.*, 2011; Bogaerts *et al*., 2012; Boulanger *et al*., 2012). The presence of the $bla_{\text{NDM-1}}$ gene was described in the members of the family *Enterobacteriaceae* from hospitals of Pakistan, where *Acinetobacter* species were not found to be the carrier (Kumarasamy *et al*., 2010; Perry *et al*., 2011); however, this study reported the incidence of NDM-1 carriage in *A. baumannii*. Ultimately, the presence of the $bla_{\text{NDM-1}}$ gene was found in six of the carbapenem resistant isolates among our study isolates of *A. baumannii.*

The global diffusion of antibiotic resistance, with the emergence and spread of MDR, sometimes panresistant bacterial clones, is now a matter of major concern with Gram-negative nonfermentative pathogens of healthcare associated infections. Examination of the antimicrobial resistance pattern in each of these cases is essential as the number of healthcare associated infections caused by MDR organisms is increasing and the antimicrobial resistance patterns and frequency of these resistances changing over time, geographical regions, and healthcare facilities. Systematic monitoring of antimicrobial resistance patterns in hospitals in a surveillance system is also essential not only to help choosing drugs for an adequate empirical therapy, but also preventing the dissemination

of MDR clones and/or plasmids carrying multiple resistance genes. Widespread dissemination of pathogens carrying antibiotic resistance genes breaching geographical boundaries due to increased human mobility undoubtedly play a pivotal role contributing to such a situation.

The overall idea behind this study was to bring to light to aware the situation of antimicrobial resistance, specifically carbapenem resistance, which is the last resort of drug for treatment of Gram-negative bacteria. Newer antibiotics will need to be discovered combating the existing carbapenems. They should have a wide range of activity as well as to be stable to inactivate enzymes produced by these type of multi-drug resistant superbugs. The most effective antibiotics in this study were colistin and polymyxin B. Thus antibiotic should be chosen judiciously and less severe infections should not be treated with carbapenem drugs to preserve the efficacy of carbapenem. The epidemiological surveillance in the major tertiary care institutions must be strengthened to monitor and control the emerging carbapenemase-producing multi-drug resistant pathogens in Bangladesh and the adjustment of the infection control measures should be considered.

As there are no promising new drugs in the pipeline to replace currently available ones for Gram-negative pathogens, and no broad-coverage vaccines are available against these agents either, the only way to mitigate their effect is to control their spread. This, however, is attainable, only if we have a clear understanding of the reasons, dynamics and complexity of the spread of these MDR nonfermentative Gram-negative organisms. To contribute to this knowledge was the purpose, we initiated our studies.

Significance and future studies:

The VIM containing *P. aeruginosa* and NDM-1 containing *A. baumannii* are MDR in nature and they are not treatable with conventional antimicrobials. Usually carbapenems are used as a last resort for treating this type of infection but unfortunately carbapenems become resistant. Only a few options are left likely colistin and polymyxin B. If all diagnostic laboratories in Bangladesh have the facility to identify MBL then this could be able to identify MDR pathogens and surveillance program will help the medical practitioners, hence the treatment will be easier and many lives will be saved.

P. aeruginosa and *A. baumannii* are very important pathogens due to their rapid development of resistance and the plasticity of their genome. In our study, we

determined the diversity of nucleotide sequences and as well as amino acid sequences in *bla*$_{VIM}$ gene in *P. aeruginosa* and our finding may help new drug development to combat with these superbugs. Further study is needed to determine the whole sequence of *bla*$_{VIM}$ gene to find out the potential mutation(s) and deduce a model of the protein-protein interaction(s) which will be responsible for particular resistance trait.

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