DETECTION OF ANTIBIOTIC RESISTANCE SPECTRUM AND RESISTANCE GENES FOR AMINOGLYCOSIDE, MACROLIDE, AND β-LACTAM ANTIBIOTICS USING WOUND SWAB SAMPLES

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Masters of Science in Biotechnology

Department of Mathematics and Natural Sciences
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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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The thesis/project titled "Detection of Antibiotic Resistance Spectrum and Resistance Genes for Aminoglycoside, Macrolide, and β-lactam Antibiotics using Wound Swab Samples" submitted by **Kazi Sarjana Safain** (Student ID: 18376008) has been accepted as satisfactory in partial fulfillment of the requirement for the degree of Masters of Science in Biotechnology on January, 2020.

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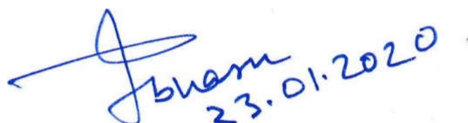

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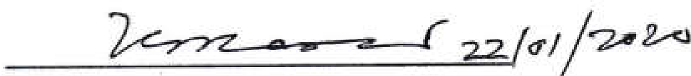

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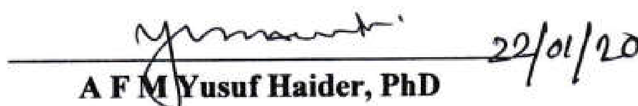

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Ethics Statement

This is to certify that the thesis titled "Detection of Antibiotic Resistance Spectrum and Resistance Genes for Aminoglycoside, Macrolide, and β-lactam Antibiotics using Wound Swab Samples" has been reviewed for and has been approved by the Biosafety, Biosecurity and Ethical Committee of Bangladesh Medical Research Council (BMRC).

Abstract

Antibiotic resistance has become a global health problem with a decrease in efficacy in treating common infections, resulting in increased sufferings, duration of hospital stay and economic burden. Infections in the wound site by multi-drug resistant pathogens play an important role in the development of chronicity and delaying wound healing. Unfortunately, information regarding resistance spectrum of the isolates and the associated genes, which are important for physicians in order to prescribe empirical treatment as well as to contain the spread of infections, is still lacking in Bangladesh. Hence, the aim of this study was to investigate antibiotic resistance in pathogenic organisms, isolated from wound sites, both phenotypically and genotypically. A total of 217 specimens were collected from outpatients affected with wound infections who visited for dressing purpose at Bangladesh Institute of Health Sciences Hospital (BIHS) and Shaheed Suhrawardy Medical College & Hospital, from January 2017 to March 2019. Identification and confirmation of the isolates were done by appropriate microbiological and biochemical methods. Furthermore, antibiogram was performed using Kirby–Bauer disk diffusion approach. PCR was performed using ESBL, macrolide and aminoglycoside resistance gene specific primers and subsequently Sanger sequencing was conducted for further confirmation of the genes. A total of 348 bacterial strains (MRSA- 17.82%, *S. aureus*- 6.32%, *S. pneumoniae*- 10.33%, *Enterococcus* spp.- 7.76%, CNS- 8.05%, *A. baumannii*- 5.75%, *E. coli*- 24.43%, *K. pneumoniae*- 12.07% and *P. aeruginosa*- 7.47%) were isolated. Co-infections were found in 25·3% of the samples and the most common association (13%) was found between MRSA and *K. pneumoniae*. Besides, antibiogram data revealed that about 54% strains were multidrug resistant (MDR). *E. coli* (96.6%) followed by MRSA (90%) isolates were found to be the most prevalent type of MDR pathogens. The MAR (multiple antibiotic resistance) index exhibited that more than half of the organisms (58.05%) had a MAR value of greater than 0.2 and for *P. aeruginosa*, the mean value (0.562) was significantly higher than other organisms tested. In addition, PCR results revealed that among the 5 ESBL genes investigated, CTXM-1 (53.71%) and NDM-1 (12.4%) were the most prevalent and these genes were predominant in *E. coli*. ErmB (38.2%) was the most common gene among the macrolide resistance genes. Also, Aac(3)-II -25.44% was the frequently detected gene among the aminoglycoside group. Moreover, the results obtained from investigation of co-resistance displayed that coexistence of CTXM-1 and ErmB (16.66%) was the most common phenomenon. Interestingly, an increasing trend in the proportion of MDR organisms was seen between the years of 2017 and 2019. Such alarming escalation of antibiotic resistance impedes the treatment and prevention of infectious diseases, resulting in increased morbidity and mortality. Therefore, nationwide surveillance as well as development of alternative strategies needs to be propagated to resolve this serious issue in the hospital settings of Bangladesh.

Keywords: Antibiotic resistance, wound infection, multidrug resistance, resistance genes, infectious diseases, hospital settings.

DEDICATION

My humble effort I dedicate to my sweet and loving

Family

And

Well-wishers

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List of Acronyms

AMR	Antimicrobial resistance
ARG	Antibiotic resistance gene
AST	Antibiotic sensitivity test
BIHS	Bangladesh Institute of Health Sciences Hospital
BLAST	Basic Local Alignment Search Tool
bp	Base pair
CDC	Centre for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CNS	Coagulase negative *Staphylococci*
CTX-M	Cefotaximase-Munich
ESBL	Extended spectrum beta lactamase
E-value	Expected value
GDP	Gross domestic product
GPP	Global Priority Pathogens
HSP	High scoring pairs
KPC	*Klebsiella pneumoniae* carbapenemase
LMIC	Low income and middle income countries
MAR	Multiple antibiotic resistance
MDR	Multidrug resistance
MHA	Mueller Hinton Agar
MIC	Minimum inhibitory concentration
MIU	Motility Indole Urease
MRSA	Methicillin Resistant *Staphylococcus aureus*
NCBI	National Centre for Biotechnology Information
NDM	New Delhi metallo-β-lactamase
NNIS	National Nosocomial Infection Surveillance
OD	Optical density
ORF	Open reading frame
OXA	Oxacillinase
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
RBC	Red blood cell
SSI	Surgical Site Infections
Tm	Melting temperature
TSI	Triple Sugar Iron
US	United States
WHO	World Health Organization
XDR	Extremely drug resistant
µl	Microliter

Chapter 1

INTRODUCTION

1.1 Background

Bacteria are considered to be the oldest form of life on earth and they appeared more than 3.5 billion years ago. The environment was extremely hostile at the time of their appearance. For their survival, they had to evolve certain strategies to manage the toxic natural elements and chemical compounds nearby of their region. In addition, it was vital to find appropriate ecological niches to avoid desiccation, high osmotic pressure, radiation, and extreme pH changes. Although microscopic, bacteria harbour a machinery that is impressively versatile in its simplicity. Meanwhile, they have adapted to most global conditions and can live in areas where only a very small number of other organisms could survive (Sütterlin, 2015). To combat against these organisms, various types of medicines and antibiotics were developed. After the discovery of penicillin by Alexander Fleming in 1928, these antibiotics are being widely used for treatment against infection. However, bacteria are developing resistance against these antibiotics while they are being applied on them. The regularly prescribed antibiotics are not strong enough to kill or inhibit those organisms. As a result these multi drug resistant (MDR) organisms are now becoming one of the major public health concerns throughout the world. Thus antibiotic resistance has become a global health problem.

Bacteria impede wound healing by several routes (Robson, 1997). An infected wound may result in prolonged debility of the patient and increased healthcare costs. Incidence rates of wound infections from international surveillance systems are available for surgical site infections (SSI), but still sparse for other wound infections. SSI are the most common nosocomial infections in patients undergoing surgery and remain a major source of postoperative morbidity. The National Nosocomial Infection Surveillance (NNIS) System, representing more than 300 hospitals in the US, reported data on SSI incidence rates between 1992 and 2000 (System, 2004). Wound infection with antimicrobial-resistant bacteria may cause further morbidity in patients and result in additional treatment costs arising from the measures instituted to avoid patient-to patient transmission (Chaix, Durand-Zaleski, Alberti, & Brun-Buisson, 1999). Avoidance of the development of resistance therefore requires increasing attention in the management of wound infections.

Antimicrobial use is the major determinant in the development of resistance (Gould, 1999). The control of outbreaks of erythromycin resistant group A *streptococci* in Finland by major reductions in the prescribing of erythromycin provides significant

support for this statement (Seppälä et al., 1997). Defining guidelines for the rational use of systemic and topical antimicrobials in wound management (both at a national level and also in individual hospitals) seems to be an important tool to limit and control the development of resistance in this area. When drawing up these guidelines, it is important to consider some general principles of antimicrobial therapy (Filius & Gyssens, 2002).

Although the circumstances of antimicrobial resistance may vary by region or country, it is clear that Asia is an epicenter of antimicrobial resistance; the prevalence of antimicrobial resistance of major bacterial pathogens has been rapidly increasing. However, public health infrastructures to control the problem are very poor in many countries. Given that more than 70% of the worldwide population lives in the Asia-Pacific region, antimicrobial resistance in this region is, literally, a global problem (Kang & Song, 2013). Evaluation of the burden of infectious diseases can be challenging because they occur at different time scales and are influenced by many factors (ie, demography, epidemiological setting, population ageing, and method of measurement) (Tacconelli & Pezzani, 2019). Periodic surveillance of resistance patterns, with linkage to associated mortality, is crucial to establish guidelines for the administration of appropriate antimicrobial agents in every Asian country. However, very little information is available regarding resistance spectrum of the isolates and; disease severity and treatment outcomes in case of multi drug resistant (MDR) pathogen infections in Bangladesh. In a study performed in Chittagong, typhoid patients were found to be unresponsive to second-line therapy (ciprofloxacin). First-line therapy was not even attempted because of existing resistance (Asna, Haq, & Rahman, 2003). Therapeutic failures like this are not rare at all. Furthermore, in relation to this, multiple studies have demonstrated irrational antibiotic prescribing by physicians, a habit of self-medication among patients, and the indiscriminate use of antibiotics in agriculture and farming in different parts of the country (I. Ahmed, Rabbi, & Sultana, 2019). However, there have been no long-term studies on trends in Antibiotic resistance on a national scale in Bangladesh. Antimicrobial resistance surveillance on a national scale is critical because it provides information on the extent of established resistance rates, as well as emerging patterns of resistance. Understanding how resistance is changing is important for (1) establishing prescribing guidelines, (2) determining investment in new therapies, and (3) improving the targeting of campaigns to reduce antimicrobial resistance. It also

provides a baseline for future analysis and comparison with other countries (Sumanth Gandra et al., 2016). Also, by studying the resistance genes that induce antibiotic resistance and the plasmids that transfer such genes, the mechanism that leads to antibiotic resistance can be elucidated. Therefore, the present study was aimed to investigate the spectrum of antibiotic resistance and to determine the genes involved for the resistance in the microorganisms causing wound infections so that appropriate strategies can be assessed to prevent such emergence, spread of resistance by horizontal transfer and development of MDR organisms.

1.2 Wound Infections: Definitions and Criteria for Diagnosis

All wounds are contaminated to a certain degree. Normally, 1000 organisms per gram of tissue are present on the surface of the skin and deep in the hair follicles and sweat glands. Colonization with β-hemolytic streptococci or more than 10^5 organisms per gram of tissue suggest clinical wound infection (Dow, Browne, & Sibbald, 1999). Chronic wound infection may be assessed by different techniques: quantitative biopsy, quantitative swab, semi quantitative swab or the rapid slide technique. Because quantitative biopsy/swab cultures are laboratory intensive, the semi quantitative swab technique is considered the most practical means of assessing bacterial burden on a routine basis. However, the definitive diagnosis of infection can only be made by the clinical presence of purulent material draining from the wound or spreading inflammation greater than the normal inflammation of healing. In burn wounds, a histologic examination of a burn wound biopsy is the most reliable means of confirming a diagnosis of invasive burn wound infection. The US Centre for Disease Control has developed definitions for nosocomial surgical wound infections and skin and soft tissue infections (Horan, Andrus, & Dudeck, 2008).

Gram-positive cocci, such as *S. aureus*, coagulase-negative staphylococci and *Streptococcus pyogenes* (group A streptococcus), are responsible for the majority of wound infections. Over the last decades there has been an increase in the documented number of infections caused by coagulase-negative staphylococci, especially with the species *Staphylococcus epidermidis*. The infection rate has been correlated with the increase in the use of prosthetic and indwelling devices and the growing number of immunocompromised patients in hospitals. Accurate identification and quantification of coagulase-negative streptococci is needed, so that the clinical disease produced by

this group of bacteria can be precisely delineated and their role as an etiologic agent can be determined. *Enterobacteriaceae* and anaerobic bacteria, such as *Bacteroides fragilis*, are also often implicated either alone or in mixed traumatic wound infections. In immunocompromised patients, one must also be concerned about organisms such as enterococci, *P. aeruginosa*, molds and yeasts. *Clostridium perfringens* infections occur in complicated open fractures or deep lacerations with gross contamination and devitalization of tissue. Gas gangrene or necrotizing fasciitis may develop in these patients. Hospital-acquired and community-acquired wound infections differ in several aspects. In the community, patients become infected with indigenous flora. Community-acquired traumatic wound infections are usually associated with a greater diversity of micro-organisms due to exposure of open wounds to inhabitants of the microbial biosphere. Hospitalized persons develop infections with species associated with areas of the hospital environment (such as sinks or drains) (Filius & Gyssens, 2002).

1.2.1 Wound infection and etiology

A wound is the result of physical disruption of the skin. Wounds can be categorized according to how they heal. Wounds heal by an ordered sequence of homeostasis, inflammation, new tissue formation (proliferation) and remodelling. The Wound Healing Society has stated that when wounds proceed through these processes in an orderly and timely manner and achieve sustained anatomic and functional integrity, they are considered acute wounds; otherwise they are considered chronic. Wounds can also be grouped according to how they are acquired (Janda, Abbott, & Brenden, 1997). The most common underlying event for all wounds is trauma. Trauma may be accidental or intentionally induced. The latter category results from surgical interventions and the use of intravascular devices. Accidentally induced wounds often result from major insults (motor vehicle accidents) or penetrating injuries (gunshot or stab wounds, and bites). Thermal burns are considered by some physicians as chronic wounds because they heal slowly compared with acute wounds. The most common chronic wounds are pressure ulcers, diabetic ulcers and venous ulcers. Tumour-associated wounds can be classified into malignant cutaneous ulcers related to the primary tumour or metastases and oncological wounds related to surgery, radiation, or chemotherapy (Filius & Gyssens, 2002).

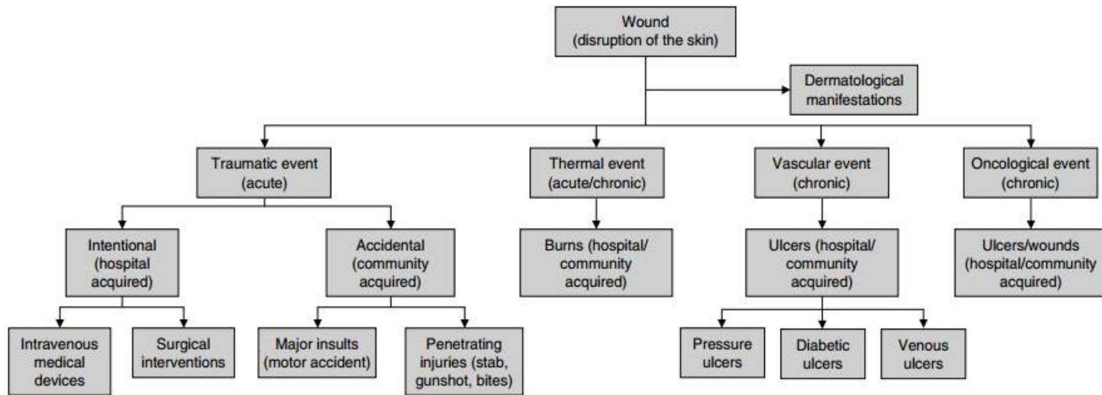

Figure 1.1: Etiology of wounds

1.3 Antibiotic Treatment

1.3.1 Historical overview

The first antimicrobial compounds to be mass produced and used on a large scale in clinical settings were the Sulfonamides or "sulpha drugs". These synthetic compounds containing a sulfonamide group, inhibit the enzyme dihydropteroate synthetase (DHPS) involved in folate biosynthesis, and were first synthesized by Alfred Bertheim and Paul Ehrlich in 1907, however Gerhard Domagk is credited for the discovery of the first commercially available sulfonamide used in the clinical setting "Prontocil", for which he later received the Nobel Prize in 1939 (Otten, 1986).

The Discovery of penicillin in 1928 by Alexander Fleming, is by many recognized as the first true antibiotic, a term coined by Selman Waksman as a compounds produced by or derived from microorganisms that in dilute concentration effectively inhibit the growth of or effectively kill other microorganisms (Waksman, 1947). Today the words antibiotics or antimicrobials are often used interchangeably for compounds used in the treatment of bacterial, protozoan or other infections by pathogenic microorganisms. The active compound of penicillin was isolated and set in production thanks to ground breaking work by Howard Florey and Ernst Boris Chain (Ligon, 2004), for which they alongside Sir Alexander Fleming received the Nobel Prize in 1945. Devastating diseases previously untreatable, such as streptococcal and chlamydial infections suddenly became treatable with the introduction of penicillin. The discovery of antibiotics sparked a new era in the treatment of infectious diseases and paved the way for modern medicine, through the golden era of antibiotics drug discovery from the 1940-1960´s.

During these decades, there was a huge expansion in the arsenal against bacterial infections through the continued discovery of new compounds. Gerhard Domagk and Alexander Fleming`s work was followed by many pioneering scientists. Especially, the work of Selman Waksman, who paved the way for new methodologies in antibiotic discovery (Waksman & Woodruff, 1940) and who was originally accredited for the discovery of streptomycin, the first treatment for one of human history`s greatest pathogens, Tuberculosis. During this relatively short period in history most of today's known classes of antibiotics were discovered. With antibiotics covering some of history`s most important human pathogens (Tuberculosis, Cholera, Malaria etc.) and it has been said that in 1969 the US Surgeon General William Stewart told the US Congress "that it was time to close the books on infectious diseases" (Spellberg, 2008).

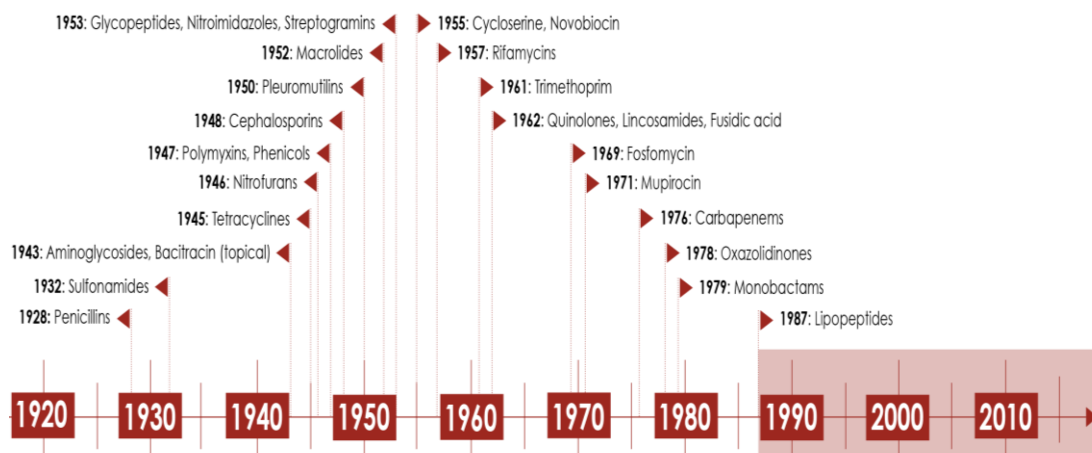

Figure 1.2: History of drug Development (Clatworthy, Pierson, & Hung, 2007)

1.3.2 Mechanisms of antibiotic action and resistance

There are several major classes of antibiotics that can be categorized based on their mode of antibacterial action. In general, antibiotics can be defined as those that inhibit cell wall synthesis, those that inhibit protein synthesis, and those that inhibit nucleic acid synthesis. The selective toxicity of antibiotics lies in the differences in cellular structures between eukaryotic and prokaryotic cells. However, differences in cellular structure among bacterial species can lead to resistance to certain antibiotics.

The definition of bacteria as resistant or susceptible is critical for clinicians. It is also very important to note the difference between intrinsic and acquired resistance to an antibiotic. Intrinsic resistance can best be described as resistance of an entire species to an antibiotic, based on inherent (and inherited) characteristics requiring no genetic

alteration. This is usually due to the absence of a target for the action of a given antibiotic or the inability of a specific drug to reach its target. For example, mycoplasmas are always resistant to β-lactam antibiotics since they lack peptidoglycan (which the β-lactams act upon). Similarly, the outer membrane of gram negative cells can prevent an antibiotic from reaching its target.

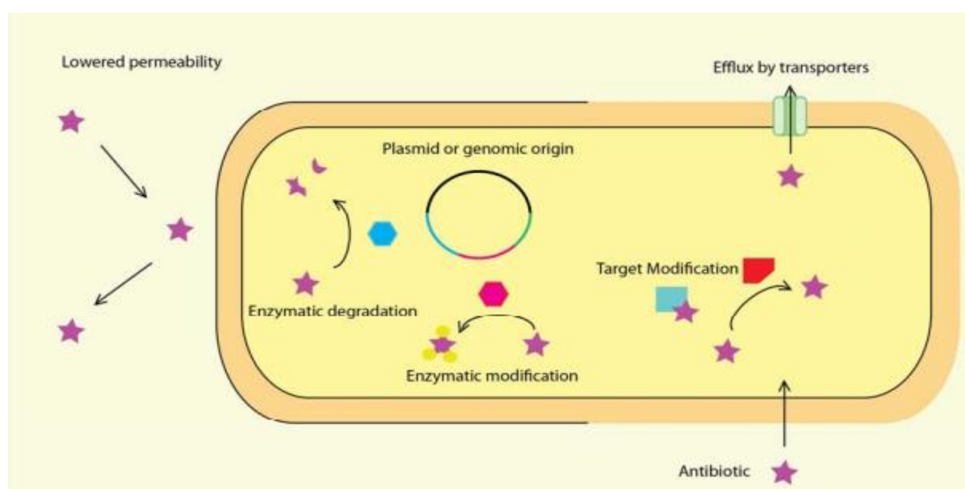

Figure 1.3: Resistance mechanisms

Acquired resistance can arise either through mutation or horizontal gene transfer. Presence of the antibiotic in question leads to selection for resistant organisms, thereby shifting the population towards resistance. The major mechanisms of acquired resistance are the ability of the microorganisms to destroy or modify the drug, alter the drug target, reduce uptake or increase efflux of the drug, and replace the metabolic step targeted by the drug.

Cell wall synthesis	**B-lactams**	**Glycopeptide**			
	Penicillin	Vancomycin			
	Cephalosporin	Avoparcin			
	Carbapenem				
Protein synthesis	**Aminoglycoside**	**Chloramphenicol**	**Tetracyclines**	**Macrolides**	**Streptogramins**
	Streptomycin			Erythromycin	Virginiamycin
	Neomycin			Azithromycin	Pristinamycin
	Kanamycin			Clarithromycin	Quinupristin-Dalfopristin
	Gentamicin				
Nucleic acid synthesis	**Sulfonamides**	**Quinolones**			
	Sulfamethoxazole-trimethoprim	Ciprofloxacin			
		Norfloxacin			

Table 1.1: Major Classes and Examples of Antibiotics

1.3.2.1 Inhibitors of Cell Wall Synthesis

There are two major groups of cell wall synthesis inhibitors, the β-lactams and the glycopeptides. As bacterial cell walls are wholly unlike the membranes of eukaryotes, they are an obvious target for selectively toxic antibiotics.

1.3.2.1.1 Resistance to β-lactams

The β-lactams include the penicillins, cephalosporins, and the carbapenems. These agents bind to the penicillin binding proteins (PBP's) that cross-link strands of peptidoglycan in the cell wall. In gram negative cells, this leads to the formation of fragile spheroplasts that are easily ruptured. In gram positive cells, autolysis is triggered by the release of lipoteichoic acid (Greenwood, Finch, Davey, & Wilcox, 2007).

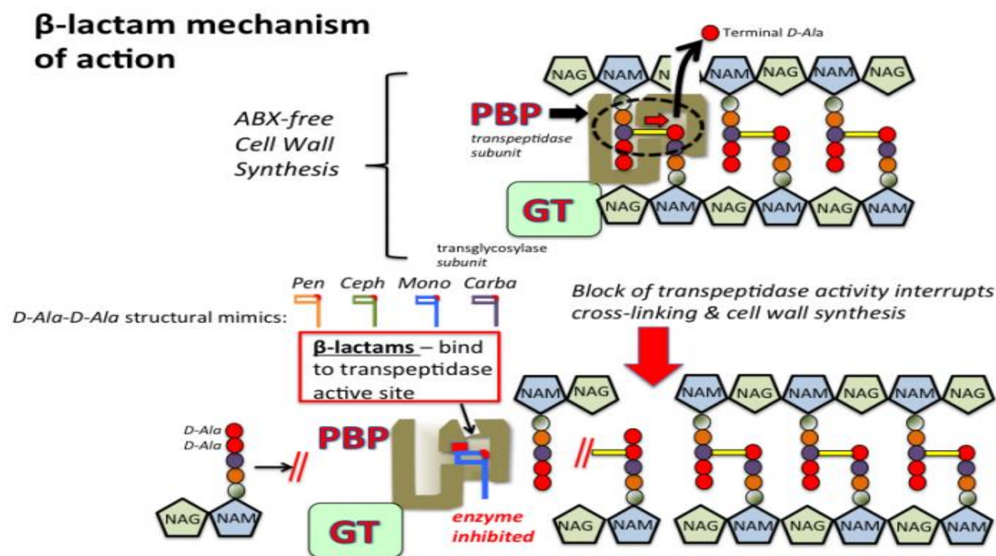

Figure 1.4: Mechanism of action of beta-lactam antibiotics. (Adapted from http://tmedweb.tulane.edu/pharmwiki/doku.php/betalactam_pharm)

The mechanism of β-lactam resistance is via the action of the β-lactamases. These enzymes catalyse hydrolysis of the β-lactam ring and, thereby, inactivate these antibiotics. Many bacteria contain chromosomally encoded β-lactamases necessary for cell wall production and it is only through over-production of these enzymes that resistance occur. β-lactamases encoded on plasmids or other transmissible elements can lead to such overproduction and, therefore, to resistance (Normark & Normark, 2002). There are also some bacteria that possess altered PBP's that result in reduced penicillin binding. Since the discovery of penicillin and resistant bacteria, various new versions

of the β-lactams have been used that have different spectrums of activity and different susceptibility to β-lactamases. Since the 1970s, several compounds, such as clavulanic acid, have been discovered that have the ability to bind irreversibly to β-lactamases and, thereby, inhibit their action. Combinations of these compounds with β-lactam drugs have been very successful in treatment of disease.

1.3.2.1.2 Resistance to glycopeptides

The glycopeptides are a group of antibiotics that include vancomycin, avoparcin, and others that bind to acyl-D-alanyl-D-alanine. Binding of this compound prevents the addition of new subunits to the growing peptidoglycan cell wall. These drugs are large molecules that are excluded from gram negative cells by the outer membrane, thus limiting their action to gram positive organisms.

Glycopeptide resistance was long thought to be rare, but has recently been shown to be quite common. Resistance in enterococci has developed through newly discovered enzymes that use D-alanyl-D-lactate in place of acyl-D-alanyl-D-alanine, allowing cell wall synthesis to continue. Other mechanisms of resistance involve the over-production of peptidoglycan precursors which overwhelm the drug (Greenwood et al., 2007).

1.3.2.2 Inhibitors of protein synthesis

There are many types of antibiotics that inhibit bacterial protein synthesis. These drugs take advantage of structural differences between bacterial ribosomes and eukaryotic ribosomes.

1.3.2.2.1 Resistance to aminoglycosides

The aminoglycoside antibiotics are a group whose mechanism of action is not completely understood. The three major groups of aminoglycosides are the streptomycins, neomycins, and kanamycins. These drugs enter bacterial cells by an active transport that involves quinones that are absent in anaerobes and streptococci, thus excluding these organisms from the spectrum of action. Streptomycins act by binding to the 30S ribosomal subunit. Kanamycins and neomycins bind to both the 50S subunit and to a site on the 30S subunit different from that of streptomycin (Greenwood et al., 2007). Activity involving initiation complexes and cell membrane proteins that contribute to cell death plays a role in the action of these antibiotics, but this is poorly understood.

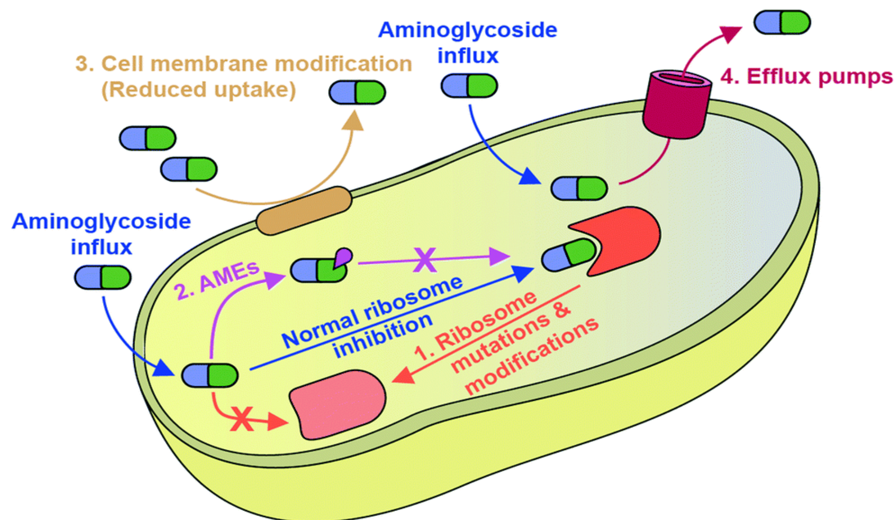

Figure 1.5: Schematic overview of mechanisms of resistance to aminoglycoside

There are three mechanisms of aminoglycoside resistance that have been identified to date. The first involves only streptomycin. Since streptomycin binds to one particular protein on the ribosome, alteration of this protein, even by a single amino acid in its structure, confers high-level resistance to the drug. The other mechanisms involve decreased uptake of the antibiotic and in one of these the cell membrane is altered, preventing active transport of the drug. In the other, one of many enzymes alters the antibiotic as it enters the cell, causing a block in further active transport.

1.3.2.2.3 Resistance to tetracyclines

The tetracyclines are another group of broad-spectrum antibiotics that inhibit bacterial protein synthesis. They are brought into the cell by active transport and, once there, bind to the 30S subunit to prevent binding of aminoacyl tRNA.

Resistance to the tetracyclines occurs via three mechanisms. First, production of a membrane efflux pump removes the drug as rapidly as it enters and there are several genes encoding these pumps. Second, several ribosome protection proteins act to prevent tetracycline from binding to the ribosome, thus conferring resistance. Third, a protein found only in *Bacteroides* spp., enzymatically inactivates tetracycline. Interestingly, efflux pump inhibitors have recently been discovered that may allow combinations of these inhibitors and tetracyclines to be used against previously resistant strains (Roberts, 1996).

1.3.2.2.4 Resistance to macrolides

The macrolides are a group of antibiotics commonly used to treat gram positive and intracellular bacterial pathogens. Erythromycin was the first of these, and several other important macrolides have been discovered since, including clarithromycin and azithromycin. Azithromycin has a longer plasma half-life which allows treatment with a single dose for some pathogens or a once daily dose for others. Clarithromycin has enhanced absorption and causes less gastrointestinal discomfort. It was originally believed that erythromycin inhibited protein synthesis by competing with amino acids for ribosomal binding sites, but newer research shows several mechanisms are involved. The macrolides are now believed to promote dissociation of tRNA from the ribosome, inhibit peptide bond formation, inhibit ribosome assembly, and prevent amino acid chain elongation.

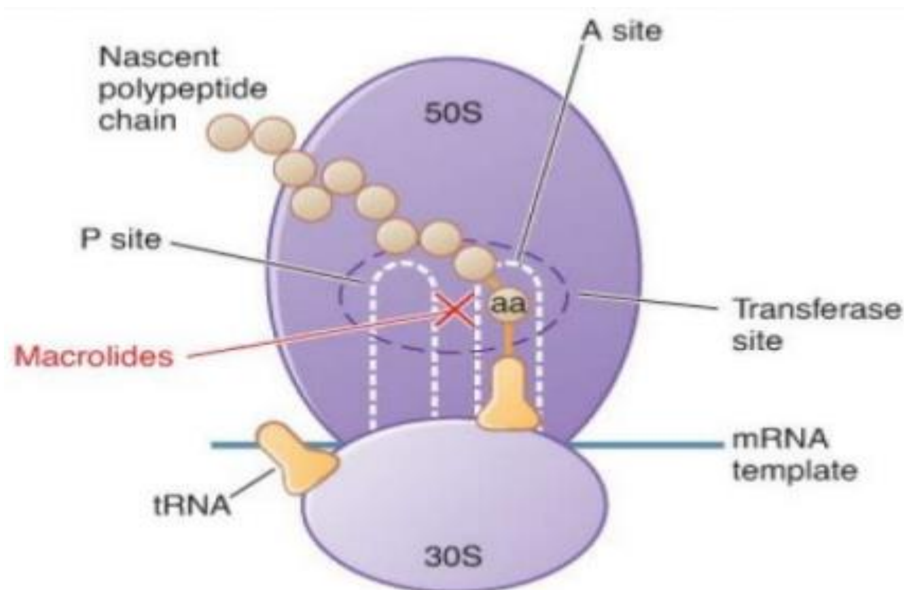

Figure 1.6: Mechanism of action of macrolide antibiotics

There are two major mechanisms of macrolide resistance. First, an efflux pump has been found that removes the drug from the cell. Second, modification of the ribosome can confer resistance. Mutations at several sites of the ribosome can allosterically prevent macrolide binding and a common alteration is dimethylation of one nucleotide on the 23S rRNA. This dimethylation not only prevents macrolide binding, but also confers resistance to lincosamide and streptogramin antibiotics (Gaynor & Mankin, 2003).

1.3.2.3 Inhibitors of nucleic acid synthesis

The sulfonamides and the diaminopyrimidines should be discussed together, in that both only indirectly inhibit nucleic acid synthesis by inhibiting folate synthesis. Folate is a coenzyme necessary for the synthesis of purines and pyrimidines. Although both types of drugs are useful on their own, they exhibit a synergistic effect when combined.

1.3.2.3.1 Resistance to sulfonamides

Sulphonamides are currently not used commonly in medicine, but the combination drug trimethoprim-sulfamethoxazole is sometimes used in the treatment of urinary tract infections. Sulphonamides serve as an analog of *p*-aminobenzoic acid. Therefore, they competitively inhibit an early step in folate synthesis. Diaminopyrimidines, of which trimethoprim is the most common, inhibit dihydrofolate reductase, the enzyme that catalyses the final step in folate synthesis. There are several resistance mechanisms microorganisms employ against each of the anti-folate drugs. For example, sulphonamides are rendered ineffective by over-production of *p*-aminobenzoic acid or production of an altered dihydropteroate synthetase. The substrate for dihydropteroate synthetase is *p*-aminobenzoic acid, and the altered form has a much lower affinity for sulphonamides than for *p*-aminobenzoic acid. Trimethoprim resistance can also result from several mechanisms, e.g., over-production of dihydrofolate reductase or production of an altered, drug-resistant form can lead to resistance. In addition, both drugs can be enzymatically inactivated, resulting in resistance (Then, 1982).

1.3.2.3.2 Resistance to quinolones

The quinolones are a chemically varied class of broad-spectrum antibiotics widely used to treat many diseases, including gonorrhoea and anthrax. Drugs in this class include nalidixic acid, norfloxacin, and ciprofloxacin. These drugs are commonly used and, worldwide, more ciprofloxacin is consumed than any other antibacterial agent. Quinolones inhibit bacterial growth by acting on DNA gyrase and topoisomerase IV, which are necessary for correct functioning of supercoiled DNA. Although quinolones target both enzymes, in gram negative organisms the primary target is DNA gyrase and, in gram positive organisms, the primary target is topoisomerase IV. There are three main mechanisms of resistance to quinolones. Resistance to some quinolones occurs with decreased expression of membrane porins. Cross-resistance to other drugs requiring these porins for activity also results from these changes. A second mechanism

of resistance is expression of efflux pumps in both gram negative and gram positive organisms and the third is alteration of the target enzymes. Several mutations have been described in both quinolone target proteins that result in reduced binding affinities (Ruiz, 2003). It is believed that high-level quinolone resistance is brought about by a series of successive mutations in the target genes, rather than a single mutation (Normark & Normark, 2002).

1.3.3 Multidrug-resistance

Resistance development in many human pathogens has been on an unprecedented scale, as resistance has evolved into multidrug resistance (MDR). This has led to increased global morbidity and mortality and we are today facing the possibility of a post antibiotic era. Especially bacterial strains belonging to the ESKAPE group of pathogens (*Enterococcus faecium*, *S. aureus*, *K. pneumoniae, Acinetobacter, P. aeruginosa and Enterobacter* are of importance to this pandemic. These pathogens encompassing both Gram negative and Gram-positive bacteria often carry MDR determining genes residing on genetic resistance island (RI) of complex evolutionary origin that are encoded on the chromosome or plasmids (Rice, 2008).

1.3.3.1 Mechanisms of MDR

The main mechanisms whereby the organism develop resistance to antimicrobial agents include enzymatic inactivation, modification of the drug targets, and reduction of intracellular drug concentration by changes in membrane permeability or by the over expression of efflux pumps. With respect to efflux pumps, they provide a self-defence mechanism by which antibiotics are actively removed from the cell. For anti-bacterials, these results in sub lethal drug concentrations at the active site that in turn may predispose the organism to the development of high-level target-based resistance. Antimicrobial drugs generally act on the microbes either by inhibiting a metabolic pathway like nucleotide synthesis which in turn leads to the inhibition of DNA/RNA synthesis and further protein synthesis and disruption of the cell membrane, or, by competing with the substrate of any enzyme involved in cell wall synthesis. Microorganisms have evolved a multitude of mechanisms to overcome the effectiveness of drugs, thereby surviving exposure to the drugs. New forms of antibiotic resistance can cross international boundaries and easily spread between continents. World health leaders have described antibiotic-resistant microorganisms as “nightmare

bacteria" that "pose a catastrophic threat" to people in every country in the world (Handzlik, Matys, & Kieć-Kononowicz, 2013).

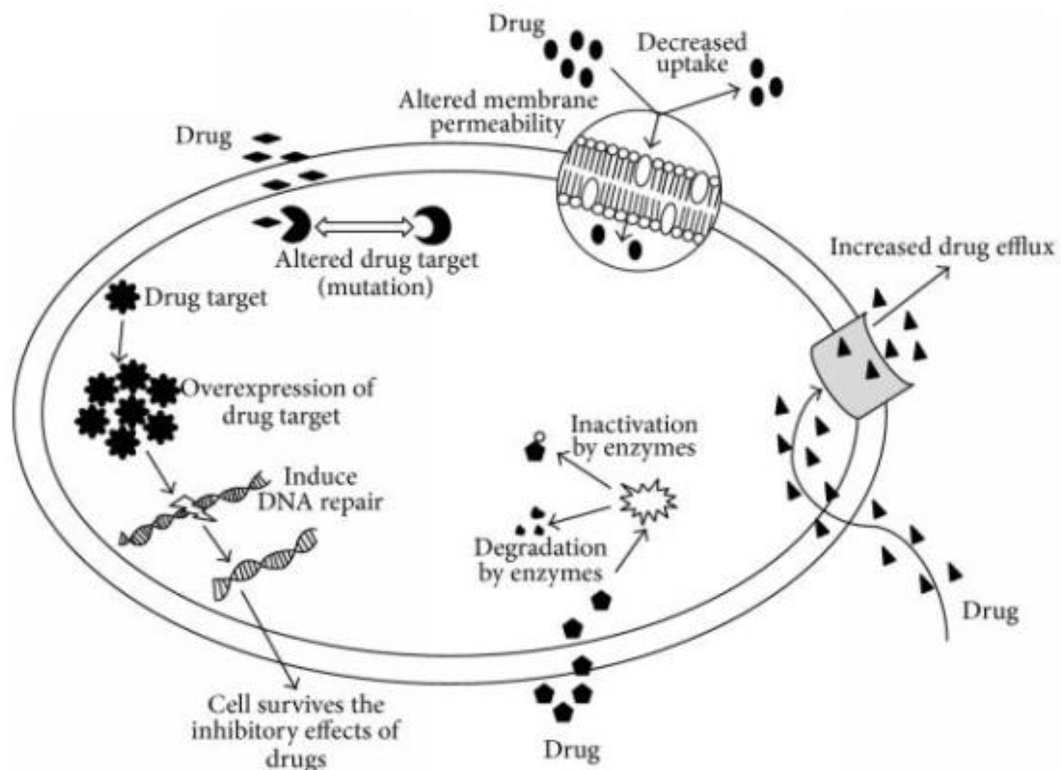

Figure 1.7: Mechanism of Multi-drug resistance

The widespread use of third generation cephalosporins and aztreonam has led to the emergence and dissemination of ESBL producing strains and their encoding genes, in particular, among *Enterobacteriaceae* associated with severe enteric illnesses (Tekiner & Özpınar, 2016).

1.4 Global economic impact of antibiotic resistance

Treatment of these resistant infections is associated with higher costs for second line drugs, additional investigations, and longer hospitalization. Other indirect costs associated with antibiotic resistance include productivity losses due to excess morbidity and premature mortality. These costs can be conceptualized as a negative externality to antimicrobial consumption accrued by all members of society, which are not reflected in the market price of antimicrobials (Shrestha et al., 2018). Moreover, it has an undesirable effect on people other than the immediate consumer of the antibiotic. This external cost is cross-sectional as it is imposed on multiple people other than the consumer itself, but also temporal in the sense that when the consequences of resistance

have appeared, the cost is also borne by the consumer. The risk of Medical Poverty Trap is more visible among the poor, illiterate and ignorant populations of underdeveloped and developing nations with low to medium resource at their disposal. The WHO data also suggests that there is lack of resources and infrastructure in the middle and low income countries. In this prevailing differential, in availability of health care at all levels (primary, secondary and tertiary level). The loss of human life is acute in spite of the fact that antibiotic resistance is a pan-global phenomenon affecting all social and income groups in community-acquired and nosocomial infections. The high cost of healthcare leads to risk of long term impoverishment, untreated morbidity and mortality. This phenomenon ultimately leads to the loss of human capital (Ahmad & Khan, 2019). The overall crude economic burden of antibiotic resistance was estimated to be at least €1.5 billion in 2007 in Europe and $55 billion in 2000 in the USA, including patient and hospital costs. Indirect patient costs were estimated on the basis of forgone earnings resulting from illness or premature death. For the European estimates, productivity losses accounted for 40% of the total estimated €1.5 billion, whereas productivity losses constituted 64% of the total estimated $55 billion for the USA. Both estimates fail to consider costs for patients or payers after discharge from the hospital (except for one outpatient follow-up visit in Europe). Excess healthcare costs for Europe were estimated based on longer hospital stays for patients with resistant infections compared to patients with susceptible infections, and one outpatient follow-up visit with a primary— care physician after hospitalization (S Gandra, Barter, & Laxminarayan, 2014). Without action, by 2050 the global economy may lose more than USD 6 trillion dollars annually because of antimicrobial resistance (AMR) – nearly 4% of Gross Domestic Product (GDP). By 2030, 24 million more people may be forced into extreme poverty because of AMR, many will come from low income countries. Thus, this will increase the number of people going hungry and suffering from malnutrition (Kang & Song, 2013).

1.5 Prevalence of Antibiotic resistance in Bangladesh

Antimicrobial resistance (AMR) poses a significant risk in terms of mortality and economic burden worldwide. However, the developing countries are more affected because of the widespread misuse of antibiotics, non-human antibiotic use, poor quality of drugs, inadequate surveillance, and factors associated with individual and national poverty (poor healthcare standards, malnutrition, chronic and repeated infections,

unaffordability of more effective and costly drugs). Furthermore, the scarcity of newer drugs means resistance must be contained before we run out of options to battle it. According to the 2014 WHO report on global surveillance of antimicrobial resistance, significant gaps in surveillance prevail, along with a lack of standards for methodology, data sharing, and coordination. However, the Southeast Asia, African, and Eastern Mediterranean regions have been identified as having major gaps (WHO, 2014).

Bangladesh, a developing country of Southeast Asia with a high degree of AMR, poses regional and global threat. In a study performed in Chittagong in 2003, typhoid patients were found to be unresponsive to second-line therapy (ciprofloxacin). First-line therapy was not even attempted because of existing resistance. Therapeutic failures like this are not rare at all. A study performed in BIRDEM General Hospital included samples collected over four years period which demonstrated that an average ESBL producing *Enterobacteriaceae* was 18 to 31% over four years. So there is considerable proportion of ESBL producing and imipenem resistant Gram negative bacteria. This study indicated high prevalence of resistant organisms to several antibiotics (Barai et al., 2017). Furthermore, in relation to this, multiple studies have demonstrated irrational antibiotic prescribing by physicians, a habit of self-medication among patients, and the indiscriminate use of antibiotics in agriculture and farming in different parts of the country (I. Ahmed et al., 2019).

1.6 Significance of antibiotic resistance surveillance in Bangladesh

Although the circumstances of antimicrobial resistance may vary by region or country, it is clear that Asia is an epicentre of antimicrobial resistance; the prevalence of antimicrobial resistance of major bacterial pathogens has been rapidly increasing. However, public health infrastructures to control the problem are very poor in many countries. Given that more than 70% of the worldwide population lives in the Asia-Pacific region, antimicrobial resistance in this region is, literally, a global problem (Kang & Song, 2013). However, there have been no long-term studies on trends in Antibiotic resistance on a national scale in Bangladesh. Antimicrobial resistance surveillance on a national scale is critical because it provides information on the extent of established resistance rates, as well as emerging patterns of resistance. Understanding how resistance is changing is important for (1) establishing prescribing guidelines, (2) determining investment in new therapies, and (3) improving the targeting of campaigns to reduce antimicrobial resistance. It also provides a baseline

for future analysis and comparison with other countries (Sumanth Gandra et al., 2016). Also, by studying the resistance genes that induce antibiotic resistance and the plasmids that transfer such genes, the mechanism that leads to antibiotic resistance and the source can be elucidated.

1.7 Aims of the study

With the major problem of multi drug resistant bacteria and the increasing rate of antibiotic resistance in Bangladesh, the overall purpose of this research was to fill in knowledge gaps concerning the existence and the mechanisms of antibiotic resistance genes. With a view to achieving this aim, the objectives of this study were set to-

- Determine the resistance pattern of the clinical isolates from wound infections
- Molecular characterization of Aminoglycosides, Extended Spectrum of β-Lactamase (ESBL) and Macrolides resistance genes among the isolates
- Understand the correlation between phenotype and genotype of resistance.

Chapter 2

METHODOLOGY

Methodology

2.1 Laboratory facility

The laboratory works of this study were conducted at the institute for developing Science and Health initiatives (ideSHi), Hosaf Tower, Mohakhali, Dhaka. The laboratory has been set up to meet international BSL-2 requirements in order to carry out molecular, immunological and genetic studies.

2.2 Duration of the study

This study was carried out from January 2019 to October 2019.

2.3 Sample collection

This retrospective analysis was conducted by reviewing records of wound swab samples that arrived at the Microbiology laboratory of the Bangladesh Institute of Health Sciences Hospital (BIHS) and Shaheed Suhrawardy Medical College & Hospital, from January 2017 to March 2019. Information about the gender and age of the patients was also provided. In total, 217 hospital and community specimens of wound submitted for routine bacterial studies were collected and analysed.

After superficial precleansing of wounds with physiologic saline, each specimen was collected by rotating a sterile, premoistened across the wound surface of a 1 cm^2 area in a zig-zag motion, from the centre to the outside of the wound. Then, the swab was placed in the tube containing the transport medium and sent to ideSHi and were processed according to standard techniques.

2.3.2 Sample Preparation and microbiological culture

Each swab was plated onto two media: blood agar and MacConkey agar. MacConkey agar plates were incubated aerobically whereas blood agar plates were incubated anaerobically at 37°C for 24 hours. Any growth was identified by morphologic aspects of the colonies.

2.3.3 Confirmation of bacterial isolates

For confirming the bacterial isolates, several methods were followed. These methods include-

- ✓ Analysis of colony morphology
- ✓ Gram staining

- ✓ Biochemical tests
- ✓ Catalase test
- ✓ Oxidase test
- ✓ Coagulase test
- ✓ Antibiotic sensitivity test

2.3.3.1 Microbial isolation and identification

Well isolated colonies were used for the phenotypic identification of antibiotic resistance.

2.3.3.2 Analysis of colony morphology

When grown on a variety of media, microorganisms exhibit differences in the macroscopic appearance of their growth. These differences are known as cultural characteristics, are used as a basis for separating microorganisms into taxonomic groups. Analysis of colony morphology helps to determine the cultural characteristics of microorganisms as an aid in identifying and classifying organisms into taxonomic groups. These demonstrate well isolated colonies and are evaluated by observing the size, pigmentation, form, margin and elevation of the colonies (Cappuccino & Sherman, 2008).

2.3.3.3 Gram staining

Grease or oil free slides are essential for the preparation of microbial smears. Grease or oil from the slides was removed by cleaning with a tissue paper. With a sterile cooled loop, isolated colonies along with a drop of normal saline were heat fixed on the centre of a glass slide. A satisfactory smear allows examination of the typical cellular arrangement and isolated cells. Then the slide with heat fixed smear was placed on staining tray. The smear was flooded with crystal violet for 1 minute. Later, the slide was gently rinsed with distilled water using a wash bottle. Then the slide was again flooded with Gram's iodine for 1 minute. It forms complex structure with crystal violet which tightly binds with the cell wall of the bacteria. The iodine solution was rinsed off with distilled water. After that the smear appears as a purple circle on the slide. The smear was then decolorized using 95% ethyl alcohol or acetone. It acts as the decolorizing agent so that it dehydrates the peptidoglycan layer through shrinking the slide was then tilted slightly and the alcohol drops were applied by drop for 5 to 10

seconds until the alcohol ran almost clear. The slide was immediately rinsed with deionized water.

Then the slide was gently flooded with safranin to counter-stain and allowed to stand for 45 seconds. It gives light red or pink colour in the cell wall of gram-negative bacteria due to retention. Slide was blotted dry with a tissue paper and placed in an upright position. The smear was then viewed under a light-microscope using oil-immersion.

2.3.3.4 Biochemical tests

Biochemical tests were done to differentiate between closely related bacteria. The biochemical tests were performed according to the methods described and applied in Microbiology Laboratory of ideSHi. The biochemical tests that were done for the confirmation of bacteria were-

- Triple Sugar Iron (TSI) test
- Citrate utilization test
- Motility Indole Urease (MIU) test

2.3.3.4.1 Triple Sugar Iron Test

With a sterilized straight inoculation needle, the top of a well-isolated colony from MacConkey agar plate was picked up. The needle containing the colony was inoculated into TSI Agar by first stabbing through the centre of the medium to the bottom of the tube and then streaking on the surface of the agar slant. The cap was left on loosely and incubated at 35°C in ambient air for 18 to 24 hours to observe carbohydrate fermentation, CO_2 and H_2S production.

TSI Slant contains agar, pH sensitive dye (phenol red), 1% sucrose, 1% lactose and 0.1% glucose, sodium thiosulphate together with ferrous sulphate or ferrous ammonium sulphate. All of these ingredients were mixed together and allowed to solidify in a test tube in a slanted angle. This slanted shape of this medium provides an array of surfaces that are either exposed to oxygen-containing air in varying degrees or not exposed to air. Bacteria fermenting any of these three sugars will produce acid as by-products. Therefore, the colour of the phenol red will turn red to yellow. Position of the colour change in the tube will determine the fermented sugar. For example, if the butt turns yellow, it represents that fermentation of glucose has occurred. If the whole agar turns yellow, it represents that all three sugars have been fermented and if it turns black, it

confirms the production of hydrogen sulphide (H_2S) gas and production of bubbles confirms Carbon dioxide (CO_2) gas formation.

2.3.3.4.2 Citrate utilization test

A well isolated colony was taken using a sterilized straight needle. The needle containing the colony was inoculated into Simmons's citrate agar by first stabbing through the centre of the medium to the bottom of the tube and then streaking on the surface of the agar slant. Then it was incubated overnight at 37°C to detect if the organism had the ability to utilize citrate. Abundant growth on the slant and a change from green to blue in the medium indicates a positive test for growth using citrate and there would be no colour change for the negative result.

2.3.3.4.3 Motility indole urease test

Motility Indole Urease (MIU) media is commonly used for the identification of gram negative bacteria of *Enterobacteriaceae* family. It is a single medium which is incorporated with three individual tests such as motility of the organism, indole production and presence of urease enzyme.

A sterile straight needle containing a well isolated colony from a freshly cultured media was inoculated in the MIU media making a single stab down the centre of the tube to about half the depth of the medium and an indole reagent paper was attached at the top of the test tube. Then it was incubated overnight at 37°C. If the organism is motile, it would typically give diffuse, hazy growths that spread throughout the medium rendering it slightly opaque. Indole production will be determined by the indole reagent paper attached at the top of the test tube. Change of the paper colour into pink indicates a positive result. To detect the presence of urease enzyme, the top of the media is observed. If the organism is urease positive, it will degrade the urea present in the medium into ammonia. This ammonia will react with water to produce ammonium hydroxide and thus the colour of the medium will turn yellow to red or pink.

2.3.3.5 Catalase test

A small amount of bacterial colony was transferred to a surface of clean, dry glass slide using a loop. A drop (1 drop ~10 µl) of 30% H_2O_2 was placed on to the slide and mixed. Within 5 to 10 seconds the rapid evolution of oxygen as evidenced by bubbling specifies positive result. A negative result is no bubble or only a few scattered bubbles.

2.3.3.6 Oxidase test

A small amount of well isolated colony from a fresh bacterial culture was picked up and rubbed onto a filter paper soaked with the substrate tetramethyl-p-phenylenediamine dihydrochloride. Inoculated area of paper was observed for a colour change to deep blue or purple within 10-30 seconds. If the colour changes, it indicates positive result and no colour change indicates negative result.

2.3.3.7 Coagulase test

Coagulase is an enzyme-like protein and causes plasma to clot by converting fibrinogen to fibrin. *S. aureus* produces two forms of coagulase: bound and free. Coagulase test is used to differentiate *S. aureus* (positive) which produce the enzyme coagulase, from *S. epidermis* and *S. saprophyticus* (negative) which do not produce coagulase. i.e. Coagulase Negative *Staphylococcus* (CNS). A small amount of bacterial colony was transferred to a surface of clean, dry glass slide using a loop. A drop (1 drop ~10 µl) of human or rabbit plasma was placed on to the slide and mixed gently. Result was read as positive for a coarse clumping of cocci visible to the naked eye within 10 seconds. On the other hand negative result was interpreted for the absence of clumping or any reaction taking more than 10 seconds to develop.

2.3.3.8 Antibiotic sensitivity test

Antibiotic sensitivity test (AST) is performed to determine the resistance pattern of particular bacterium. The test is carried out by antibiotic disk diffusion method on Mueller-Hinton agar (MHA) media. Well isolated colonies from MacConkey agar plate and blood agar plates were picked up by a sterilized loop and suspended in 0.8% normal saline and then vortex was done for uniform mixing. Using aseptic technique, a sterile swab was dipped into the bacterial suspension in normal saline. The swab was then used to make bacterial lawn on the MHA (blood agar was used for *Streptococcus* spp.) by gently rubbing it on the surface. To obtain uniform growth, the plate was swabbed in one direction, after completion the swabbing direction was changed by rotating the plate to 45° for 4 times. This helps to disperse the organism throughout the plate.

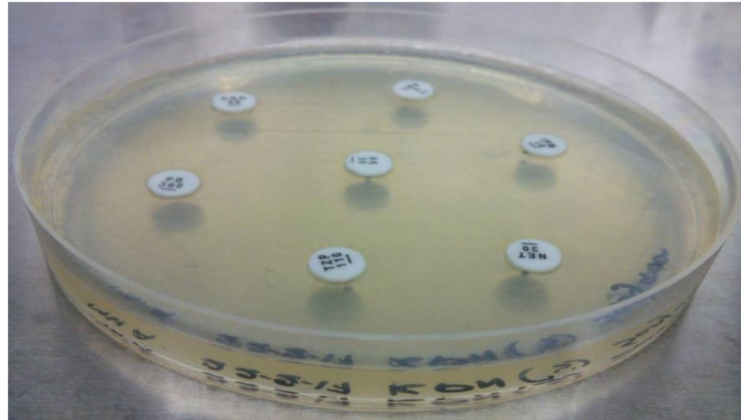

Figure 2.1: Placement of antibiotic discs on MHA containing bacterial lawn culture

The plate was then allowed to dry for approximately 5 minutes. The required antibiotic disks were then placed using one flame-sterilized forceps on the lawned surface of the MHA. The disks were gently pressed using the forceps to the agar to ensure that the discs are attached to the agar. Plates were incubated overnight at an incubation temperature of 37 °C (98.6 °F). Following overnight incubation, inhibition zone sizes of each antibiotic was measured by taking the lowest diameter of the clear zone.

In this study, specific commercially available antibiotics were used. The antibiotics were selected for the Antimicrobial Sensitivity Test as those are specified for the detection of gram positive and gram negative organisms according to CLSI (Clinical and Laboratory Standards Institute) Guideline as modified in 2016. The antibiotic disks which were used for AST along with their zone diameter for gram positive (Table 2.1) and gram negative organisms (Table 2.2) are given below:

SL	Antibiotics Name	Short Name	Concentration	Result Interpretation		
				Sensitive	**Intermediate**	**Resistant**
1	Ampicillin	AMP	10 µg	**≥17**	**14-16**	**≤13**
2	Tetracycline	TET	30 µg	**≥15**	**12-14**	**≤11**
3	Azithromycin	AZM	15 µg	**≥13**		**≤12**
4	Cefixime	CFM	5 µg	**≥19**	**16-18**	**≤15**
5	Ceftriaxone	CRO	30 µg	**≥23**	**20-22**	**≤19**
6	Ciprofloxacin	CIP	5 µg	**≥21**	**16-20**	**≤15**
7	Gentamycin	CN	10 µg	**≥15**	**13-14**	**≤12**
8	Imipenem	IMP	10 µg	**≥23**	**20-22**	**≤19**
9	Meropenem	MEM	10 µg	**≥23**	**20-22**	**≤19**
10	Netilmicin	NET	30 µg	**≥15**	**13-14**	**≤12**
11	Pipercilin+ Tazobactam	TZP	10 µg	**≥21**	**18-20**	**≤17**
12	Polymyxin B	PB	15 µg	**≥14**	**12-13**	**≤11**
13	Tobramycin	TOB	10 µg	**≥15**	**13-14**	**≤12**
14	Chloramphenicol	C	10 µg	**≥18**	**13-17**	**≤12**

Table 2.1: Zone diameter interpretation chart for Enterobactericeae (Ref: Clinical and Laboratory Standards Institute (CLSI), Zone Diameter for Enterobactericeae)

SL	Antibiotics Name	Short Name	Concentration	Result Interpretation		
				Sensitive	Intermediate	Resistant
1	Penicillin	P	10 µg	**≥29**		**≤28**
2	Clindamycin	CD	2 µg	**≥21**	**15-20**	**≤14**
3	Cefoxitin	FOX	30 µg	**≥22**		**≤21**
4	Vancomycin	VAN	30 µg	**≥17**	**15-16**	**≤14**
5	Chloramphenicol	C	30 µg	**≥18**	**13-17**	**≤12**
6	Rifampicin	RIF	5 µg	**≥20**	**17-19**	**≤16**
7	Linezolid	LZ	30 µg	**≥21**		**≤20**
8	Gentamycin	CN	10 µg	**≥15**	**13-14**	**≤12**
9	Azithromycin	AZM	15 µg	**≥18**	**14-17**	**≤13**
10	Tetracycline	TET	30 µg	**≥19**	**15-18**	**≤14**
11	Netilmicin	NET	30 µg	**≥15**	**13-14**	**≤12**
12	Ciprofloxacin	CIP	5 µg	**≥21**	**16-20**	**≤15**
13	Levofloxacin	LEV	5 µg	**≥19**	**16-18**	**≤15**
14	Tobramycin	TOB	10 µg	**≥15**	**13-14**	**≤12**

Table 2.2: Zone diameter interpretation chart for Gram positive organisms (Staphylococcus spp, Enterococcus spp and Streptococcus spp) (Ref: Clinical and Laboratory Standards Institute (CLSI), Zone Diameter for Staphylococcus spp, Enterococcus spp and Streptococcus spp)

2.3.3.9 Preservation of bacteria

To use the bacteria for a long period of time we needed to preserve them in a proper condition. We had to make sure that there were no characteristic changes in the strains. Accordingly, bacterial strains were stored for short term as well as for long term preservation.

2.3.3.9.1 Short term preservation

For daily or weekly use of the bacteria short term preservation method was followed. There is a chance of the bacterial colony to get mutated when the culture is old because the competition for nutrition and space increases between the bacteria in the same colony. So the bacteria try to develop to get the most benefits. In this study, the bacteria were preserved for short time period in agar plates such as Nutrient agar and MacConkey. Agar plates were used for this purpose. A single colony of bacteria was taken in a loop and was streaked on the agar plates by quadrant streak method to get more single colonies. The plates were incubated overnight at 37°C to get the colonies. Following day, the plates were sealed with Parafilm® and stored at 4°C refrigerator for the next use. The Parafilm® helps to avoid any contamination in the plate while storing and also it helps to maintain the moisture in the plate which prevents the bacterial colonies from drying. Using this method, the strains can be stored in the refrigerator for

a month. For this study, the strains were sub-cultured on seventh day after culturing to maintain healthy bacterial colonies.

2.3.3.9.2 Long term preservation

Long term bacterial preservation is important because it helps to store the strain as stock sample. The bacteria could be revived again for later use if the short term preserved bacteria are very old with possibility of mutation. For this study, long term preservation of bacteria was done in a medium containing skim milk, tryptone, glucose, and glycerine (called STGG medium) and it was stored at -80°C in Ultra Low Temperature (ULT) freezer. It is an inexpensive medium and remains stable for at least 6 months after sterilization. After every 6 months, the bacteria were thawed from the freezer, streaked on the agar plate and incubated overnight at 37°C to culture the bacteria. Next day, the bacterial colonies were ready for use or a few single colonies were picked up and inoculated in fresh STGG medium and restored in the ULT freezer for further preservation.

2.4 Detection of antibiotic resistance genes

In order to confirm the presence of antibiotic resistance genes (Aminoglycoside, Macrolide and ESBL) in the bacterial isolates, molecular techniques such as PCR (polymerase chain reaction) and sequencing were done.

2.4.1 Designing a PCR primer

The DNA sequences of several antibiotic resistance genes were retrieved from the nucleotide database of National centre for Biotechnology Information (NCBI). The retrieved FASTA sequence was then used to design specific primers using Primer-BLAST.

2.4.1.1 Software

Primer-3 plus, Primer-Blast.

Primer-Blast is a widely used program for designing PCR primers (PCR = "Polymerase Chain Reaction"). PCR is an essential and ubiquitous tool in genetics and molecular biology. Primer-Blast can also design hybridization probes and sequencing primers. PCR is used for many different goals. Consequently, primer blast has many different

input parameters that can be controlled and that tell exactly what characteristics make good primers for certain goals.

Primer-BLAST URL: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

2.4.1.2 Method

- ⇨ **http://www.ncbi.nlm.nih.gov/tools/primer-blast/** was browsed
- ⇨ Sequences were pasted
- ⇨ Parameters were changed if necessary
- ⇨ **Get primers** was clicked on and few minutes were required to get the result.

Figure 2.2: Uploading query sequence into Primer-BLAST

List of Aminoglycoside resistance gene primers selected for the study

Gene	Primer name	Sequence (5’-->3’)	Amplicon size (bp)
aac(3)-II	aac(3)-II_F	ATATCGCGATGCATACGCGG	877
	aac(3)-II_R	GACGGCCTCTAACCGGAAGG	
aac(6)-Ib	aac(6)-Ib_F	TTGCGATGCTCTATGAGTGGCTA	482
	aac(6)-Ib_R	CTCGAATGCCTGGCGTGTTT	
aac(6)-II	aac(6)-II_F	CGACCATTTCATGTCC	541
	aac(6)-II_R	GAAGGCTTGTCGTGTTT	
ant(3)-I	ant(3)-I_F	CATCATGAGGGAAGCGGTG	787
	ant(3)-I_R	GACTACCTTGGTGATCTCG	
aph(3)-VI	aph(3)-VI_F	ATGGAATTGCCCAATATTATT	780
	aph(3)-VI_R	TCAATTCAATTCATCAAGTTT	
armA	armA_F	CCGAAATGACAGTTCCTATC	846
	armA_R	GAAAATGAGTGCCTTGGAGG	
rmtB	rmtB_F	ATGAACATCAACGATGCCCTC	769
	rmtB_R	CCTTCTGATTGGCTTATCCA	

Table 2.3: Primers specific to aminoglycoside resistance genes and their amplicon size

List of Macrolide resistance gene primers selected for the study

Gene	Primer name	Sequence (5’-->3’)	Amplicon size (bp)
ErmA	ErmA-F	CTTCGATAGTTTATTAATATTAGT	645
	ErmA-R	TCTAAAAAGCATGTAAAAGAA	
ErmB	ErmB-F	GGA ACA TCT GTG GTA TGG CG	425
	ErmB-R	CAT TTA ACG ACG AAA CTG GC	
ErmC	ErmC-F	CAA ACC CGT ATT CCA CGA TT	295
	ErmC-R	ATC TTT GAA ATC GGC TCA GG	
ErmF	ErmF-F	TGT TCA AGT TGT CGGTTG TG	260
	ErmF-R	CAG GAC CTA CCT CAT AGA CA	

Table 2.4: Primers specific to macrolide resistance genes and their amplicon size

List of Extended spectrum beta lactamase (ESBL) gene primers selected for the study

Gene	Primer name	Sequence (5'-->3')	Amplicon size (bp)
KPC	KPC-1A	CTGTCTTGTCTCTCATGGCC	795
	KPC-1B	CCTCGCTGTGCTTGTCATCC	
Oxa48	Oxa48-F	GCGTGGTTAAGGATGAACAC	400
	Oxa48-R	CATCAAGTTCAACCCAACCG	
VIM1	VIM1F	AGTGGTGAGTATCCGACAG	241
	VIM1R	ATGAAAGTGCGTGGAGAC	
NDM	NDM-F	GGTTTGGCGATCTGGTTTTC	621
	NDM-R	CGGAATGGCTCATCACGATC	
CTX-M1	CTX-M1-3F	AATCACTGCGCCAGTTCACGCT	841
	CTX-M1-R2	AGCCGCCGACGCTAATACA	

Table 2.5: Primers specific to ESBL genes and their amplicon size

2.4.2 Bacterial DNA extraction

DNA was extracted from the isolates of different organisms. The procedure performed in order to extract high quality DNA from the bacterial isolates is given below-

- One separated single colony was taken from MacConkey agar media and blood agar media by using a sterile platinum loop
- It was suspended in a 1.5 ml Microcentrifuge tube containing 100 µl autoclaved PBS then waited for 1-2 minutes
- The suspension was then vortexed for 15 seconds until homogenization
- The tube was heated at Water bath (WiseBath, USA) for 10 minutes
- It was immediately kept in ice for 1 minute
- The suspension was then centrifuged at 13000 rpm for 10 minutes
- The supernatant was transferred into another RNase and DNase free microcentrifuge tube
- The separated supernatant was used as the PCR template and the pellet was discarded. The extracted DNA was stored at -20°C for later use.

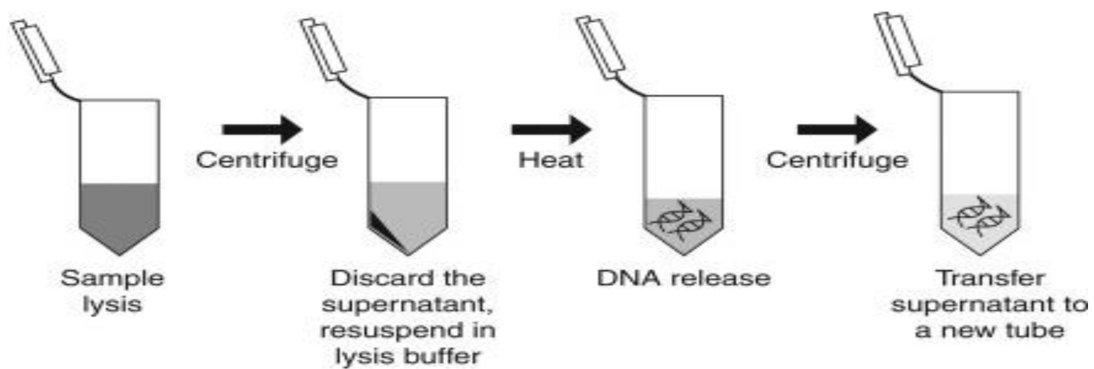

Figure 2.3: Bacterial DNA extraction using boiling method (Barbosa, Nogueira, Gadanho, & Chaves, 2016)

2.4.3 Measurement of DNA concentration and purity

DNA concentration was measured with EON spectrophotometer using Take 3 plate. Approximately 2 µl Nuclease Free water was used as blank and 2 µl of DNA sample was loaded on the Take 3 plate and OD (optical density) was measured with spectrophotometer. The concentration was measured in ng/µl. The purity was checked using OD ratio at 260 nm/280 nm reading. The result was evaluated with GEN5 Software.

2.4.4 Polymerase chain reaction for the amplification of extracted DNA

These isolates demonstrating antibiotic resistance were screened for the presence of several antibiotic resistance gene using gene-specific Polymerase Chain Reaction. PCR is a powerful technique in molecular biology that uses Taq DNA Polymerase enzyme to amplify the quantity of a DNA sample. At first, high temperatures in the denaturation phase denature the double stranded template into single strands. Then in the annealing phase, short oligonucleotides called primers bind to the template strands. After that, the Taq Polymerase binds to the primers and starts forming new strands by adding complementary nucleotides and thus forming double stranded DNA again. This occurs in the renaturation phase.

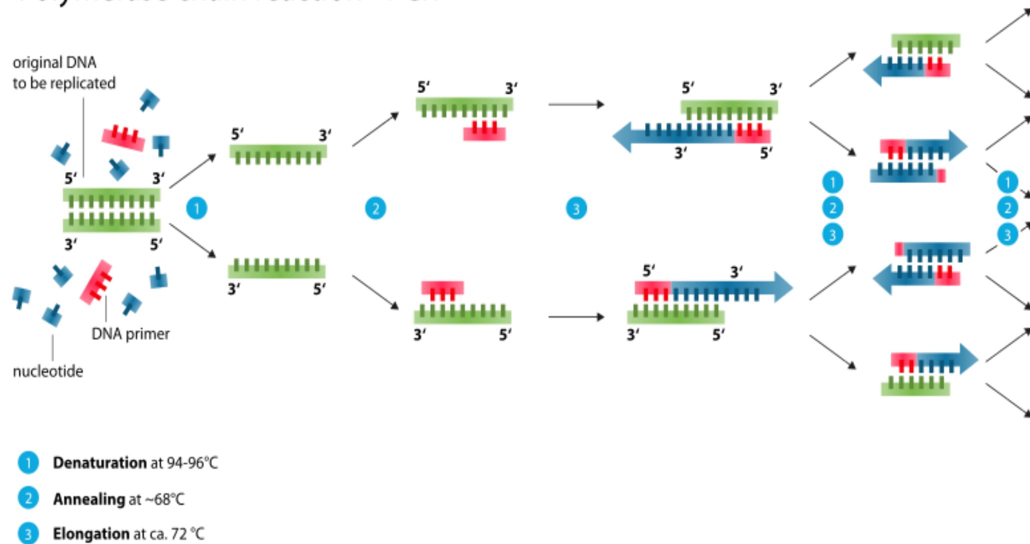

Figure 2.4: Principle of Polymerase chain reaction

(Source: https://www.onlinebiologynotes.com/polymerase-chain-reaction-pcr-principle-procedure-steps-types-application/)

2.4.5 Conventional PCR

PCR was performed using a T100 ™ thermal cycler (Bio-Rad, USA). The final reaction volume was 10 µl which contains-

- 1 µl 10X PCR buffer
- 0.3 µl 50mM $MgCl_2$
- 0.2 µl of 10mM dNTPs mixture
- 0.5 µl forward and reverse primers
- 0.05 µl of Taq polymerase
- 5.45 µl Nuclease Free Water
- 2 µl of template DNA

Components	Amount
10 mM dNTPs	0.2 µl
10X buffer	1 µl
50mM $MgCl_2$	0.3 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Taq polymerase	0.05 µl
Nuclease free water	5.45 µl
Template DNA	2 µl
Total volume	**10 µl**

Table 2.6: PCR master mix

Taq Polymerase was added right before loading the sample in the PCR machine (Infinigen, USA). All the steps were performed on the PCR cooler rack.

PCR condition	Temperature	Time	
Initial denaturation	95°C	5minutes	
Denaturation	95°C	30 seconds	35 Cycles
Annealing	~55°C (Depending on primer Tm)	30 seconds	35 Cycles
Extension	72°C	45 seconds	35 Cycles
Final extension	72°C	6 minutes	
	10°C	∞	

Table 2.7: Thermal cycling profile

The annealing temperature (55°C) is related to the melting temperature (Tm) of the primers and must be determined for each primer pair used in PCR. During the extension step (72°C), the polymerase extends the primer to form a nascent DNA strand. This process is repeated multiple times (35 cycles) and because each new strand can also serve as a template for the primers, the region of interest is amplified exponentially. The final step of the PCR is generally a longer, single temperature step (6 min at 72°C) that allows for the completion of any partial copies and the clearance of all replication machinery from the nascent DNA. Once the PCR is complete, the thermal cycler is set to 4°C to 10°C to maintain product integrity until such time as the tubes can be removed from the machine.

2.4.6 Agarose gel preparation and electrophoresis

Agarose gel electrophoresis is a standard laboratory procedure used to separate amplified PCR product into bands based on size. Amplified DNA is applied in wells in the gel close to the negative electrode. In the presence of an electrical field, negatively charged DNA moves toward the positive pole through the small holes that make up the

gel matrix. These holes allow the shorter fragments of DNA to migrate faster than their longer counterparts. Once the reaction is complete, the length of the amplified DNA can be accurately determined by comparing with a DNA ladder.

Amplified PCR products were analysed by electrophoresis on 1% agarose gel. 0.50 g agarose (Ultrapure, Invitrogen, USA) was dissolved in 50 ml 1X TAE buffer heating it in a microwave oven for about 1 to 2 minutes. The mixture was then allowed to cool down skin tolerable temperature. Then, 1 µl of DNA gel stain SYBR Safe (Invitrogen, USA) was added and homogenously mixed before solidification of the gel. The gel was then poured on the gel casting tray previously set with the comb and allowed to solidify. While pouring the melted gel mix solution into the gel tray, care was taken so that no bubbles were formed.

2.4.6.1 Detection of PCR product using agarose gel electrophoresis

5 µl of the PCR product was mixed with 2 µl of loading dye and was loaded into the individual wells of the gel. A ladder of size 1Kb plus (Invitrogen, USA) was used to ensure amplification of the desired gene and measure the exact product size which was estimated to be within 1,500 bp. Amplified PCR products were electrophoresed at 100 volts for 40 minutes. The separated DNA bands were observed on a Gel documentation system (Bio-Rad, USA) under Ultraviolet light.

2.4.7 PCR purification of PCR products

PCR purification is a convenient system for fast and reliable purification of single- or double-stranded DNA fragments from PCR and other enzymatic reactions. DNA was purified from PCR product by using QIAquick PCR Product purification kit. Fragments ranging from 100 bp to 10 kb are purified from primers, nucleotides, polymerases, and salts using QIAquick spin columns in a microcentrifuge tube.

- 5 volumes of PB Buffer (Binding buffer) was added to 1 volume of PCR product and mixed by pipetting
- A column was placed in a provided collection tube and the mixture was transferred to the column
- 1 to 2 minutes incubation for binding
- The mixture was centrifuged at 13,000 rpm or 17,000xg for 1 minute
- The flow through was discarded and the column was placed back into the same collection tube

- 750 µl PE Buffer (Wash buffer) was added to the column and waited for 1 minute
- It was then centrifuged at 13,000 rpm or 17,000 xg for 1 minute
- The flow through was discarded and the column was placed back in the same tube
- The column was once more centrifuged at 13,000 rpm or 17,000xg for 1 minute to remove residual wash buffer
- The column was placed in an autoclaved 1.5ml microcentrifuge tube
- 15 µl of Nuclease free water was added to the center of the membrane of the column
- Centrifuged at 14,000rpm for 2 minutes
- The column was discarded and 1.5 ml microcentrifuge tube was taken
- The purified PCR product was stored at 4 °C

2.4.8 Gel purification of PCR products

Another way of purifying the DNA product from agarose gel is gel purification. Gel purification allows isolation and purification of DNA fragments based on size. After observing the presence of the desired DNA in the PCR amplicon, DNA was purified from the agarose gel using QIAGEN gel extraction kit. Before purification, approximately 30 µl of PCR products along with the loading dye were loaded and electrophoresis was done. For getting smoother separation of DNA bands, the gel was run at 80 volts for 1.5 hours. The procedure performed in order to purify gel is given below:

- The DNA fragment was excised from the agarose gel with a clean, sharp scalpel. The size of the gel slice was minimized by removing extra agarose.
- Then the excised gel was weighed in an Electronic balance machine (Mega, Japan standard).
- 3 volume of buffer QG was added to 1 volume of gel.
- To dissolve the gel properly, it was incubated at 50°C for 10 minutes with periodic vortexing.
- After the gel has dissolved, the colour of the mixture was observed if it was yellow or not. If the colour turns orange or violet, 10 µl of 3 M sodium acetate is mixed.

- Then, 1 gel volume of isopropanol was added to the sample to increase the yield of DNA fragments.
- Then a QIA quick spin column was placed in a provided 2 ml collection tube.
- For binding of DNA, the sample solution was added to the QIA quick column and then centrifuged at 17,900 g for 1 minute.
- The flow through was then discarded.
- After discarding the flow- through, 0.5 ml of QG buffer was added and centrifuged for 1 minute to remove all the traces of agarose. This step is only required when the DNA will subsequently be used for direct sequencing.
- Later, for washing 750 µl of PE buffer was added to the QIA quick column and centrifuged for 1 minute at 17,900 x G.
- It was centrifuged twice and the flow through was discarded to remove the whole of ethanol as it will precipitate the DNA. Ethanol is PCR inhibitor and it will precipitate the product, that's why it is done twice. Residual ethanol from buffer PE will not be completely removed unless the flow through is discarded before this additional centrifugation.
- The QIA quick column was placed into a clean 1.5 ml microcentrifuge tube directly onto the white membrane without touching.
- To elute DNA, 50 µL of nuclease free water was added to the centre of the QIA quick membrane which was then centrifuged for 1 minute at maximum speed. Water is added according to the concentration.
- The column was then discarded and the supernatant containing the extracted PCR product was stored at +4°C for downstream use.

2.5 Nucleotide sequencing (Sanger sequencing method)

DNA sequencing is the process of reading nucleotide bases in a DNA molecule. During Sanger sequencing, DNA polymerase copy single stranded DNA templates by adding nucleotides to a growing chain. During Sanger sequencing, DNA polymerases copy single-stranded DNA templates by adding nucleotides to a growing chain (extension product). Chain elongation occurs at the 3' end of a primer, an oligonucleotide that anneals to the template. The deoxynucleotides added to the extension product are selected by base-pair matching to the template. The extension product grows by the formation of a phosphodiester bridge between the 3'-hydroxyl group on the primer and

the 5'-phosphate group of the incoming deoxynucleotide (Watson et al. 1987). Growth occurs in the 5' -> 3' direction. DNA polymerases can also incorporate analogues of nucleotide bases. The dideoxy method of DNA sequencing developed by Sanger takes advantage of this characteristic by using 2', 3'-dideoxynucleotides as substrates. When dideoxynucleotides are incorporated at the 3' end of the growing chain, chain elongation is terminated selectively at A, C, G, or T. This is because once the dideoxynucleotide is incorporated, the chain lacks a 3'-hydroxyl group so further elongation of the chain is prevented. The DNA samples were sent at IEDCR (Institute of Epidemiology, Disease Control and Research) for sequencing. The calculation of cycle sequencing was obtained according to the measured template concentration.

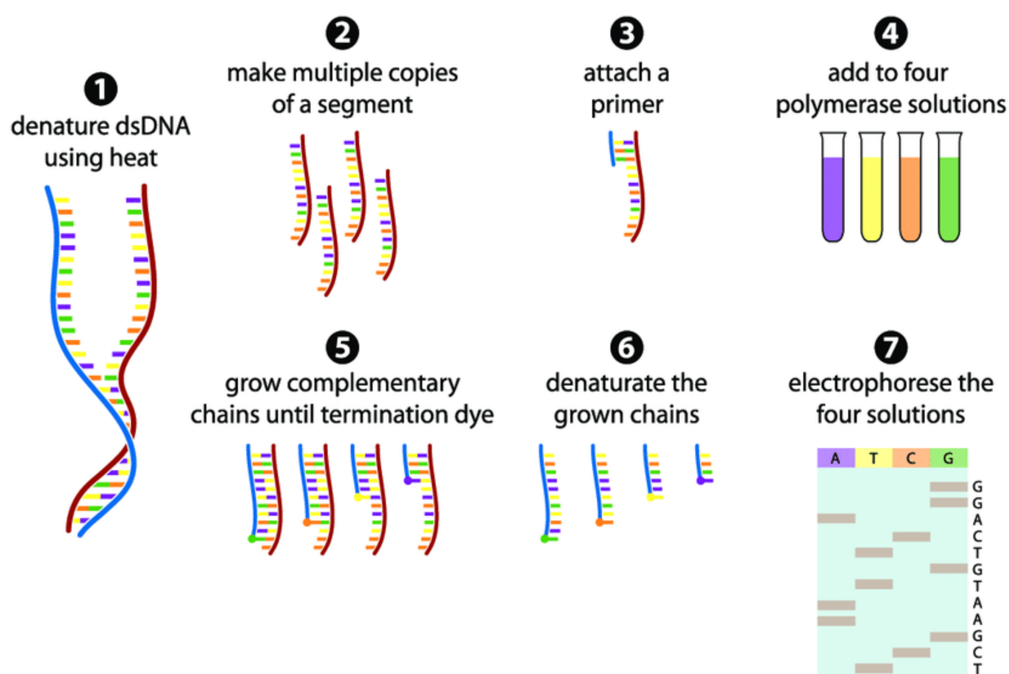

Figure 2.5 Sanger sequencing method (Gauthier, 2008)

Components	Amount
5X PCR sequencing buffer	2.0 µl
Big dye(2.5X)	0.25 µl
Primers	0.3 µl
PCR Product	1 µl
Nuclease Free Water	6.5 µL

Table 2.8: Master cycle components with amount

The tubes containing the template were spun, and 10-20 ng/µL (depending on the concentration) of each of the purified PCR products were added to the 8-tube PCR strip.

Then nuclease free water was added to the mixture to make the total volume 10 µL. The PCR tubes were centrifuged at 4000 rpm for 3 minutes. Then, the PCR strip was placed in the Mastercycler® gradient (Cat. No. 4095-0015, USA Scientific) Thermal Cycler and subjected to following thermal cycling profile: pre-denaturation at 96°C for 3 minutes; 25 cycles of denaturation at 96°C for 10 seconds, annealing at 55°C for 7 seconds and extension at 60°C for 4 minutes.

PCR condition	Temperature	Time	
Initial denaturation	96°C	3 minutes	
Denaturation	96°C	10 seconds	30 Cycles
Annealing	55°C	7 seconds	
Extension	60°C	4 minutes	
Final extension	4°C	∞	

Table 2.9: Thermal cycling profile for cycle sequencing

After completion of cycle sequencing, the reaction plate was centrifuged at 4100 rpm for 2 minutes. Then, 45 µl of SAM solution and 10 µl of X-terminator (Applied Biosystems, USA) were added per 10 µl volume. Both of the solution aid in removal of impurities by desalting as salt interferes with electro-kinetic injection and elimination of remaining labeled ddNTPs, thus, minimizing background noise produced by dye blobs in the sequencing results. Before addition, the X-terminator solution was vortexed properly at maximum speed for at least 30 seconds, until it became homogenous. As it was difficult to pipette the highly dense X-terminator solution out from the bottom of its container, wide bore micropipette tips were used. Later, the reaction plate was sealed and vortexed for half an hour. The mixture was then centrifuged at 4100 g for 2 minutes and the supernatant collected for capillary electrophoresis. 10 µl of supernatant was transferred to a fresh sequencing tube. Before placing the sequencing tubes into the capillary electrophoresis instrument, it was covered with Septa mat. Rest of the supernatant was stored at +4°C for later use.

2.5.1 Sequence analysis

Sequencing data were analysed by Chromas Lite 2.4 software to identify the sequence alignments for showing identity and detecting mutations. The obtained sequence was subjected to further analysis using Basic Local Alignment Search Tool (BLAST) for finding sequence similarity with sequences already reported in online databases.

Basic Local Alignment Search Tool (BLAST): https://blast.ncbi.nlm.nih.gov/Blast.cgi

2.5.2 Basic Local Alignment Search Tool (BLAST)

BLAST is an algorithm that can compare and align a query nucleotide or protein sequence with a number of sequences contained in its database. It finds regions of local similarity between the sequences by calculating the statistical significance of matches. It is both rapid and sensitive and hence is used by millions of biologists. It is available online at the National Centre for Biotechnology Information (NCBI) website (Lobo, 2008).

2.5.2.1 Method

- ⇨ https://blast.ncbi.nlm.nih.gov/Blast.cgi was browsed
- ⇨ Sequences were pasted
- ⇨ Parameters were changed if necessary
- ⇨ **BLAST** was clicked on and few minutes were required to get the result.

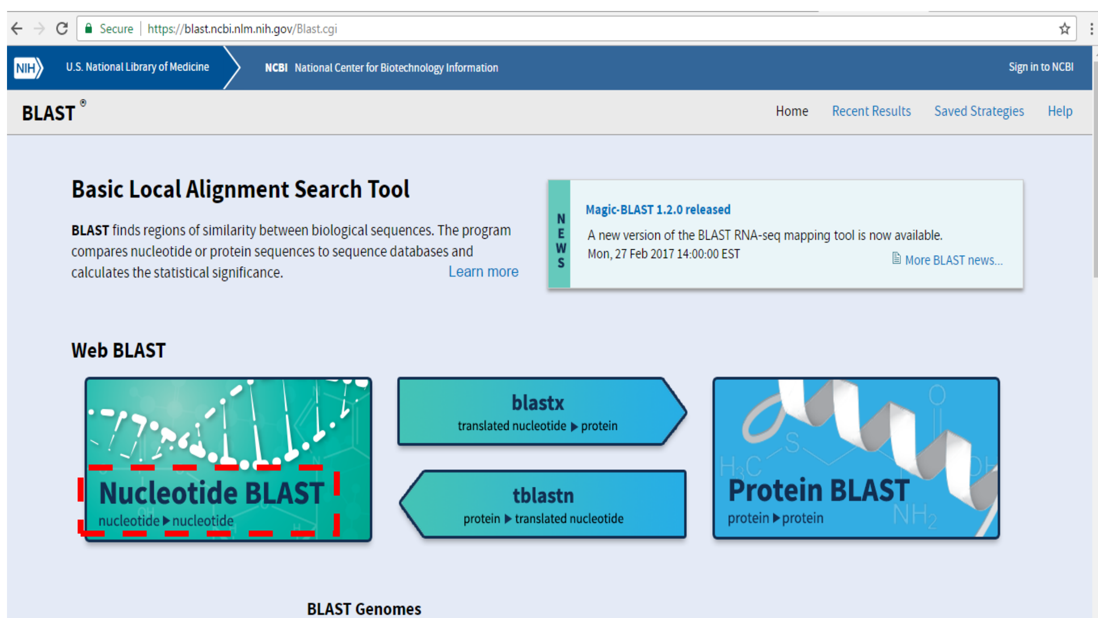

Figure 2.6: BLAST Homepage

Figure 2.7: Entering query sequence into BLASTn

Chapter 3
RESULTS

Result

3.1 Identification and confirmation of bacterial isolates

The isolates were confirmed using different laboratory tests. The test results were observed and recorded.

3.1.1 Colony characteristics on different agar plates

Growth were observed for colonies in MacConkey and blood agar plates.

3.1.1.1 Colony characteristics of Gram negative organisms

As observed on the plates after the 18-24 hour incubation period, *E. coli*, and *K. pneumoniae*. turned the media red indicating an acidic environment below pH 6.8 due to their fermentation of lactose within the media, while *A. baumannii* and *P. aeruginosa* generated a colourless media indicative of producing a basic environment, demonstrative of non-lactose fermenting species.

Bacterial isolates	**Colony characteristics of Gram negative organisms**					
	Size	**Margin**	**Elevation**	**Pigment**	**Form**	**Consistency**
K. pneumoniae	Medium	Entire	Convex	Pink	Circular	Smooth
E. coli	Medium	Entire	Convex	Pink	Circular	Smooth
A. baumannii	Small	Entire	Umbonate	Transparent	Irregular	Smooth
P. aeruginosa	Small	Entire	Umbonate	Transparent	Irregular	Smooth

Table 3.1: Colony characteristics of the isolates on MacConkey agar medium

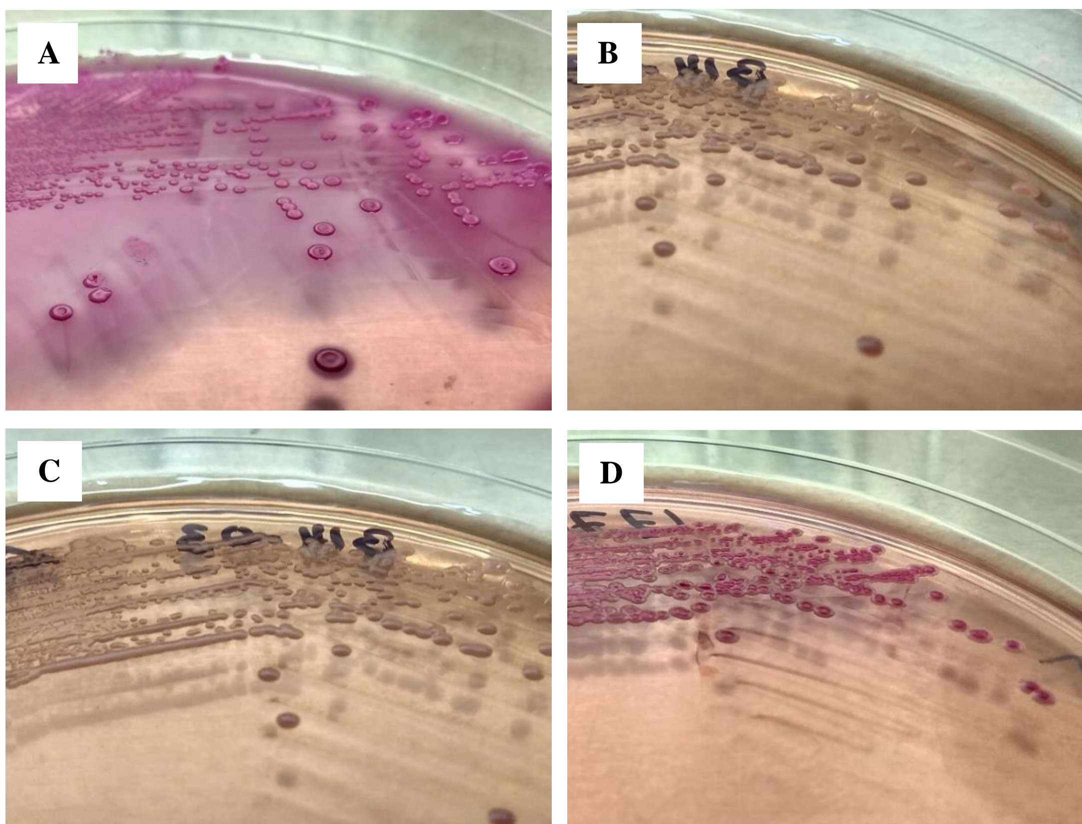

Figure 3.1: Colonies of ***A.*** *E. coli,* ***B.*** *P. aeruginosa,* ***C.*** *A. baumannii and* ***D.*** *K. pneumoniae on MacConkey agar plate.*

3.1.1.2 Colony characteristics of Gram positive organisms

After 24-48 hours incubation, isolates in anaerobic condition on Blood agar plate, the colony appearance and morphology were observed for the confirmation of bacterial strains. Several hemolytic and non-hemolytic organisms were identified allowing the detection of hemolysis (destroying the RBC) by cytolytic toxins secreted by some bacteria, such as certain strains of *Streptococcus, Enterococcus* and *S. aureus.*

Bacterial isolates	Colony characteristics of Gram positive organisms					
	Size	**Margin**	**Elevation**	**Pigment**	**Form**	**Consistency**
S. aureus	Medium	Entire	Convex	Golden yellow or white	Circular	Smooth
***Enterococcus* spp.**	Small	Entire	Raised	Watery	Circular	Smooth
S. pneumoniae	Small	Entire	Raised	Greenish	Circular	Smooth

Table 3.2: Colony characteristics of the isolates on Blood agar medium

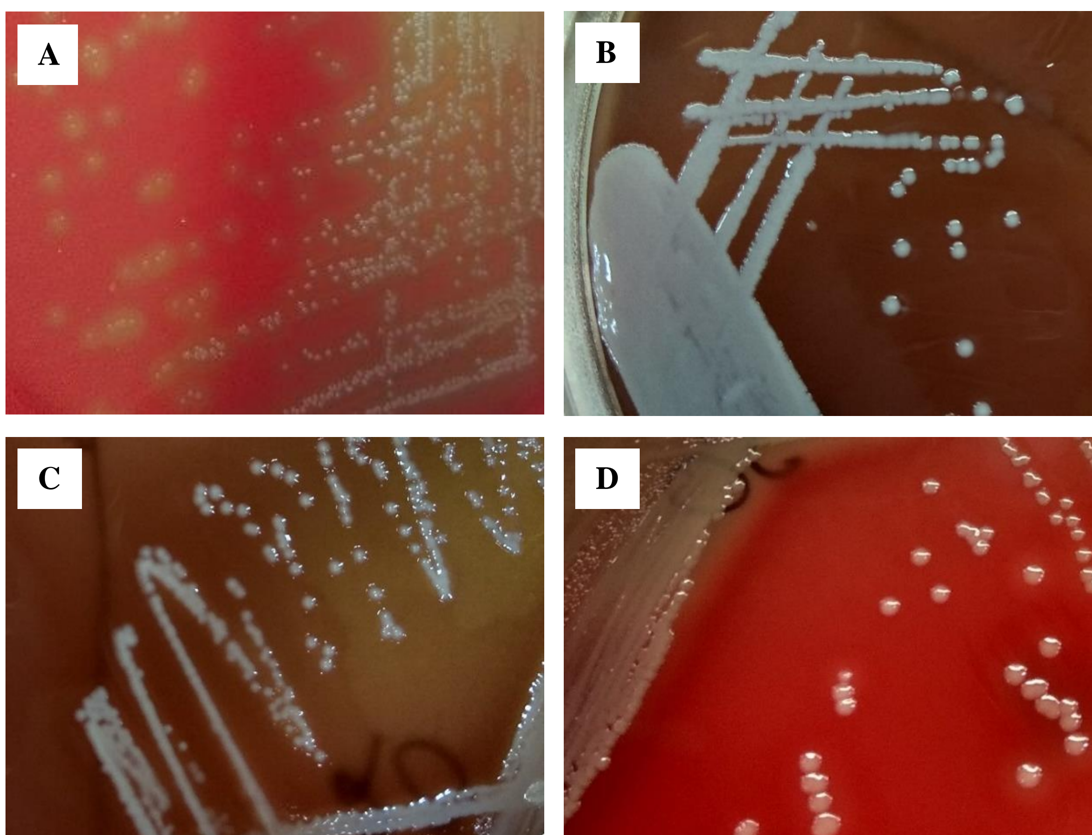

Figure 3.2: Colonies of ***A.*** *S. pneumoniae,* ***B.*** *CNS,* ***C.*** *Enterococcus spp. and* ***D****. S. aureus on blood agar plate.*

3.1.2 Gram Staining results

Gram staining result of the isolates were observed under microscopic oil emersion lens for the confirmation of cellular morphology and arrangement. Gram negative isolates retained the pink colour, denoting that they were gram negative rods; whereas gram positive isolates retained purple colour.

Bacterial isolates	Shape	Stained colour	Category
K. pneumonia	Rod	Pink	Gram Negative
E. coli	Rod	Pink	Gram Negative
P. aeruginosa	Rod	Pink	Gram Negative
A. baumannii	Rod	Pink	Gram Negative
S. aureus	Cocci	Violet	Gram Positive
S. pneumoniae	Cocci	Violet	Gram Positive
Enterococcus spp.	Cocci	Violet	Gram Positive

Table 3.3: Gram staining results of the isolated organisms

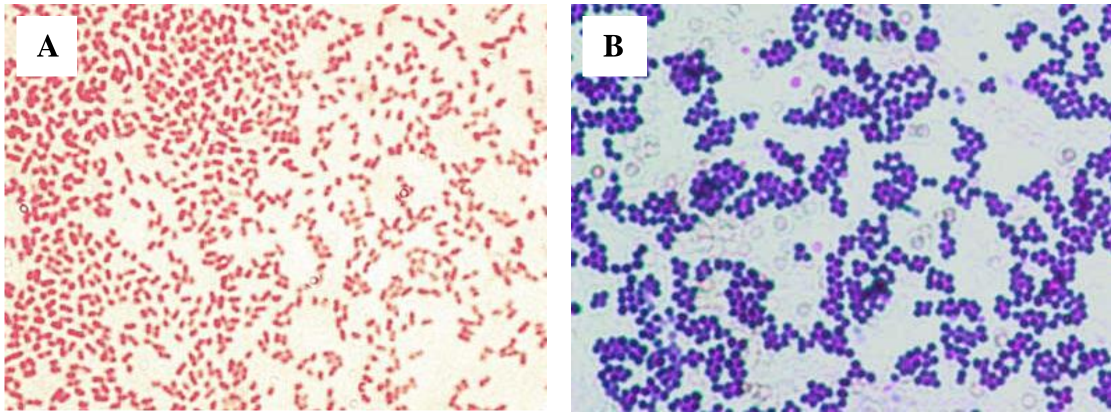

*Figure 3.3: Microscopic view of **A.** Gram negative K. pneumoniae and **B.** Gram positive S. aureus.*

3.1.3 Biochemical test results

3.1.3.1 Triple sugar iron test

Triple sugar iron (TSA) is a differential medium containing lactose, sucrose, a small amount of glucose (dextrose), ferrous sulphate and the pH indicator phenol red. This is used to differentiate enteric bacteria based on the ability to reduce sulphur and ferment carbohydrates. The following result shows that *K. pneumoniae* and *E. coli* were able to ferment lactose and/or sucrose and turned the slant into acidic and also all of them were able to ferment glucose which turned the butt of the TSI tube acidic. These organisms were capable of producing gas too after fermentation of the sugars. On the other hand, the other three Gram negative organisms could not ferment any sugar, so no change was observed for both slant and butt of the test tube.

Bacterial isolates	**Characteristics**			
	Slant	**Butt**	**Gas production**	**H_2S Production**
K. pneumoniae	Acid	Acid	+	-
E. coli	Acid	Acid	+	-
P. aeruginosa	Alkaline	Alkaline	+	-
A. baumannii	Alkaline	Alkaline	-	-

Table 3.4: TSI Test results of the organisms

3.1.3.2 Citrate utilization test

Citrate agar is used to test an organism's ability to utilize citrate as a source of energy. The medium contains citrate as the sole carbon source and inorganic ammonium salt ($NH_4H_2PO_4$) as the sole source of nitrogen. The following result shows that only *E. coli* could not utilise citrate, and the rest of the organisms utilized citrate and as a result; the colour of the agar turned green to blue.

Organisms	**Reaction**
K. pneumoniae	+
E. coli	-
P. aeruginosa	+
A. baumannii	+

Table 3.5: Citrate utilization test results of the organisms

3.1.3.3 Motility indole urease test

MIU Medium Base is formulated to detect motility, urease and indole production in single tube. Motile organisms show either diffused growth or turbidity extending away from stab inoculation line while non motile organisms grow along the stab line. Organisms that utilize urea, produce ammonia which makes the medium alkaline, showing pink-red colour by change in the phenol red indicator. Indole is produced from tryptophan present in casein enzymic hydrolysate. The indole produced combines with the aldehyde present in the Kovac's reagent to form a red complex. On basis of the characteristics of motility, indole and urease, the four Gram negative organisms were characterized which is shown in the following table:

Organisms	**Characteristics**		
	Motility	**Indole**	**Urease**
K. pneumoniae	-	-	+
E. coli	+	+	-
P. aeruginosa	+	-	-
A. baumannii	-	-	-

Table 3.6: MIU test results of the organisms

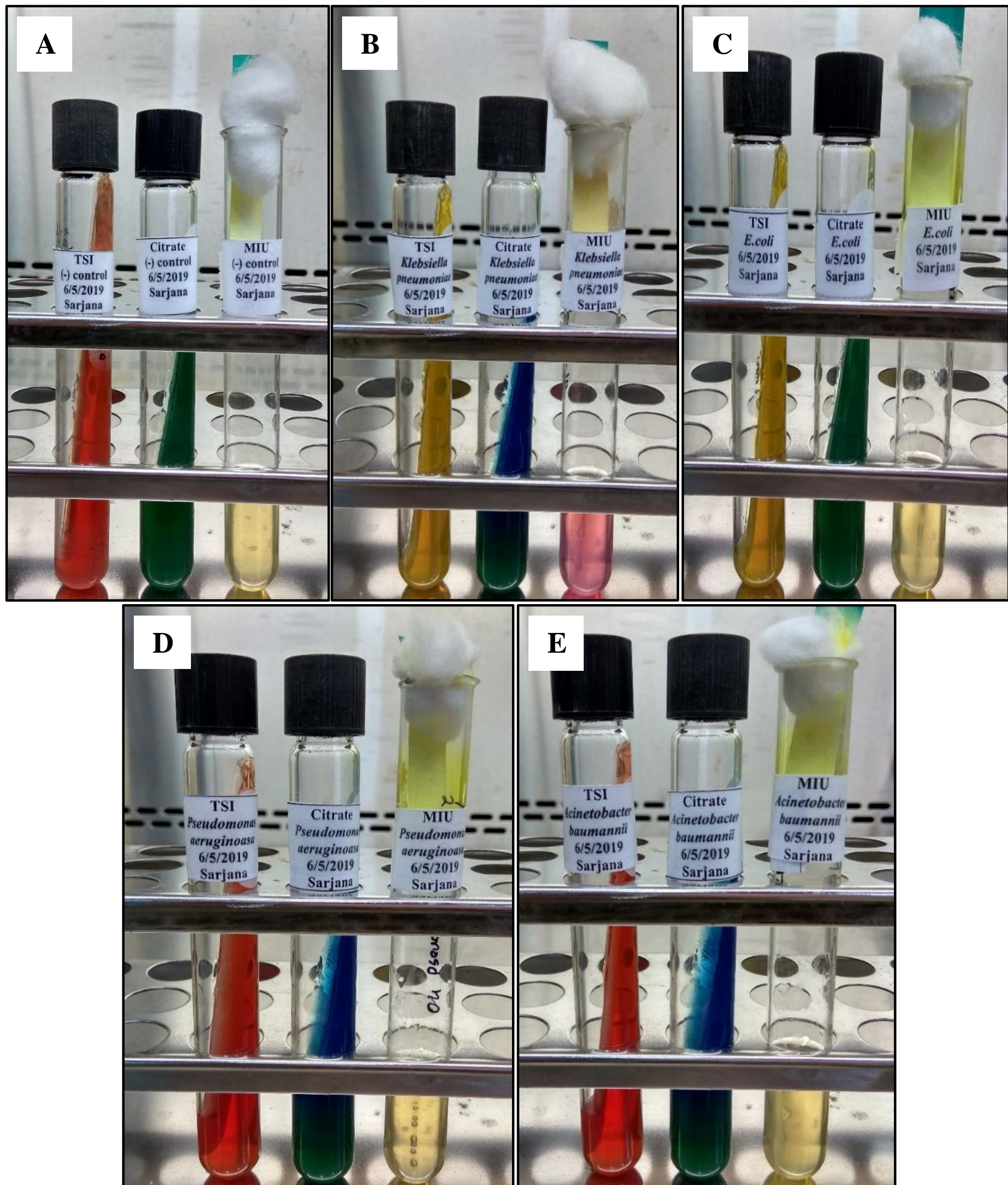

Figure 3.4: Biochemical test results of ***A.*** *Negative control,* ***B.*** *K. pneumoniae,* ***C.*** *E. coli,* ***D.*** *P. aeruginosa and* ***E.*** *A. baumannii in TSI, citrate and MIU tubes.*

3.1.3.4 Catalase test result

The positive catalase test results suggested the strains were likely aerobic bacteria, which possess the catalase enzyme as observed in both these species through their rapid effervescence of CO_2 bubbles. Catalase test results of the Gram negative isolates showed positive results which confirmed that these isolates were gram negative microorganisms and belonged to *Enterobacteriaceae* family. In case of gram positive organisms, *Enterococcus* spp. and *S. pneumoniae* gave negative results.

Organisms	Catalase test results
E. coli	(+)
K. pneumoniae	(+)
P. aeruginosa	(+)
A. baumannii	(+)
Enterococcus spp.	(-)
S. aureus	(+)
S. pneumoniae	(-)

Table 3.7: Catalase test results of the organisms

Figure 3.5: Results of catalase positive test of S. aureus (left) and catalase negative test of S. pneumoniae (right)

3.1.3.5 Oxidase test results

Oxidase test is done only for gram negative organisms. The conversion of the light blue filter paper to deep blue/purple by both species once more, demonstrated their capacity for utilizing oxygen as the terminal electron acceptor in the bacterial electron transport chain. Only *P. aeruginosa* showed positive reaction which indicates that it is oxidase positive.

Organisms	Oxidase test results
E. coli	(-)
K. pneumoniae	(-)
P. aeruginosa	(+)
A. baumannii	(-)

Table 3.8: Oxidase test results of the organisms

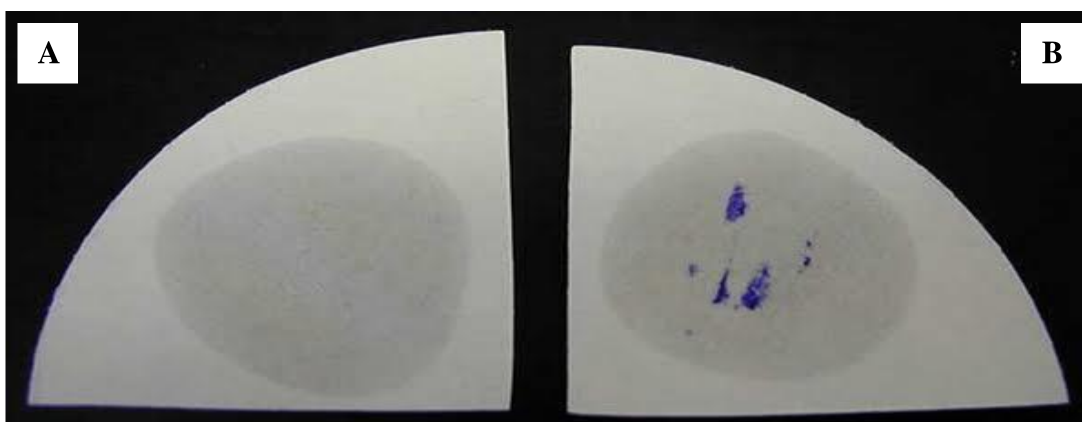

*Figure 3.6: **A.** E. coli, K. pneumoniae and A. baumannii are showing negative oxidase test. **B.** P. aeruginosa strain showing positive to oxidase and thus inoculum turns colourless to blue.*

3.1.3.6 Coagulase test result

Macroscopic clumping in 10 seconds or less in coagulated plasma drop and no clumping in saline or water drop indicates positive result and also indicates that the organism auto agglutinates**.**

Organisms	Coagulase test results
CNS	(-)
S. aureus	(+)

Table 3.9: Coagulase test results of the organisms

3.2 Rate of isolation

A total of 348 microorganisms were isolated from 217 wound swabs. Amid those microbes, there were 9 distinct species of bacteria identified. The percentage of isolated species are represented in Figure 3.7. Among the enlisted 12 global priority pathogens (GPP) according to World Health Organization (WHO), we found 9 bacterial species from GPP list including *A. baumannii* (n=20), *P. aeruginosa* (n=26), *K. pneumoniae* (n=42) and *E. coli* (n=85) as the Priority 1 (Critical) pathogens, followed by MRSA (n=62), *S. aureus* (n=22), and *Enterococcus* spp. (n=27) as the Priority 2 (High) pathogens. Among the Priority 3 (Medium) pathogens list, *S. pneumoniae* (n=36) strains were analysed. Additionally, CNS (n=28) which is not enlisted in any of the priority list, was also found and investigated. The data shows that the most commonly isolated species in wound samples is *E. coli* (24.43%) followed by MRSA- 17.82%, *K. pneumoniae*.- 12.07% and *S. pneumoniae*- 10.33%.

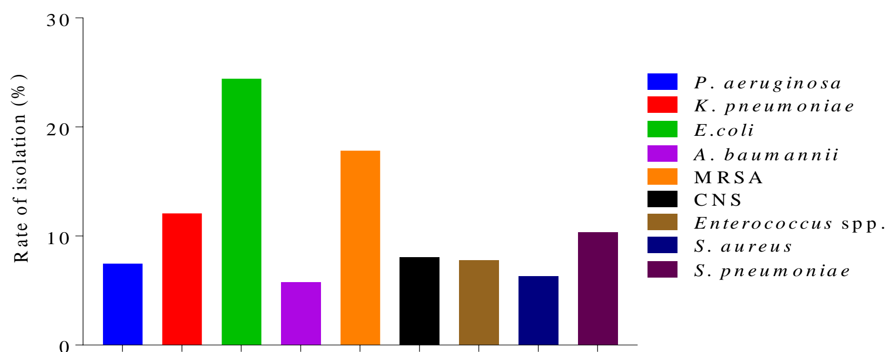

Figure 3.7: Percentage of different species of microorganism isolated from wound swab samples.

3.3 Rate of polymicrobial infection

Polymicrobial infection involve multiple pathogens causing a severe infection. Most of the cases, combination of two pathogens were found and very rarely three or more pathogens. In this study, polymicrobial infection was found in 55 (25·3%) of the infected wounds and was mainly constituted by two species; three species were the maximum number of species isolated per sample and represented only 3·4% of the total polymicrobial infections. Figure 3.8 shows the most common bacterial combinations in wound polymicrobial infection. The predominant combination of species found in polymicrobial infections was MRSA and *K. pneumoniae* (13%), MRSA and *Enterococcus* spp. (11%), CNS and *P. aeruginosa* (7%) and MRSA and *E. coli* (5.45%). The most common association was MRSA and *K. pneumoniae* (13%).

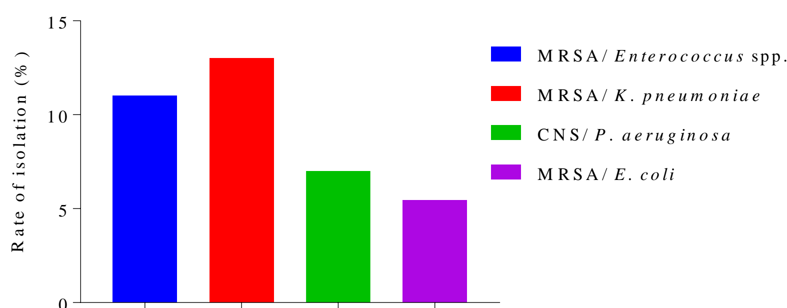

Figure 3.8: Percentage of the most common bacterial associations found in the infected wounds analysed

3.4 Antibiotic susceptibility test results

Antibiotic Sensitivity Test gives us an idea about the resistivity or sensitivity pattern of an organism. This allows us to choose an effective antibiotic against that organism

during an infection. To find out the resistivity pattern, AST was done. Due to the large number of strains and antibiotics tested, these results are summarized in terms of percentage of resistant populations against individual drugs, instead of displaying the raw data in the form of tables, they have been illustrated graphically.

3.4.1 Antibiotic resistance pattern for Gram negative organisms

The bar charts in the figure 3.9 (A-D) represents antibiotic resistance percentage of each Gram negative species isolated from wound swabs. The data gives us the idea that *P. aeruginosa*, *K. pneumoniae* and *E. coli* have higher antibiotic resistance percentage. The situation is worst for *P. aeruginosa* and *K. pneumoniae* isolates which exhibited an elevated resistance to all antibiotics tested. Overall, *P. aeruginosa* showed the highest resistance to majority of the antibiotics investigated. This species showed higher resistance not only to first line drugs, second line drugs were also found to be ineffective (Carbanicilin-48%, Netilmycin- 45%, Trimethoprim+ Sulphamethoxazole- 41%). A dreadful resistance pattern was also seen for *K. pneumoniae* isolates for second line antibiotics including Carbanicilin-23%, Amikacin- 7%, Trimethoprim+ Sulphamethoxazole-17%. In addition, *E. coli* isolates were shown to be highly resistant to β-lactams (Ampicillin-89%, Imipenem-56%). These organisms also showed an accelerated patterns for Erythromycin, Azithromycin and Nalidixic acid, showing resistance frequencies of 89%, 58% and 59%, respectively. Whereas, all of the *A. baumannii* strains were found to be completely sensitive to many of the first line drugs including imipenem, tetracycline, tobramycine, norfloxacin and azithromycin. Colistin was found to be the most effective drug against Gram negatives.

3.4.2 Antibiotic resistance pattern for isolated Gram positive bacteria

The antibiotic resistance pattern of Gram positive bacteria are determined on MHA. Blood agar plate is only used for *S. pneumoniae*. Amongst the Gram positive pathogenic organisms, the antibiogram data manifests an alarming resistance level for MRSA which were 100% resistant against cefoxitin, 85.5% against erythromycin, and 81% resistant against penicillin. None of the tested antibiotics were found to be effective against MRSA strains. Surprisingly, CNS exhibited higher percentage of resistance to most of the drugs tested. In addition, low percentage of resistance to vancomycin were observed for most of the Gram positives whereas, 19% *Enterococcus* spp were vancomycin resistant.

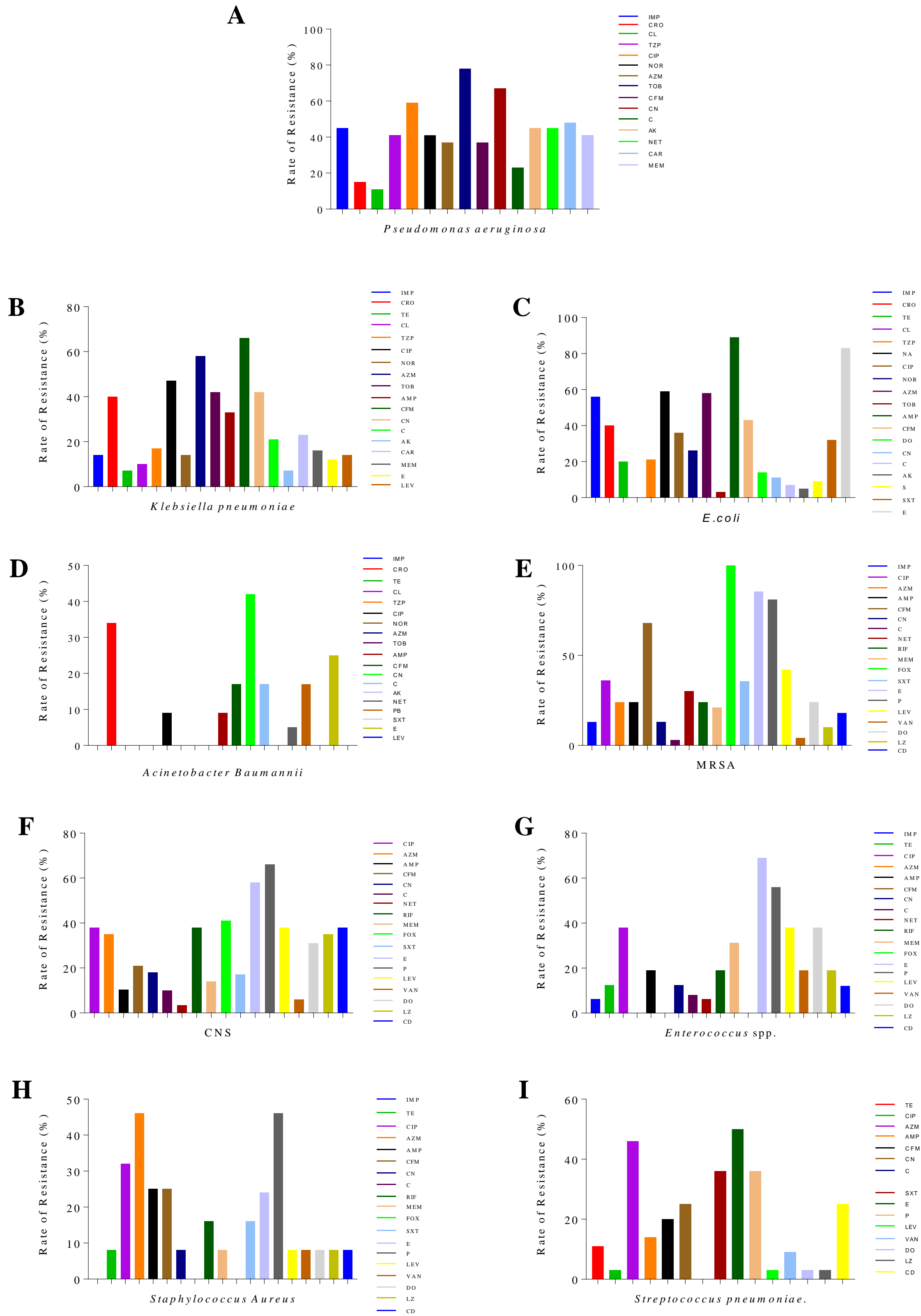

Figure 3.9: Disk diffusion base rates of resistance of isolated organisms to different antibiotics, A. P. aeruginosa, B. K. pneumoniae, C. E. coli, D. A. baumannii, E. MRSA, F. CNS, G. Enterococcus spp., H. S. aureus, I. S. pneumoniae

3.5 Spectrum of Multi-Drug Resistance

If an organism is resistant to three or more antibiotics that microorganism is referred as multi drug resistant (MDR) organism. The figure below shows the percentage of MDR organism isolated from each species. *E. coli* and MRSA are the topper of the list having a percentage of 96.6 and 90 respectively. *S. pneumoniae* scores the least percentage amongst all other organisms which is 55.62%.

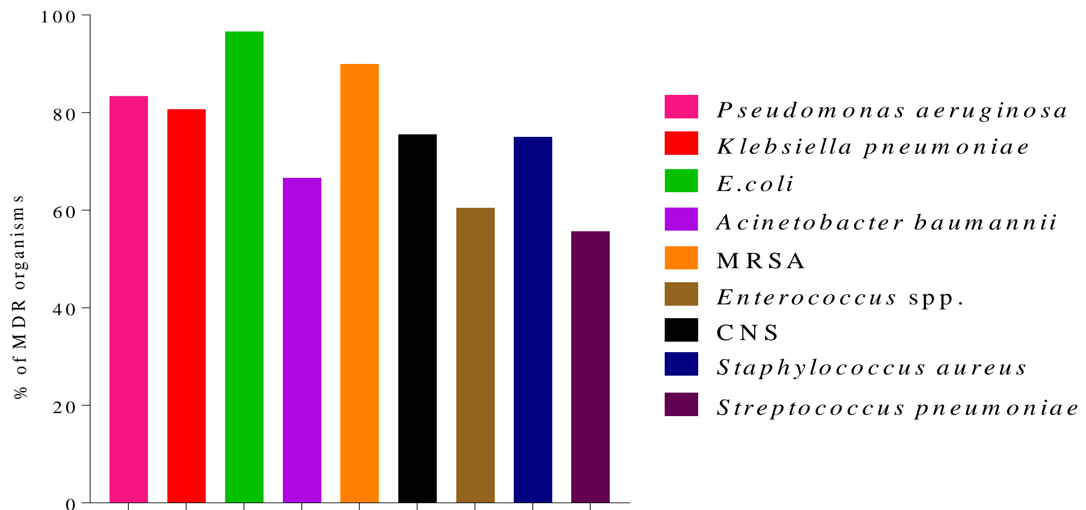

Figure 3.10: Resistance frequency of the indicated clinical isolates

3.6 Antibiotic resistance profile and MAR index

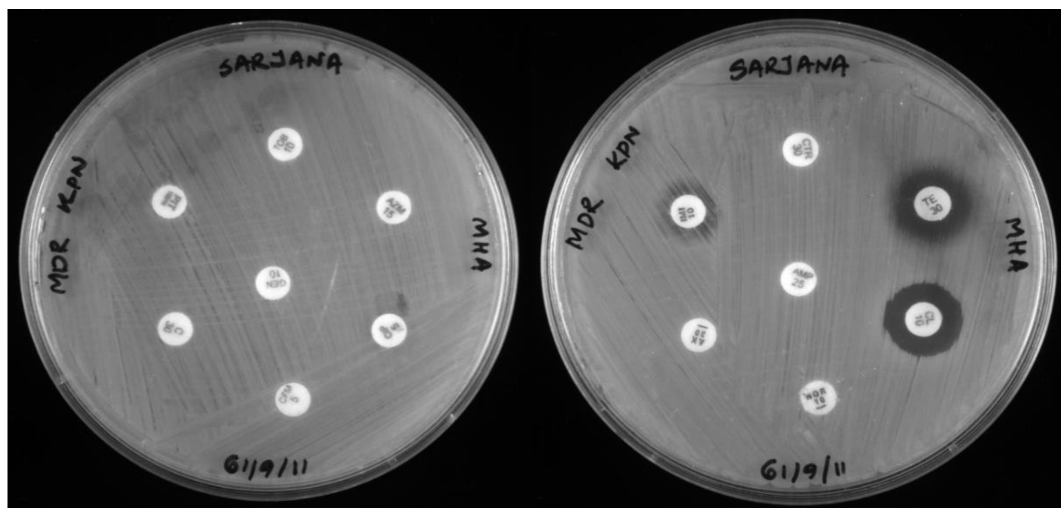

Figure 3.11: Determining the resistance pattern of one of the MDR K. pneumoniae on MHA

MAR index is calculated as the ratio of number of antibiotics to which organism is resistant to total number of antibiotics to which the organism is exposed (Krumperman, 1983).

Formula for calculation of MAR index is,

MAR index= **a/b**,

Where,

a= the number of antibiotics to which the isolate was resistant

b= the number of antibiotics to which the isolate was exposed.

MAR Index (Range)	***P. aeruginosa* *n* *(%)***	***K. pneumoniae* *n* *(%)***	***E. coli* *n* *(%)***	***A. baumannii* *n* *(%)***	***MRSA* *n* *(%)***	***CNS* *n* *(%)***	***E. spp* *n* *(%)***	***S. aureus* *n* *(%)***	***S. pneumoniae* *n* *(%)***	**Total *n* *(%)***
<0.2	5 (19.23)	10 (23.8)	56 (65.89)	9 (45)	0	9 (32.14)	13 (48.15)	14 (63.62)	17 (47.22)	133 (38.22)
0.2	0	0	0	0	3 (4.84)	3 (10.75)	0	0	7 (19.44)	13 (3.73)
>0.2 Mean±SD	21 (80.77) 0.562± 0.18	32 (76.2) 0.38± 0.15	29 (34.11) 0.32± 0.133	11 (55) 0.312± 0.089	58 (93.59) 0.4± 0.11	16 (57.11) 0.45± 0.11	14 (51.85) 0.38± 0.14	8 (36.38) 0.36± 0.11	12 (33.34) 0.34± 0.059	201 (58.05)
Total	26	42	85	20	62	28	27	22	36	348

Table 3.10: Distribution of MAR index values within identified organisms

Table 3.10 shows the antibiotic resistance profile and Multiple Antibiotic Resistance (MAR) index of several isolated organisms. The proportion of isolates with Multiple Antibiotic Resistance (MAR) index with 0.2 and greater than 0.2 was 3.73% and 58.05%, respectively; while those with MAR index less than 0.2 was 38.22% showing that a greater proportion of the isolates are likely to be from a high risk source. A large proportion of MRSA (93.59%), *P. aeruginosa* (80.77%) and *K. pneumoniae* (76.2%) isolates exhibited MAR index value of greater than 0.2, whereas the highest mean value (0.562) of MAR index was observed for *P. aeruginosa*. Besides, *A. baumannii, E. coli* and *S. pneumoniae* isolates were seen to have lower mean MAR index of 0.312, 0.32, 0.34, respectively.

3.7 Genotypic detection

In the phenotypic detection experiments, many organisms were found to be resistant to multiple antibiotics. Therefore, we were interested to know whether those organisms harbour any antibiotic resistance genes or not. To address this issue, we focused on major three antibiotic resistance classes (ESBL, macrolide and aminoglycoside resistance genes). In order to detect whether the PCR was successful, the amplified DNA was run in 1% agarose gel. To detect the size of the DNA band, the GeneRuler 1Kb Plus DNA Ladder by Invitrogen was used. DNA ladder is a solution of DNA

molecules of different known lengths. When run alongside an unknown PCR product in an agarose gel, the ladder allows estimating the size of the unknown fragment by comparing it to the closest band in the ladder lane. Amplified products were analysed with gel electrophoresis and interpreted visually.

3.7.1 ESBL gene detection

Of the total of five ESBL genes examined, three genes (CTXM-1, NDM-1 and OXA-48) were detected in this study. CTXM-1 was the most frequently detected antibiotic resistance gene in this entire study.

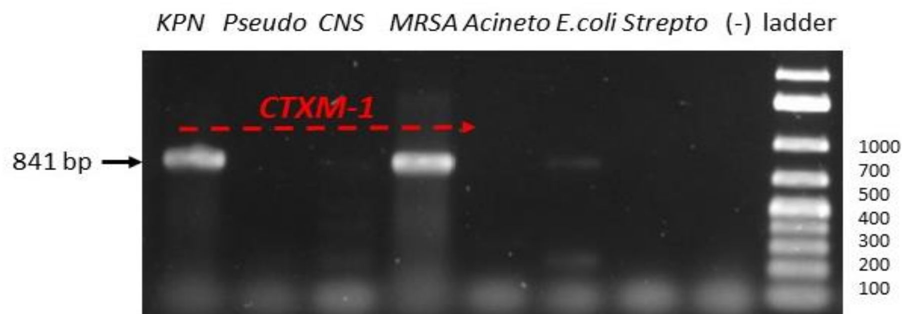

Figure 3.12: PCR amplification products of CTXM-1 gene homologues resolved on Gel red stained 1% agarose gel. The desired band size for the gene specific primer is 841 bp.

3.7.2 Macrolide resistance gene detection

Among the four macrolide resistance gene specific primers used, three (ErmB, ErmC and ErmF) were detected. ErmB was the most prevalent gene detected among the macrolide resistance genes investigated in this study.

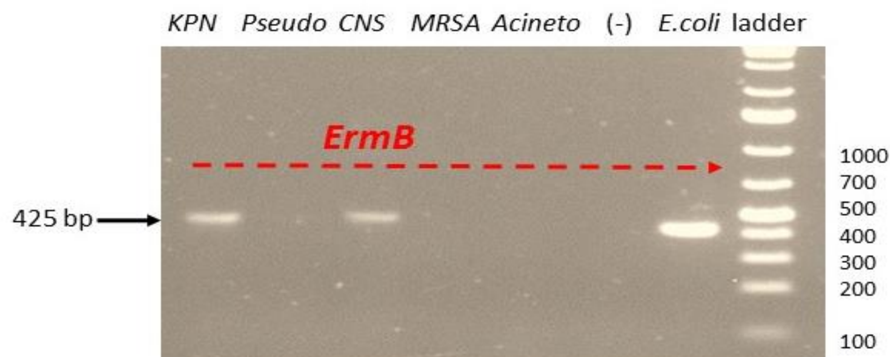

Figure 3.13: PCR amplification products of ErmB gene homologues resolved on Gel red stained 1% agarose gel. The desired band size for the gene specific primer is 425 bp.

3.7.3 Aminoglycoside resistance gene detection

Five aminoglycoside resistance genes were detected among the seven primer sets. Aac(3)-II was the predominant gene detected among aminoglycoside resistance genes.

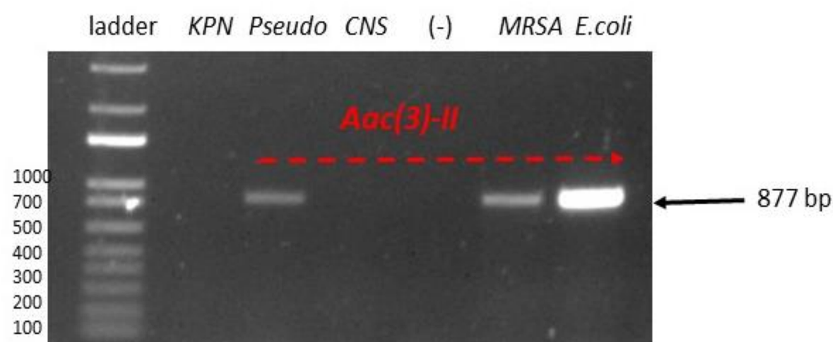

Figure 3.14: PCR amplification products of aac(3)-II gene homologues resolved on Gel red stained 1% agarose gel. The desired band size for the gene specific primer is 877 bp.

3.8 Sequencing data

The desired DNA bands for specific antibiotic resistance genes were subjected to sequencing. The sequencing data were analysed by Chromas Lite 2.1 software. Chromas Lite is a handy and reliable application designed to read chromatogram files generated by sequencers. The software is able to process and export sequences without altering the original form of the data. This tool generates a four colour chromatogram showing the result of sequencing run. Different bases are represented in different colours that are defined below:

1. Adenosine= Green

2. Guanine= Black

3. Cytosine= Blue

4. Thymine= Red

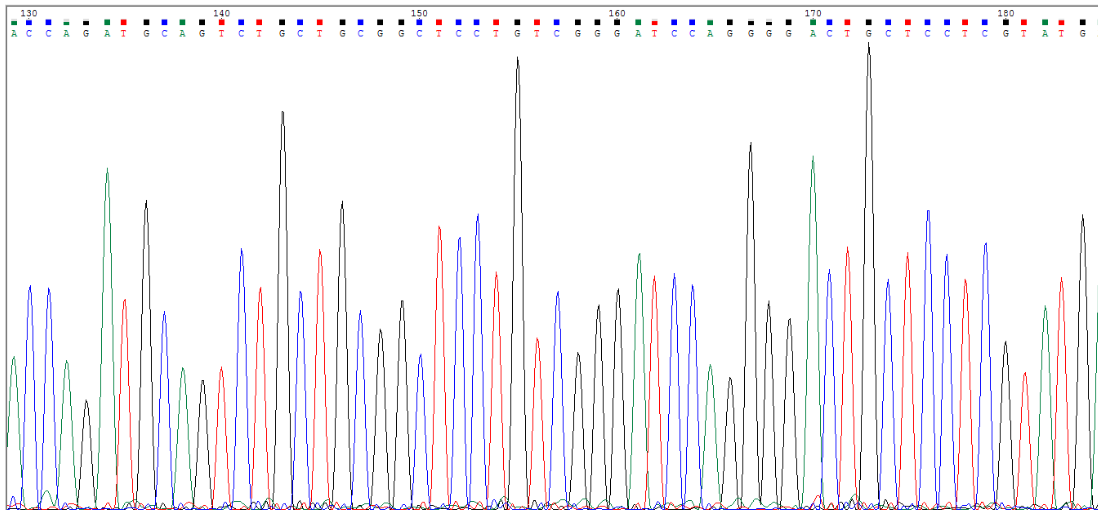

Figure 3.15: Diagrammatic representation of a part of the chromatogram of sequence.

3.8.1 Analysis of the obtained sequence

A graphic summary of the BLAST results for the target sequence was obtained where the top red bar indicated the query sequence. Database hits are shown aligned to the query, below the red bar. Of the aligned sequences, the most similar are shown closest to the query. In this case, all the hits are high scoring database matches that align to most of the query sequence. Alignment score is the summation of each specified aligned pair of bases or residues and their nulls in the alignment and the higher the alignment score, the better the alignment is. Here high alignment score indicates a significant alignments.

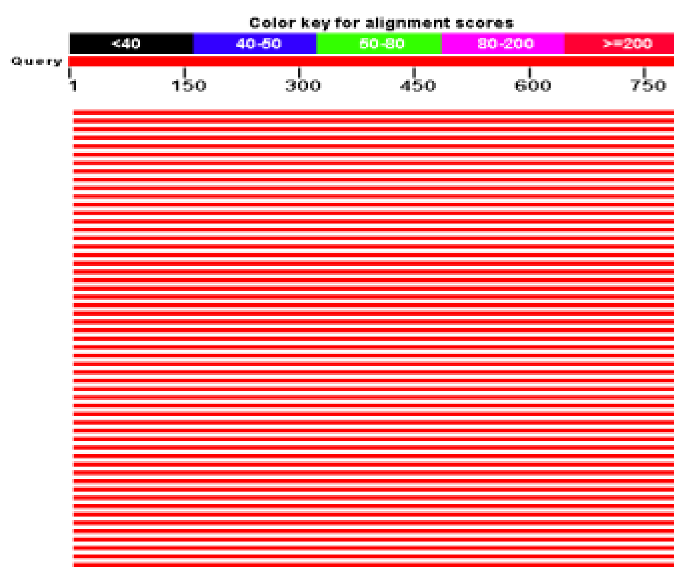

Figure 3.16: Graphic Summary of BLASTn result using the sequenced PCR product

The Descriptions table provides a summary of the database sequences identified by BLAST to be similar to the input query. Two selection controls at the top of the table, “All” and “None”, allow for the quick selection and de-selection of matched database sequences. From left to right, the descriptions table columns provide information where Max Score is based on the overall score of HSPs (high scoring pairs) between sequences, similar to Expect Value. The higher the Max Score, the better the alignment between the hit and the query. Total Score is obtained by the sum of scores from all HSPs from the same database sequence. Query Coverage is the amount of the query sequence, expressed as a percent that overlaps the subject sequence. E (expected) Value describes the chance of randomly achieving the same alignment in a database of a particular size. An E Value is used to describe the significance (instead of a P value) of each sequence alignment hit to the query and Max Ident (Maximum Identity) is the highest percent identity for a set of aligned segments to the same subject sequence.

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Klebsiella pneumoniae isolate 23 plasmid pCTXM15_DHQP1400954, complete sequence	1424	1424	98%	0.0	99%	CP016925.1
Escherichia coli blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-15, complete CD	1424	1424	98%	0.0	99%	NG_048935.1
Escherichia coli 1125509 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-163, co	1424	1424	98%	0.0	99%	NG_048948.1
Klebsiella pneumoniae KK08 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-156	1424	1424	98%	0.0	99%	NG_048940.1

Figure 3.17: Top BLAST hits that produced significant alignments with query sequence

After BLASTn analysis, it was found that the resistant bacterial sequence matched with CTXM-1 gene.

Klebsiella pneumoniae KK08 blaCTX-M gene for class A extended-spectrum beta-lactamase CTX-M-156, complete CDS
Sequence ID: ref|NG_048940.1| Length: 876 Number of Matches: 1

Range 1: 42 to 832 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1424 bits(771)	0.0	785/791(99%)	5/791(0%)	Plus/Plus

```
Query  9    GGC-ACCGTCACGCTGTTGTTAGG-AGTGTGCCGCTGTATGCGCAAACGGCGGACGTACA  66
Sbjct  42   GGCAACCGTCACGCTGTTGTTAGGAAGTGTGCCGCTGTATGCGCAAACGGCGGACGTACA  101
Query  67   GCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAA  126
Sbjct  102  GCAAAAACTTGCCGAATTAGAGCGGCAGTCGGGAGGCAGACTGGGTGTGGCATTGATTAA  161
Query  127  CACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCAC  186
Sbjct  162  CACAGCAGATAATTCGCAAATACTTTATCGTGCTGATGAGCGCTTTGCGATGTGCAGCAC  221
Query  187  CAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTT  246
Sbjct  222  CAGTAAAGTGATGGCCGCGGCCGCGGTGCTGAAGAAAAGTGAAAGCGAACCGAATCTGTT  281
Query  247  AAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAA  306
Sbjct  282  AAATCAGCGAGTTGAGATCAAAAAATCTGACCTTGTTAACTATAATCCGATTGCGGAAAA  341
Query  307  GCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGA  366
Sbjct  342  GCACGTCAATGGGACGATGTCACTGGCTGAGCTTAGCGCGGCCGCGCTACAGTACAGCGA  401
Query  367  TAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTT  426
Sbjct  402  TAACGTGGCGATGAATAAGCTGATTGCTCACGTTGGCGGCCCGGCTAGCGTCACCGCGTT  461
Query  427  CGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACAC  486
Sbjct  462  CGCCCGACAGCTGGGAGACGAAACGTTCCGTCTCGACCGTACCGAGCCGACGTTAAACAC  521
Query  487  CGCCATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCG  546
Sbjct  522  CGCCATTCCGGGCGATCCGCGTGATACCACTTCACCTCGGGCAATGGCGCAAACTCTGCG  581
Query  547  GAATCTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACATGGAT  606
Sbjct  582  GAATCTGACGCTGGGTAAAGCATTGGGCGACAGCCAACGGGCGCAGCTGGTGACATGGAT  641
```

Figure 3.18: One of the matched sequence with CTXM-1 gene

The quality of the alignment is represented by the Score (S).The score of an alignment is calculated as the sum of substitution and gap scores. Substitution scores are given by a look-up table (PAM, BLOSUM) whereas gap scores are assigned empirically. The significance of each alignment is computed as an E-value (Expectation value). The number of different alignments with scores equivalent to or better than S that are expected to occur in a database search by chance. The lower the E-value, the more significant the score. Hence, a sequence with low E-value is considered a good match as it is unlikely to occur by chance.

After doing the nucleotide analysis, maximum identity and low E-values were found. These maximum identity and lower E-value suggested that sequences were homologous and the amplified sequence belonged to the CTXM-1 subclass of ESBL genes.

3.9 Resistance gene profiles among different isolates

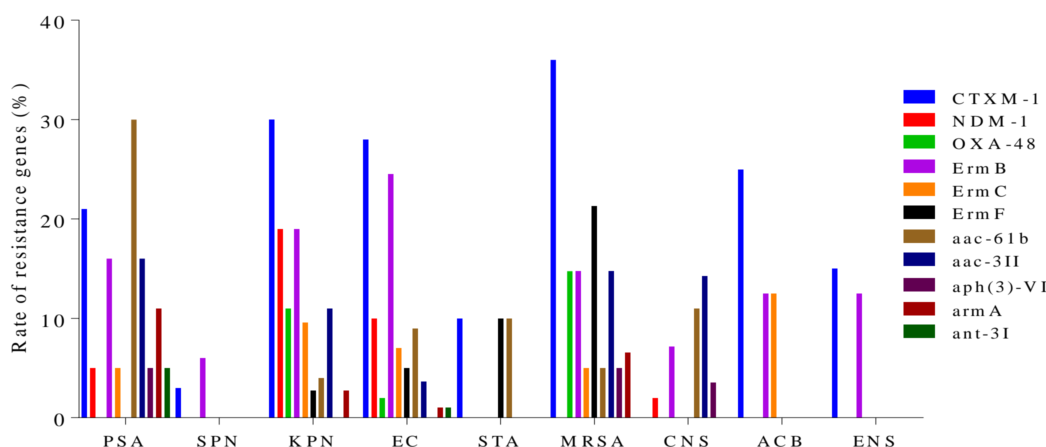

Figure 3.19: Distribution of CTXM-1, NDM-1, OXA-48, ErmB, ErmF, ErmC, aac(6)-Ib, aac(3)-II, aph(3)-VI, armA and ant-3I genes among isolates. In the figure, PSA= P. aeruginosa, SPN= S. pneumoniae, KPN= K. pneumoniae, EC= E. coli, STA=S. aureus, ACB= A. baumannii and ENS= Enterococcus spp.

To observe the resistance gene spectrum among different isolates, the percentage of antibiotic resistance genes were assessed in respect to different organisms. Figure 3.19 shows that *E. coli* strains had been found as the predominant bacterial agent carrying all of the resistance genes except aph(3)-VI. MRSA, *K. pneumoniae* and *P. aeruginosa* were detected as the next prevalent organisms which exhibited a higher percentage of resistance genes, in particular CTXM-1, ErmB, aac(6)-Ib and aac(3)-II. The most frequently identified ESBL gene was CTXM-1 (Amber class-A type), which was present in almost every isolates with the highest frequencies found in MRSA (36%), *K. pneumoniae* (30%) and *E. coli* (28%), although none of the CNS strains showed to harbor this prevalent gene. NDM-1, which is a metallo-β-lactamase of Amber class-B was also shown to be commonly detected among ESBL genes. The main reservoir of this gene was found to be *K. pneumoniae* (19%). Among the Amber class-D type ESBL genes, OXA-48 was predominantly present in 14.75% MRSA strains. The commonly detected macrolide resistance gene, ErmB was found to be prevalent in *E. coli* (24.50%), *K. pneumoniae* (19%) and *P. aeruginosa* (16%). In addition, 30% *P. aeruginosa*, 11% CNS and 10% *S. aureus* were found to prevalently possess aac(3)-II, the frequently detected aminoglycoside resistance gene. *S. pneumonia* and *Enterococcus* spp. were found to harbor only 2 of these resistance genes, with ErmB being the commonly harbored resistance gene in both of these least resistance gene harboring isolates.

3.10 Phenotypic and genotypic discrepancies

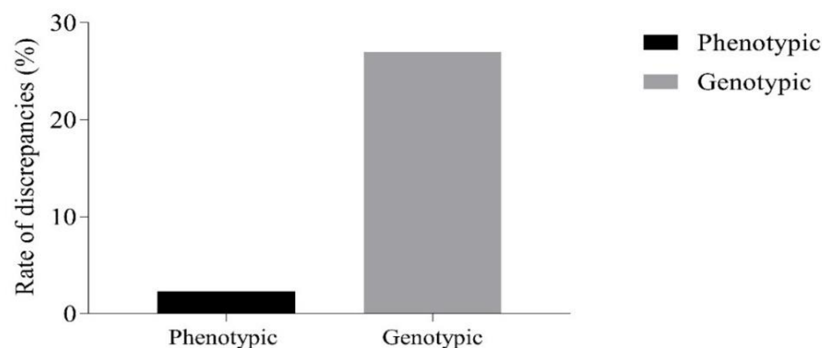

Figure 3.20: Phenotypic and genotypic inconsistencies among isolated organisms

The antibiotic-resistant determinants associated with resistance were detected by PCR. Of the 430 isolates, 115 (27%) isolates did not harbor any of the genes tested even though they were phenotypically resistant. The reason behind this might be the genes responsible for resistance wasn't investigated in this study. Also the isolates were sensitive to higher concentration of drugs. Minimum inhibitory concentration (MIC) may give a better understanding in this case. On the contrary, 10 (2.33%) of the total isolates were observed to be phenotypically inconsistent although resistance genes for these organisms were detected. Probably the detected genes are functionally inactive and further whole genome sequencing might be useful.

3.11 Kinetics of antibiotic resistance over three years

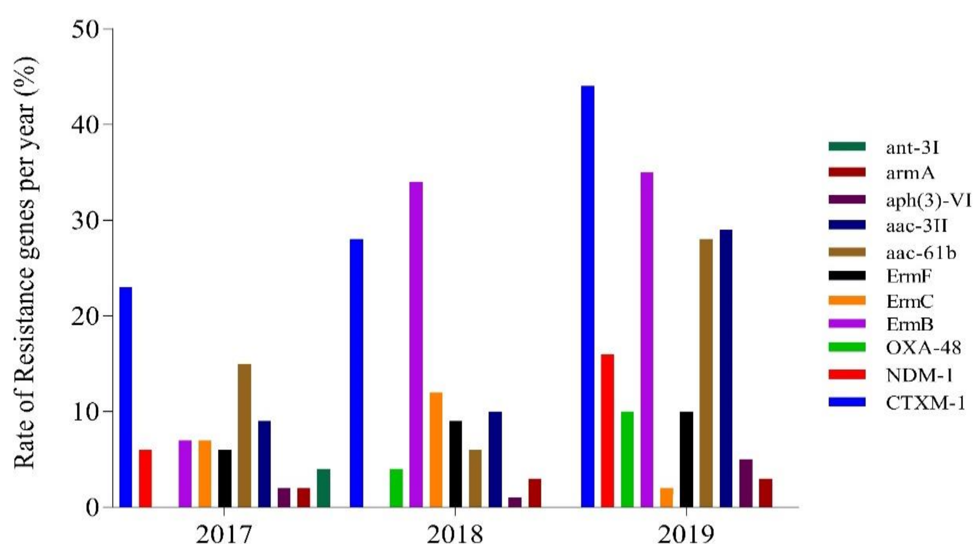

Figure 3.21: Kinetics of the percentage of detected antibiotic resistance genes between 2017 and 2019

To observe the antibiotic resistance trend of the pathogenic organisms enlisted in the priority list declared by WHO, the percentage of MDR organisms and also the detected resistance genes from 2017 to 2019 were calculated and analyzed. Figure 3.21 shows that the relative proportion of MDR strains harboring antibiotic resistance genes is broadly increasing over time. CTXM-1, ErmB, aac(3)-II and aac(6)-Ib have been the prevailing detected resistance gene throughout these years. Among the ESBL genes, percentage of CTXM-1 and NDM-1 has notoriously increased from 2017 to 2019. CTXM-1, which is apparently the predominant resistance gene detected over the past three years, the percentage of this gene has increased by 21% from 2017 to 2019. OXA-48 remained undetected in 2017 and appeared for the first time in 2018, however the detection rate of this gene has doubled in the subsequent year. However, no isolates carrying VIM and KPC genes were identified in these years. With regard to macrolide resistance genes, percentage of ErmB and ErmF has substantially risen. On the other contrary, rate of ErmC has significantly dropped by 10% in 2019. On the other hand, we could not detect the other type of macrolide resistance gene (ErmA) over the three year period. A trend toward decreasing aminoglycoside resistance was seen for ant(3)-I gene, which was only determined in 2017, aph(3)-VI and armA remained relatively stable for the past three years, ranging from 0-5%, and the other two aminoglycoside resistance genes named aac(6)-Ib and aac(3)-II showed steady escalation over the same period. rmtB and aac(6)-II among the aminoglycoside resistance genes were not found in any of the strains within these years. This data demonstrates an alarming escalation kinetics of resistance throughout these years.

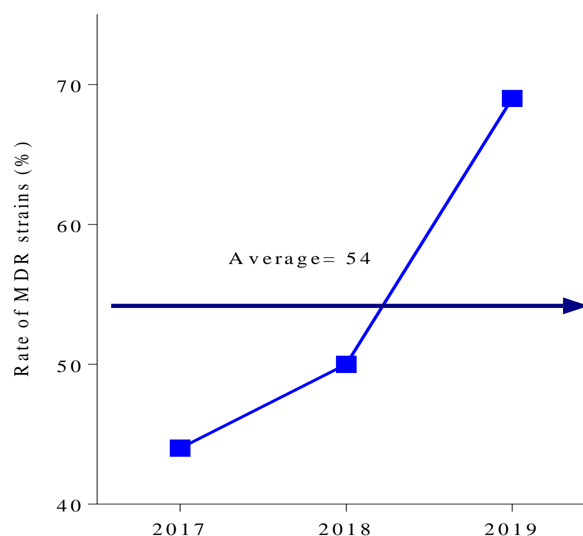

Figure 3.22: Year wise kinetics of the rate of MDR organisms

Similarly, a significant increasing trend in the proportion of MDR organisms was identified over the study period. In 2017, the percentage of MDR organisms was 44%. In 2018, the rate jumped by 6%; and in 2019, 69% organisms which is greater than the average MDR percentage (54%) were found to be MDR indicating a steeper line in the graph for growing antibiotic resistance trend as shown in figure 3.22.

3.12 Co-existence of multiple antibiotic resistance genes

Results obtained from investigation of co-resistance in isolates are displayed in Table 3.11. A total of 102 strains revealed to possess co-resistance genes. *E. coli* (n=40), *P. aeruginosa* (n=26) and MRSA (n=17) strains exhibited to be the predominant organisms in harboring co-resistance genes. Coexistence of CTXM-1 and ErmB (n=17) was frequently observed in *E. coli* (n=10) isolates, while combination of genotypes of CTXM-1 and aac(3)-II (n=10) was found in 5 *E. coli* isolates. A disappointing resistance pattern was seen for an *E. coli* isolate which showed to have genotypic resistance pattern combinations of a series of five genes (ErmB, ErmF, aac(6)-Ib, ant(3)-I, CTXM-1). A *P. aeruginosa* strain was seen to contain multiple ESBL genes (CTXM-1, NDM-1 and OXA-48), with the addition of aac(6)-Ib, an aminoglycoside resistance gene. Both ErmB and ErmC were carried by 6 isolates, 7 isolates harbored ErmB and ErmF. 11 strains were found to harbor multiple aminoglycoside resistance genes with aac(3)-II being most common gene.

Co-existence of resistance genes	***E. coli***	***P. aeruginosa***	***K. pneumoniae***	**MRSA**	***A. baumannii***	***S. aureus***	**CNS**	**No. of isolates carrying co-localized resistance genes**
CTXM-1, ErmB	10		4	3				17
CTXM-1, ErmC	3			2	1			6
CTXM-1, ErmF	3	4						7
ErmB, ErmC	4	2						6
ErmB, ErmF	3			3	1			7
ErmB, ErmC, OXA-48	1			1				2
ErmB, OXA-48		2						2
CTXM-1, aac(6)-Ib	1	2	4			1		8
CTXM-1, NDM-1, aac(3)-II, ErmB	1			1				2
ErmB, ErmF, aac(6)-Ib	1							1
ErmB, aac(3)-II, aph(3)-VI, aac(6)-Ib		1					1	2
ErmB, ErmF, aac(6)-Ib, ant(3)-I, CTXM-1	1							1
ErmB, CTXM-1, aac(3)-II	1	2						3
ErmF, CTXM-1, aac(6)-Ib			1			1		2
Aac(6)-Ib, aac(3)-II	2	1	1					4
Aac(6)-Ib, aac(3)-II, CTXM-1	1			1				2
OXA-48, armA		1						1
OXA-48, armA, aac(6)-Ib, aac(3)-II				1				1
CTXM-1, NDM-1, OXA-48, aac(6)-Ib		1						1
CTXM-1, NDM-1, aac(6)-Ib		2	2					4
NDM-1, aac(3)-II	1	1						2
NDM-1, aac(6)-Ib			1					1
Aph(3)-VI, aac(3)-II, CTXM-1				2				2
armA, aac(3)-II, CTXM-1				1				1
CTXM-1, aac(3)-II	5	4		1				10
OXA-48, aac(3)-II		1		1				2
NDM-1, aac(3)-II	2	2					1	5
Total	**40**	**26**	**13**	**17**	**2**	**2**	**2**	**102**

Table 3.11: Rate of co-resistance genotypes frequently observed in organisms

Chapter 4

DISCUSSION

Discussion

The study demonstrates the antibiotic resistance spectrum of the bacterial isolates from wound infection and the genes responsible for the resistance for ESBL, macrolide and aminoglycoside resistance. All the specimens were collected from two tertiary hospitals of Dhaka, Bangladesh and to my best knowledge this is the first antibiotic resistance study on wound infection isolates in Bangladesh. Bangladesh is still lacking this type of data regarding resistance spectrum of isolates of clinical specimens (especially wound infection) as well as molecular biology related information of these resistant strains barely exists. To address this critical knowledge gap, we speculated that resistance levels might be apparent in wound infected patients as evidenced by antibiotic resistance gene (ARG) abundances over time. Major focal points of the study included (a) in vitro antibiotic resistance/sensitivity assay for bacterial pathogens, (b) determination of spectrum of multi drug resistance and MAR index value, , (c) coinfection patterns, (d) determination of the liable resistance genes, (e) co-existence of multiple antibiotic resistance genes, (f) and also investigation of the kinetics of resistance over the three years study period. Knowing the resistance pattern would help clinicians to avoid unnecessary use of antibiotics to treat wound infection patients which also might help control of the rapid emergence of antibiotic resistant strains. On top of that, the data collected from this report could provide an up-to-date overview of the present situation of antibiotic resistance in Bangladesh which will assist in fighting the pathogens more effectively.

Wound sites are highly susceptible to infections and coinfections with more than one pathogen is very frequent. Several studies have reported antibiotic resistance spectrum in infections on multiple sites such as wound, respiratory tract, urinary tract and in other susceptible sites (Ladhani & Gransden, 2003; Valencia, Kirsner, & Kerdel, 2004; J. WANG & XIAO, 2008). Wounds and other lesions are prone to contamination with a multitude of organisms from the body surfaces and environment; the contaminating organisms are at first generally present in relatively small numbers, as originally introduced, and need not subsequently multiply. These sites can serve as the source for the MDR pathogens. So the susceptible organisms can come into juxtaposition with MDR one which helps to spread the drug resistant genes to susceptible one. In spite of technological advances that have been made in surgery and wound management, wound infection has been regarded as the most common nosocomial infection especially in

patients undergoing surgery (Ahmed, 2012) and wound colonization with pathogenic organisms normally results in aggressive management of the wound complicated by a greatly limited choice of therapeutic antibiotic. Thus the infection in wound sites pose bigger threat as this phenomenon helps in spreading the MDR genes not only for wound sites but also for nosocomial pathogens. Avoidance of the development of resistance therefore requires increasing attention in the management of wound infections (Filius & Gyssens, 2002). So the information on the spectrum of their reistance, source and mode of dissemination is crucial for treating and controlling the spread of MDR infections which in turn will reduce the disease severity, monetary burden and overall can lessen the morbidity and mortality rate.

In recent decades, antibiotic resistance has become a global health problem. The decreasing effectiveness of antibiotics in treating common infections has quickened in recent years and with the emergence of untreatable strains of carbapenem resistant *Enterobacteriaceae,* we are almost at the dawn of a pre-antibiotic era. In high-income countries, continued high rates of antibiotic use in hospitals, the community and agriculture have contributed to selection pressure that has sustained resistant strains, forcing a shift to more expensive and more broad-spectrum antibiotics. On the other hand, in low income and middle-income countries (LMICs), antibiotic use is being increased with rising incomes, high rates of hospitalization and high prevalence of hospital infections. Thus, resistance arises as a consequence of mutations in microbes and selection pressure from antibiotic usage that provides a competitive advantage for mutant strains, whereas suboptimum antibiotic doses help stepwise selection of resistance. (Laxminarayan et al., 2013). Furthermore, the comprehensive use of empirical therapy referred by specialists is very common in some developing and resource limited countries like Bangladesh. Hence, antibiotic resistance is expanding and still no faster method is established to detect such resistance. As a result, antibiotics are frequently prescribed without any diagnostic tests or sometimes used for viral infections such as fever and sore throat. A cross-sectional study perfomed in Chittagong revealed that third generation and fourth generation antibiotics were frequently used which is not less than 45% and most of the antibiotics were used for the common cold and fever (40.01%) whereas in microbial infection (17.01%). That study also presented that among the prescribed patients, 73% patients were concerned about the course of antibiotics, effect of antibiotics and 27% were not concern about it (Chowdhury, Kubra,

Islam, Rahman, & Mehedy, 2015). More terrifying is that there are > 350,000 new cases with 70,000 deaths due to an antibiotic therapeutic impasse annually. Cases of MDR and XDR in Bangladesh are of extreme significance in overall public health management (Noor & Munna, 2015). Though this is a major concern, no long term study related to antimicrobial resistance in wound infections and the responsible genes has yet been initiated in Bangladesh. Thus, this is the first study in Bangladesh to show phenotypic and genotypic evidence of bacterial resistance to aminoglycoside, macrolide resistance and ESBL genes in wound infected sites and hence, making antimicrobial susceptibility surveillance and testing more crucial in selecting empiric regimen or definitive treatment for physicians.

The study analyzed a total of 348 clinical bacterial strains which were isolated from patients with wound infections. Among the enlisted 12 global priority pathogens (GPP) according to World Health Organization (WHO) analyzed (Asokan & Vanitha, 2018), 8 of the total 9 bacterial species isolated during this study are enlisted in GPP list including *A. baumannii* (n=20), *P. aeruginosa* (n=26), *K. pneumoniae* (n=42) and *E. coli* (n=85) as the Priority 1 (Critical) pathogens, followed by MRSA (n=62), *S. aureus* (n=22) and *Enterococcus* spp. (n=27) as the Priority 2 (High) pathogens. Among the Priority 3 (Medium) pathogens list, *S. pneumoniae* (n=36) strains were. Additionally, CNS (n=28) which is not though listed in the priority list, was found very frequently in this study considering the fact that even the non-pathogenic organisms can act as hub of antibiotic resistance gene. The hospitalized and immunocompromised patients with underlying diseases is the main target of these bacteria. Estimates are that from 3% to 5% of patients leave the hospital having acquired infections, depending on case mix, hospital size, and other multiple factors (Schaberg, Culver, & Gaynes, 1991).The frequent isolation rate of the global priority pathogens from the participants of this study supports the idea of urgent containment of these pathogens in hospital settings. Not only the wound infection pathogens but also controlling other pathogenic organisms from multiple infection sites will help in recovery and management of the other infections.

The study identified that Gram-positive organisms accounted for 50.58% of isolates, compared to Gram-negative isolates that accounted for 49.72%. *E. coli* (24.43%) and *S. aureus* (24.14%) were found to be the major microbial pathogens responsible for the wound infections. This finding is in line with a previous study which identified *E.*

coli as the major pathogen in the wound infection, followed by *S. aureus* in a different setup (Naher, Afroz, & Hamid, 2018). According to Centre for Disease Control and Prevention (CDC), *S. aureus* is the most common organism associated with surgical wound infections. Thus, our results confirm the usual most prevalent microorganisms found in infected wounds (de Bessa et al., 2015). The organisms causing nosocomial infections have changed in medical practice over the years. Whereas gram positive organisms were the predominant organisms involved in these infections, gram negative organisms are now being isolated at an increasing rate. This shift may result from the greater complexity of the structure of the gram negative bacteria cell wall that made it to have intrinsic resistance to most antibacterial agents in use in the hospitals (Damen, Faruk, & Dancha, 2015). This is shown in the high prevalence of gram negatives such as *E. coli*, *P. aeruginosa*, *K. pneumoniae* and *A. baumannii* isolated in our study.

In our study, 25.35% of the wounds displayed polymicrobial infections and the most common association was found between MRSA and *K. pneumoniae*. Similarly, another study reported 27·2% of the wounds that showed polymicrobial infections (Bessa, Fazii, Di Giulio, & Cellini, 2015). Since wound colonization is most frequently polymicrobial, involving numerous microorganisms that are potentially pathogenic, any wound is at some risk of becoming infected and resitance gene spread in close proximity in case of coinfection. It is known that interspecies interactions consist mostly in bacterial synergy that enhances survival, therefore hampering the infection eradication. Moreover, microorganisms have the ability to establish themselves and proliferate as a biofilm, both in monomicrobial and polymicrobial biofilms, which are often considered to be a further complication that has a significant contribution to the lack of successful antibiotic treatment. Because of this problem, researchers are seeking for new alternative therapies useful to enhance wound healing, such as laser therapy (Bessa et al., 2015).

Furthermore, antibiogram study was designed to know the condition of antibiotic resistance or sensitivity patterns of major wound infection bacterial pathogens to avoid unnecessary use of antibiotics to treat patients. The bacterial isolates were examined for their susceptibility pattern to the most commonly used antibiotics in therapy. As described in result section, the in vitro antibiotic resistance patterns were concerning in the sense that most of the isolates except *S. pneumaniae*, *Enterococcus* spp. and *A.*

baumannii exhibited alarming levels of resistance against the majority of available first line antibiotics. The antibiogram data manifests an alarming resistance level for MRSA, *K. pneumoniae* and *P. aeruginosa* which displayed resistance not only against all the first-line of antibiotics but also against most of the second-line of antibiotics used. . In a study performed in Chittagong, typhoid patients were found to be unresponsive to second-line therapy (ciprofloxacin). First-line therapy was not even attempted because of existing resistance (Asna, Haq, & Rahman, 2003). Therapeutic failures like this are not rare at all. The current study revealed that for gram positive organisms, the most effective antibiotic was found to be vancomycin, whereas colistin, the last resort antibiotic, was the most active antimicrobial agent for gram negatives. While resistance rates to older antibiotics like chloramphenicol and trimethoprim–sulfamethoxazole, was comparatively less. Similar findings were observed in single-center studies in India, with increasing susceptibility to older antibiotics like ampicillin, chloramphenicol, and trimethoprim–sulfamethoxazole. These changes are likely due to the replacement of these drugs as an empiric treatment option for wound infection with newer drugs such as aminoglycosides, macrolides and β-lactams. These findings suggest that these drugs can no longer be considered an empiric treatment option for suspected wound infections; rather, physicians may be able to use older drugs again or third-generation cephalosporins (Gandra et al., 2016). On the other hand, vancomycin was the most effective antibiotic against gram positives. For instance, the results from other studies that explored vancomycin to be the most effective against CNS and *S. aureus* (Ghadiri, Vaez, Khosravi, & Soleymani, 2012).

Approximately, 54% isolates were classified as MDR in our study. *E. coli* (96.6%) was found to be the most prevalent MDR organism, followed by MRSA (90%) and *P. aeruginosa* (83.4%). Similarly, Meng Wang et al., showed that the most frequently isolated MDR bacteria in patients with hospital acquired infection (HAI) were extended-spectrum beta-lactamase (ESBL)-producing *E. coli* (37.7%), MDR *P. aeruginosa* (19.5%) and MDR *A. baumannii* (18.2%) (M. Wang et al., 2019). Whereas, we found that, according to MAR index, the mean value was the highest (0.5) for *P. aeruginosa* strains. Similarly, Osundiya, Oladele, & Oduyebo found that MAR index value for *P. aeruginosa* strains *was* 0.4 (Osundiya, Oladele, & Oduyebo, 2013). Currently, *P. spp.* represents a leading cause of hospital-acquired bacteremia, accounting for 4% of all cases. In one study, 58.6% of the isolates were confirmed MDR

P. aeruginosa (Rossolini & Mantengoli, 2005). In another study, out of 119 MDR positive samples, burn wounds showed the highest percentage 43 (36%), while least percentage 4 (3%) of MDR *P. aeruginosa* was found in surgical wounds (Nikokar et al., 2013). Therefore, the adequate management of *such MDR pathogens* should be considered as a significant challenge for clinicians.

Antibiotic resistant strains were further analysed using molecular techniques such as polymerase chain reaction (PCR) followed by PCR product sequencing. ESBL genes were chosen in such a way that it covers all the Amber classes of carbapenemases (Amber class A-D). Interestingly, the polymerase chain reaction and sequencing result revealed the presence of ESBL (CTXM-1, NDM-1 and OXA-48), macrolide resistance genes (ErmB, ErmC and ErmF) and aminoglycoside resistance (aac-6Ib, aac-3II, ant-3I, aph-3VI, armA) genes. In this study, *E. coli* strains had been found as the predominant bacterial agent carrying almost all of the resistance genes. This finding is in line with other studies where *E. coli* isolated from wastewater was capable of transferring resistance genes and producing antibiotic resistant phenotypes. *E. coli* isolates producing various resistance enzymes have emerged worldwide as an important cause of various infections. CTX-M-15 is often associated with OXA-1, TEM-1 beta-lactamases, and Aac(6)-Ib-cr, a variant of an aminoglycoside modifying enzyme that is responsible for reduced susceptibility to both aminoglycosides and fluoroquinolones (Literak et al., 2010). The plasmids containing different resistance genes in *E. coli* play an important role in the multiple antibiotic resistant transfer. Carbapenem-resistant *Enterobacteriaceae* (CRE) are particularly problematic, given the frequency with which *Enterobacteriaceae* cause infections and the potential for widespread transmission of carbapenem resistance via mobile genetic elements (Gupta, Limbago, Patel, & Kallen, 2011). The most important carbapenemases among *Enterobacteriaceae* clinical isolates are KPC (Amber class-A) and NDM (Amber class-B). While KPC-producing organisms are rarely reported in Asian countries (Nordmann, Cuzon, & Naas, 2009; Yang et al., 2010). NDM-producing organisms are prevalent among *Enterobacteriaceae* in India and Pakistan, even in community-onset infections (Kumarasamy et al., 2010) and is the most dangerous metallo-β-lactamase. A positive finding is that NDM was detected comparatively less than other ESBL genes investigated in this study. Among the 16 genes investigated, CTXM-1 was the most frequently detected gene and *E. coli* was found to be the main reservoir of this most

prevalent gene. The international dissemination of CTX-M ESBL genes and ST131 over the last decade has been described as a pandemic. CTX-M-producing *E. coli* and ST131 have emerged as a significant cause of both community-onset and hospital-acquired infections in Asian countries, including Korea (Park et al., 2012), Taiwan (Chung et al., 2012), China (Cao et al., 2011), Hong Kong (Ho, Lo, Lai, Chow, & Yam, 2012), Japan (Matsumura et al., 2012), Malaysia (W. S. Ho et al., 2012), and Thailand (Kiratisin, Apisarnthanarak, Laesripa, & Saifon, 2008), and the incidence of serious infections due to CTX-M-producing *E. coli* likely will continue to increase.

This study demonstrated phenotypic and genotypic discrepancies for some strains. Disk diffusion approach may vary for different strains, in such cases MIC would give a better understanding for phenotypic inconsistencies. In addition, genotypic discrepancies were also discovered for some strains and the reason behind this might be the genes responsible for resistance wasn't investigated in this study. Other studies have also found such discrepancies for certain strains (Nyberg et al., 2007). The isolates that remain undetected with the screening recommendations from CLSI are problematic, since failing to detect ESBL, aminoglycoside and macrolide resistance enzymes can have serious consequences when treating patients.

It was observed in this study that the relative proportion of MDR strains harboring antibiotic resistance genes is broadly increasing over time. CTXM-1, ErmB and aac(6)-Ib have been the prevailing detected resistance gene throughout these years. Similarly, an increasing trend in the proportion of MDR organisms was identified over the study period. The percentage of MDR organisms has nearly doubled in these 3 years. Other studies confirm that the relative proportion of resident bacteria containing antibiotic resistance gene (ARG) is broadly increasing over time, ranging from 2 to 15 times higher in 2008 compared with 1970-1979 levels (Knapp, Dolfing, Ehlert, & Graham, 2009). Antibiotic resistance genotyping demonstrated the presence of multiple antibiotic resistance genes among many isolates (29.31%) with one *E. coli* isolate harboring 5 genes encoding identical resistance phenotypes. A study performed in Department of Microbiology, Dhaka Medical College investigated that more than one carbapenemase gene was present in 45% of the isolates (Begum & Shamsuzzaman, 2016). Similarly Lin Li et al,. showed that coexistence of ESBL and/or PMQR genes to be identified in chicken isolates. Two *E. coli* isolates carried blaTEM-1, blaCTX-M and

qnrS, while two others carried blaCTX-M, qnrS and aac(6)-1b (Li et al., 2014). Therefore, the accumulation of multiple antibiotic resistance mechanisms in these clinical isolates raises alarm on potential spread of extensively drug-resistant organisms in healthcare settings.

An important limitation of this study must be mentioned. This study was carried out using only ESBL, macrolide and aminoglycoside gene specific primers. Additionally, other antibiotic resistance genes such as tetracyclines, quinolones and other resistance genes should have been investigated in these isolates as they were found to be resistant to these antibiotics phenotypically. Along with this, evaluation of atypical resistance genes in these pathogenic organisms should also be done. Also, in this preliminary study, Disk-diffusion susceptibility testing was performed whereas determination of the minimal inhibitory concentration (MIC) was not performed which reflects resistance condition more accurately. It would be necessary to expand investigations to include more hospitals, both within the capital city and the broader territories for active surveillance by increasing the sample size. Now-a-days, whole genome sequencing using Next generation sequencing is performed for such surveillance studies which would provide a clearer insight of such resistance, it could not have done due to resource limitation and specialist expertise. Moreover, follow up studies are necessitous to find out whether these genes are plasmid-mediated or not. On top of that, further research and in-depth analysis are still required.

In summary, highly pathogenic MDR strains were detected from wound infected sites in tertiary hospitals of the capital of Bangladesh, which can also contribute to other hospital acquired infections. It is to be noted however that, these observations underscore the need for urgent steps to arrest the increasing incidence of resistance to the antibiotics in the hospital settings. A tailored antibiotic treatment is in demand to tackle this major issue and also regular surveillance should be carried out to monitor the susceptibility of these pathogens and choose appropriate regimens both for prophylaxis and treatment of surgical wound infections. Continuous dialogue between the laboratory results and the attending physicians is strongly advised in keeping with preventing and controlling surgical wound infections at minimal cost. This will encourage rational use of antimicrobial agents and help in curbing the menace of resistance to these agents.

Bibliography:

1. Sütterlin, S., *Aspects of Bacterial Resistance to Silver*. 2015, Acta Universitatis Upsaliensis.
2. Robson, M.C., *Wound infection: a failure of wound healing caused by an imbalance of bacteria.* Surgical Clinics of North America, 1997. **77**(3): p. 637-650.
3. System, N.N.I.S., *National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992 through June 2004, issued October 2004.* Am J Infect Control, 2004. **32**: p. 470-485.
4. Chaix, C., et al., *Control of endemic methicillin-resistant S. aureus: a cost-benefit analysis in an intensive care unit.* Jama, 1999. **282**(18): p. 1745-1751.
5. Gould, I., *A review of the role of antibiotic policies in the control of antibiotic resistance.* Journal of Antimicrobial Chemotherapy, 1999. **43**(4): p. 459-465.
6. Seppälä, H., et al., *The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland.* New England Journal of Medicine, 1997. **337**(7): p. 441-446.
7. Filius, P.M.G. and I.C. Gyssens, *Impact of increasing antimicrobial resistance on wound management.* American journal of clinical dermatology, 2002. **3**(1): p. 1-7.
8. Kang, C.-I. and J.-H. Song, *Antimicrobial resistance in Asia: current epidemiology and clinical implications.* Infection & chemotherapy, 2013. **45**(1): p. 22-31.
9. Tacconelli, E. and M.D. Pezzani, *Public health burden of antimicrobial resistance in Europe.* The Lancet Infectious Diseases, 2019. **19**(1): p. 4-6.
10. Asna, S., J.A. Haq, and M.M. Rahman, *Nalidixic acid-resistant Salmonella enterica serovar Typhi with decreased susceptibility to ciprofloxacin caused treatment failure: a report from Bangladesh.* Japanese journal of infectious diseases, 2003. **56**(1): p. 32-33.
11. Ahmed, I., M.B. Rabbi, and S. Sultana, *Antibiotic resistance in Bangladesh: A systematic review.* International Journal of Infectious Diseases, 2019.
12. Gandra, S., et al., *Trends in antibiotic resistance among major bacterial pathogens isolated from blood cultures tested at a large private laboratory*

network in India, 2008–2014. International Journal of Infectious Diseases, 2016. **50**: p. 75-82.

13. Janda, J., S. Abbott, and R. Brenden, *Overview of the etiology of wound infections with particular emphasis on community-acquired illnesses.* European Journal of Clinical Microbiology and Infectious Diseases, 1997. **16**(3): p. 189-201.
14. Dow, G., A. Browne, and R. Sibbald, *Infection in chronic wounds: controversies in diagnosis and treatment.* Ostomy/wound management, 1999. **45**(8): p. 23-7, 29-40; quiz 41-2.
15. Horan, T.C., M. Andrus, and M.A. Dudeck, *CDC/NHSN surveillance definition of health care–associated infection and criteria for specific types of infections in the acute care setting.* American journal of infection control, 2008. **36**(5): p. 309-332.
16. Otten, H., *Domagk and the development of the sulphonamides.* Journal of Antimicrobial Chemotherapy, 1986. **17**(6): p. 689-690.
17. Waksman, S.A., *What is an antibiotic or an antibiotic substance?* Mycologia, 1947. **39**(5): p. 565-569.
18. Ligon, B.L. *Penicillin: its discovery and early development*. in *Seminars in pediatric infectious diseases*. 2004. Elsevier.
19. Waksman, S.A. and H.B. Woodruff, *The soil as a source of microorganisms antagonistic to disease-producing bacteria.* Journal of bacteriology, 1940. **40**(4): p. 581.
20. Spellberg, B., *Dr. William H. Stewart: mistaken or maligned?* Clinical infectious diseases: an official publication of the Infectious Diseases Society of America, 2008. **47**(2): p. 294-294.
21. Clatworthy, A.E., E. Pierson, and D.T. Hung, *Targeting virulence: a new paradigm for antimicrobial therapy.* Nature chemical biology, 2007. **3**(9): p. 541.
22. Greenwood, D., et al., *Antimicrobial chemotherapy*. 2007: OUP Oxford.
23. Normark, B.H. and S. Normark, *Evolution and spread of antibiotic resistance.* Journal of internal medicine, 2002. **252**(2): p. 91-106.
24. Roberts, M.C., *Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution.* FEMS microbiology reviews, 1996. **19**(1): p. 1-24.

25. Gaynor, M. and A.S. Mankin, *Macrolide antibiotics: binding site, mechanism of action, resistance.* Current topics in medicinal chemistry, 2003. **3**(9): p. 949-960.
26. Then, R.L., *Mechanisms of resistance to trimethoprim, the sulfonamides, and trimethoprim-sulfamethoxazole.* Reviews of infectious diseases, 1982. **4**(2): p. 261-269.
27. Ruiz, J., *Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection.* Journal of Antimicrobial Chemotherapy, 2003. **51**(5): p. 1109-1117.
28. Rice, L.B., *Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE*. 2008, The University of Chicago Press.
29. Handzlik, J., A. Matys, and K. Kieć-Kononowicz, *Recent advances in multi-drug resistance (MDR) efflux pump inhibitors of Gram-positive bacteria S. aureus.* Antibiotics, 2013. **2**(1): p. 28-45.
30. Tekiner, İ.H. and H. Özpınar, *Occurrence and characteristics of extended spectrum beta-lactamases-producing Enterobacteriaceae from foods of animal origin.* brazilian journal of microbiology, 2016. **47**(2): p. 444-451.
31. Shrestha, P., et al., *Enumerating the economic cost of antimicrobial resistance per antibiotic consumed to inform the evaluation of interventions affecting their use.* Antimicrobial Resistance & Infection Control, 2018. **7**(1): p. 98.
32. Ahmad, M. and A.U. Khan, *Global economic impact of antibiotic resistance: A review.* Journal of global antimicrobial resistance, 2019.
33. Gandra, S., D. Barter, and R. Laxminarayan, *Economic burden of antibiotic resistance: how much do we really know?* Clinical microbiology and infection, 2014. **20**(10): p. 973-980.
34. Barai, L., et al., *Antibiotic Resistance: Situation Analysis In a Tertiary Care Hospital of Bangladesh.* Bangladesh Journal of Microbiology, 2017. **34**(1): p. 15-19.
35. Cappuccino, J.G. and N. Sherman, *Microbiology: a laboratory manual*. Vol. 9. 2008: Pearson/Benjamin Cummings Boston, MA.
36. Lobo, I., *Basic local alignment search tool (blast).* Nature Education, 2008. **1**(1): p. 215.
37. Laxminarayan, R., et al., *Antibiotic resistance—the need for global solutions.* The Lancet infectious diseases, 2013. **13**(12): p. 1057-1098.

38. Adedeji, W., *The treasure called antibiotics.* Annals of Ibadan postgraduate medicine, 2016. **14**(2): p. 56.

39. Ventola, C.L., *The antibiotic resistance crisis: part 1: causes and threats.* Pharmacy and therapeutics, 2015. **40**(4): p. 277.

40. Percival, S., P. Bowler, and D. Russell, *Bacterial resistance to silver in wound care.* Journal of hospital infection, 2005. **60**(1): p. 1-7.

41. Podschun, R. and U. Ullmann, *Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors.* Clinical microbiology reviews, 1998. **11**(4): p. 589-603.

42. Schaberg, D.R., D.H. Culver, and R.P. Gaynes, *Major trends in the microbial etiology of nosocomial infection.* The American journal of medicine, 1991. **91**(3): p. S72-S75.

43. Ahmed, M.I., *Prevalence of nosocomial wound infection among postoperative patients and antibiotics patterns at teaching hospital in Sudan.* North American journal of medical sciences, 2012. **4**(1): p. 29.

44. Filius, P.M.G. and I.C. Gyssens, *Impact of increasing antimicrobial resistance on wound management.* American journal of clinical dermatology, 2002. **3**(1): p. 1-7.

45. Percival, S., P. Bowler, and D. Russell, *Bacterial resistance to silver in wound care.* Journal of hospital infection, 2005. **60**(1): p. 1-7.

46. Chowdhury, M.M.H., et al., *Indiscriminate uses of antibiotics as a threat to public health demand implementation of effective drug practices and enhancement of public awareness in Bangladesh.* European Journal of Scientific Research, 2015. **133**(2): p. 187-195.

47. Noor, R. and M.S. Munna, *Emerging diseases in Bangladesh: current microbiological research perspective.* Tzu Chi Medical Journal, 2015. **27**(2): p. 49-53.

48. Adedeji, W. (2016). The treasure called antibiotics. *Annals of Ibadan postgraduate medicine, 14*(2), 56.

49. Ahmad, M., & Khan, A. U. (2019). Global economic impact of antibiotic resistance: A review. *Journal of global antimicrobial resistance*.

50. Ahmed, I., Rabbi, M. B., & Sultana, S. (2019). Antibiotic resistance in Bangladesh: A systematic review. *International Journal of Infectious Diseases*.

51. Ahmed, M. I. (2012). Prevalence of nosocomial wound infection among postoperative patients and antibiotics patterns at teaching hospital in Sudan. *North American journal of medical sciences, 4*(1), 29.

52. Akinkunmi, E. O., Adesunkanmi, A.-R., & Lamikanra, A. (2014). Pattern of pathogens from surgical wound infections in a Nigerian hospital and their antimicrobial susceptibility profiles. *African health sciences, 14*(4), 802-809.

53. Asna, S., Haq, J. A., & Rahman, M. M. (2003). Nalidixic acid-resistant Salmonella enterica serovar Typhi with decreased susceptibility to ciprofloxacin caused treatment failure: a report from Bangladesh. *Japanese journal of infectious diseases, 56*(1), 32-33.

54. Barai, L., Saha, M. R., Rahman, T., Khandaker, T., Dutta, S., Hasan, R., & Haq, J. A. (2017). Antibiotic Resistance: Situation Analysis In a Tertiary Care Hospital of Bangladesh. *Bangladesh Journal of Microbiology, 34*(1), 15-19.

55. Begum, N., & Shamsuzzaman, S. (2016). Emergence of carbapenemase-producing urinary isolates at a tertiary care hospital in Dhaka, Bangladesh. *Tzu Chi Medical Journal, 28*(3), 94-98.

56. Bessa, L. J., Fazii, P., Di Giulio, M., & Cellini, L. (2015). Bacterial isolates from infected wounds and their antibiotic susceptibility pattern: some remarks about wound infection. *International wound journal, 12*(1), 47-52.

57. Bowler, P., Duerden, B., & Armstrong, D. G. (2001). Wound microbiology and associated approaches to wound management. *Clinical microbiology reviews, 14*(2), 244-269.

58. Cappuccino, J. G., & Sherman, N. (2008). *Microbiology: a laboratory manual* (Vol. 9): Pearson/Benjamin Cummings Boston, MA.

59. Chaix, C., Durand-Zaleski, I., Alberti, C., & Brun-Buisson, C. (1999). Control of endemic methicillin-resistant Staphylococcus aureus: a cost-benefit analysis in an intensive care unit. *Jama, 282*(18), 1745-1751.

60. Chowdhury, M. M. H., Kubra, K., Islam, M. T., Rahman, M. M., & Mehedy, M. E. (2015). Indiscriminate uses of antibiotics as a threat to public health demand implementation of effective drug practices and enhancement of public awareness in Bangladesh. *European Journal of Scientific Research, 133*(2), 187-195.

61. Chung, H.-C., Lai, C.-H., Lin, J.-N., Huang, C.-K., Liang, S.-H., Chen, W.-F., . . . Wang, J.-L. (2012). Bacteremia caused by extended-spectrum-β-lactamase-

producing Escherichia coli sequence type ST131 and non-ST131 clones: comparison of demographic data, clinical features, and mortality. *Antimicrobial agents and chemotherapy, 56*(2), 618-622.

62. Clatworthy, A. E., Pierson, E., & Hung, D. T. (2007). Targeting virulence: a new paradigm for antimicrobial therapy. *Nature chemical biology, 3*(9), 541.

63. Damen, J. G., Faruk, S., & Dancha, C. (2015). Aerobic Bacteria Isolates of Septic Wound Infections and their Antibiogram in North Central Nigeria.

64. Davis, I., Richards, H., & Mullany, P. (2005). Isolation of silver- and antibiotic-resistant Enterobacter cloacae from teeth. *Oral microbiology and immunology, 20*(3), 191-194.

65. de Bessa, J., de Carvalho Mrad, F. C., Mendes, E. F., Bessa, M. C., Paschoalin, V. P., Tiraboschi, R. B., . . . Bastos Netto, J. M. (2015). Antibiotic prophylaxis for prevention of febrile urinary tract infections in children with vesicoureteral reflux: a meta-analysis of randomized, controlled trials comparing dilated to nondilated vesicoureteral reflux. *The Journal of urology, 193*(5S), 1772-1777.

66. Dow, G., Browne, A., & Sibbald, R. (1999). Infection in chronic wounds: controversies in diagnosis and treatment. *Ostomy/wound management, 45*(8), 23-27, 29-40; quiz 41-22.

67. Filius, P. M. G., & Gyssens, I. C. (2002). Impact of increasing antimicrobial resistance on wound management. *American journal of clinical dermatology, 3*(1), 1-7.

68. Gandra, S., Barter, D., & Laxminarayan, R. (2014). Economic burden of antibiotic resistance: how much do we really know? *Clinical microbiology and infection, 20*(10), 973-980.

69. Gandra, S., Mojica, N., Klein, E. Y., Ashok, A., Nerurkar, V., Kumari, M., . . . Das, B. R. (2016). Trends in antibiotic resistance among major bacterial pathogens isolated from blood cultures tested at a large private laboratory network in India, 2008–2014. *International Journal of Infectious Diseases, 50*, 75-82.

70. Gaynor, M., & Mankin, A. S. (2003). Macrolide antibiotics: binding site, mechanism of action, resistance. *Current topics in medicinal chemistry, 3*(9), 949-960.

71. Ghadiri, H., Vaez, H., Khosravi, S., & Soleymani, E. (2012). The antibiotic resistance profiles of bacterial strains isolated from patients with hospital-

acquired bloodstream and urinary tract infections. *Critical care research and practice, 2012*.

72. Gould, I. (1999). A review of the role of antibiotic policies in the control of antibiotic resistance. *Journal of antimicrobial Chemotherapy, 43*(4), 459-465.

73. Greenwood, D., Finch, R., Davey, P., & Wilcox, M. (2007). *Antimicrobial chemotherapy*: OUP Oxford.

74. Gupta, N., Limbago, B. M., Patel, J. B., & Kallen, A. J. (2011). Carbapenem-resistant Enterobacteriaceae: epidemiology and prevention. *Clinical infectious diseases, 53*(1), 60-67.

75. Handzlik, J., Matys, A., & Kieć-Kononowicz, K. (2013). Recent advances in multi-drug resistance (MDR) efflux pump inhibitors of Gram-positive bacteria S. aureus. *Antibiotics, 2*(1), 28-45.

76. Horan, T. C., Andrus, M., & Dudeck, M. A. (2008). CDC/NHSN surveillance definition of health care–associated infection and criteria for specific types of infections in the acute care setting. *American journal of infection control, 36*(5), 309-332.

77. Janda, J., Abbott, S., & Brenden, R. (1997). Overview of the etiology of wound infections with particular emphasis on community-acquired illnesses. *European Journal of Clinical Microbiology and Infectious Diseases, 16*(3), 189-201.

78. Kang, C.-I., & Song, J.-H. (2013). Antimicrobial resistance in Asia: current epidemiology and clinical implications. *Infection & chemotherapy, 45*(1), 22-31.

79. Knapp, C. W., Dolfing, J., Ehlert, P. A., & Graham, D. W. (2009). Evidence of increasing antibiotic resistance gene abundances in archived soils since 1940. *Environmental science & technology, 44*(2), 580-587.

80. Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., . . . Irfan, S. (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *The Lancet infectious diseases, 10*(9), 597-602.

81. Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N., . . . Goossens, H. (2013). Antibiotic resistance—the need for global solutions. *The Lancet infectious diseases, 13*(12), 1057-1098.

82. Li, L., Wang, B., Feng, S., Li, J., Wu, C., Wang, Y., . . . Zeng, M. (2014). Prevalence and characteristics of extended-spectrum β-lactamase and plasmid-

mediated fluoroquinolone resistance genes in Escherichia coli isolated from chickens in Anhui province, China. *PloS one, 9*(8), e104356.

83. Li, Q., Chang, W., Zhang, H., Hu, D., & Wang, X. (2019). The role of plasmids in the multidrug-resistant linked transfer of ESBLs-producing Escherichia coli isolated from wastewater treatment plants. *Frontiers in microbiology, 10*, 633.

84. Ligon, B. L. (2004). *Penicillin: its discovery and early development.* Paper presented at the Seminars in pediatric infectious diseases.

85. Literak, I., Dolejska, M., Janoszowska, D., Hrusakova, J., Meissner, W., Rzyska, H., . . . Cizek, A. (2010). Antibiotic-resistant Escherichia coli bacteria, including strains with genes encoding the extended-spectrum beta-lactamase and QnrS, in waterbirds on the Baltic Sea Coast of Poland. *Appl. Environ. Microbiol., 76*(24), 8126-8134.

86. Lobo, I. (2008). Basic local alignment search tool (blast). *Nature Education, 1*(1), 215.

87. Naher, A., Afroz, S., & Hamid, S. (2018). Cockroach associated foodborne pathogens: Distribution and antibiogram. *Bangladesh Medical Research Council Bulletin, 44*(1), 30-38.

88. Nikokar, I., Tishayar, A., Flakiyan, Z., Alijani, K., Rehana-Banisaeed, S., Hossinpour, M., . . . Araghian, A. (2013). Antibiotic resistance and frequency of class 1 integrons among Pseudomonas aeruginosa, isolated from burn patients in Guilan, Iran. *Iranian journal of microbiology, 5*(1), 36.

89. Noor, R., & Munna, M. S. (2015). Emerging diseases in Bangladesh: current microbiological research perspective. *Tzu Chi Medical Journal, 27*(2), 49-53.

90. Nordmann, P., Cuzon, G., & Naas, T. (2009). The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. *The Lancet infectious diseases, 9*(4), 228-236.

91. Normark, B. H., & Normark, S. (2002). Evolution and spread of antibiotic resistance. *Journal of internal medicine, 252*(2), 91-106.

92. Otten, H. (1986). Domagk and the development of the sulphonamides. *Journal of Antimicrobial Chemotherapy, 17*(6), 689-690.

93. Percival, S., Bowler, P., & Russell, D. (2005). Bacterial resistance to silver in wound care. *Journal of hospital infection, 60*(1), 1-7.

94. Podschun, R., & Ullmann, U. (1998). Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical microbiology reviews, 11*(4), 589-603.

95. Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE: The University of Chicago Press.

96. Roberts, M. C. (1996). Tetracycline resistance determinants: mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS microbiology reviews, 19*(1), 1-24.

97. Robson, M. C. (1997). Wound infection: a failure of wound healing caused by an imbalance of bacteria. *Surgical Clinics of North America, 77*(3), 637-650.

98. Rossolini, G., & Mantengoli, E. (2005). Treatment and control of severe infections caused by multiresistant Pseudomonas aeruginosa. *Clinical Microbiology and Infection, 11*, 17-32.

99. Ruiz, J. (2003). Mechanisms of resistance to quinolones: target alterations, decreased accumulation and DNA gyrase protection. *Journal of Antimicrobial Chemotherapy, 51*(5), 1109-1117.

100. Schaberg, D. R., Culver, D. H., & Gaynes, R. P. (1991). Major trends in the microbial etiology of nosocomial infection. *The American journal of medicine, 91*(3), S72-S75.

101. Seppälä, H., Klaukka, T., Vuopio-Varkila, J., Muotiala, A., Helenius, H., Lager, K., . . . Resistance, F. S. G. f. A. (1997). The effect of changes in the consumption of macrolide antibiotics on erythromycin resistance in group A streptococci in Finland. *New England Journal of Medicine, 337*(7), 441-446.

102. Shrestha, P., Cooper, B. S., Coast, J., Oppong, R., Thuy, N. D. T., Phodha, T., . . . Lubell, Y. (2018). Enumerating the economic cost of antimicrobial resistance per antibiotic consumed to inform the evaluation of interventions affecting their use. *Antimicrobial Resistance & Infection Control, 7*(1), 98.

103. Spellberg, B. (2008). Dr. William H. Stewart: mistaken or maligned? *Clinical infectious diseases: an official publication of the Infectious Diseases Society of America, 47*(2), 294-294.

104. Sütterlin, S. (2015). *Aspects of Bacterial Resistance to Silver.* Acta Universitatis Upsaliensis.

105. System, N. N. I. S. (2004). National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992 through June 2004, issued October 2004. *Am J Infect Control, 32*, 470-485.

106. Tacconelli, E., & Pezzani, M. D. (2019). Public health burden of antimicrobial resistance in Europe. *The Lancet infectious diseases, 19*(1), 4-6.

107. Tekiner, İ. H., & Özpınar, H. (2016). Occurrence and characteristics of extended spectrum beta-lactamases-producing Enterobacteriaceae from foods of animal origin. *brazilian journal of microbiology, 47*(2), 444-451.

108. Then, R. L. (1982). Mechanisms of resistance to trimethoprim, the sulfonamides, and trimethoprim-sulfamethoxazole. *Reviews of infectious diseases, 4*(2), 261-269.

109. Ventola, C. L. (2015). The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and therapeutics, 40*(4), 277.

110. Waksman, S. A. (1947). What is an antibiotic or an antibiotic substance? *Mycologia, 39*(5), 565-569.

111. Waksman, S. A., & Woodruff, H. B. (1940). The soil as a source of microorganisms antagonistic to disease-producing bacteria. *Journal of bacteriology, 40*(4), 581.

112. Wang, M., Wei, H., Zhao, Y., Shang, L., Di, L., Lyu, C., & Liu, J. (2019). Analysis of multidrug-resistant bacteria in 3223 patients with hospital-acquired infections (HAI) from a tertiary general hospital in China. *Bosnian journal of basic medical sciences, 19*(1), 86.

113. Yang, Q., Wang, H., Sun, H., Chen, H., Xu, Y., & Chen, M. (2010). Phenotypic and genotypic characterization of Enterobacteriaceae with decreased susceptibility to carbapenems: results from large hospital-based surveillance studies in China. *Antimicrobial agents and chemotherapy, 54*(1), 573-577.

114. Barbosa, C., Nogueira, S., Gadanho, M., & Chaves, S. (2016). DNA extraction: finding the most suitable method *Molecular Microbial Diagnostic Methods* (pp. 135-154): Elsevier.

115. Gauthier, M. G. (2008). *Simulation of polymer translocation through small channels: A molecular dynamics study and a new Monte Carlo approach.* University of Ottawa (Canada).

116. Asokan, G. V., & Vanitha, A. (2018). WHO global priority pathogens list on antibiotic resistance: an urgent need for action to integrate One Health data. *Perspectives in public health, 138*(2), 87-88.

117. Osundiya, O., Oladele, R., & Oduyebo, O. (2013). Multiple antibiotic resistance (MAR) indices of Pseudomonas and Klebsiella species isolates in Lagos University Teaching Hospital. *African Journal of Clinical and Experimental Microbiology, 14*(3), 164-168.

118. Naparsteck, M., & O'Brien, T. (1991). An Interview with Tim O'Brien. *Contemporary Literature, 32*(1), 1-11.

119. Baquero, F. (1996). Trends in antibiotic resistance of respiratory pathogens: an analysis and commentary on a collaborative surveillance study. *Journal of Antimicrobial Chemotherapy, 38*(suppl_A), 117-132.

120. Fridkin, S. K., Steward, C. D., Edwards, J. R., Pryor, E. R., McGowan Jr, J. E., Archibald, L. K., . . . Hospitals, P. I. C. A. R. E. (1999). Surveillance of antimicrobial use and antimicrobial resistance in United States hospitals: project ICARE phase 2. *Clinical infectious diseases, 29*(2), 245-252.

121. WANG, J., & XIAO, Y.-h. (2008). Mohnarin Report 2006-2007: Bacterial Distribution and Resistance in Bloodstream Infections [J]. *Chinese Journal of Nosocomiology, 9*.

122. Valencia, I. C., Kirsner, R. S., & Kerdel, F. A. (2004). Microbiologic evaluation of skin wounds: alarming trend toward antibiotic resistance in an inpatient dermatology service during a 10-year period. *Journal of the American Academy of Dermatology, 50*(6), 845-849.

123. Ladhani, S., & Gransden, W. (2003). Increasing antibiotic resistance among urinary tract isolates. *Archives of disease in childhood, 88*(5), 444-445.

124. Nyberg, S. D., Österblad, M., Hakanen, A. J., Huovinen, P., Jalava, J., & Resistance, F. S. G. f. A. (2007). Detection and molecular genetics of extended-spectrum beta-lactamases among cefuroxime-resistant Escherichia coli and Klebsiella spp. isolates from Finland, 2002–2004. *Scandinavian journal of infectious diseases, 39*(5), 417-424.

125. Cao, X., Cavaco, L. M., Lv, Y., Li, Y., Zheng, B., Wang, P., . . . Aarestrup, F. M. (2011). Molecular characterization and antimicrobial susceptibility testing of Escherichia coli isolates from patients with urinary tract infections in 20 Chinese hospitals. *Journal of clinical microbiology, 49*(7), 2496-2501.

126. Ho, P.-L., Lo, W. U., Lai, E. L., Chow, K.-H., & Yam, W.-C. (2012). Escherichia coli O25b-ST131 is an important cause of antimicrobial-resistant infections in women with uncomplicated cystitis. *Journal of Antimicrobial Chemotherapy, 67*(10), 2534-2535.

127. Matsumura, Y., Yamamoto, M., Nagao, M., Hotta, G., Matsushima, A., Ito, Y., . . . Ichiyama, S. (2012). Emergence and spread of B2-ST131-O25b, B2-ST131-O16 and D-ST405 clonal groups among extended-spectrum-β-lactamase-producing Escherichia coli in Japan. *Journal of Antimicrobial Chemotherapy, 67*(11), 2612-2620.

128. Kiratisin, P., Apisarnthanarak, A., Laesripa, C., & Saifon, P. (2008). Molecular characterization and epidemiology of extended-spectrum-β-lactamase-producing Escherichia coli and Klebsiella pneumoniae isolates causing health care-associated infection in Thailand, where the CTX-M family is endemic. *Antimicrobial agents and chemotherapy, 52*(8), 2818-2824.

129. Ho, W. S., Balan, G., Puthucheary, S., Kong, B. H., Lim, K. T., Tan, L. K., . . . Thong, K. L. (2012). Prevalence and characterization of multidrug-resistant and extended-spectrum beta-lactamase-producing Escherichia coli from pediatric wards of a Malaysian hospital. *Microbial Drug Resistance, 18*(4), 408-416.

130. Krumperman, P. H. (1983). Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. *Appl. Environ. Microbiol., 46*(1), 165-170.

APPENDIX-I

Media composition

The media composition used in this research is given below. All the media were autoclaved at 121°C for 30 minutes for sterilization.

1. MacConkey Agar (Difco™)

Ingredients	Amount (g/L)
Peptone	17.0
Proteose Peptone	3.0
Lactose	10.0
Bile Salts No. 3	1.5
Sodium Chloride	5.0
Agar	13.5
Neutral Red	0.03
Crystal Violet	0.001

2. Blood Agar

Ingredients	Amounts (g/L)
Tryptone	15g
Phytone or Soytone	5g
NaCl	5g
Agar	15g
Defibrinated sheep red blood	5ml
Distilled water	1 litre

3. Mueller Hinton Agar (Oxoid, England)

Ingredients	Amount (g/L)
Beef, dehydrated infusion from	300.0
Casein hydrolysate	17.5
Starch	1.5
Agar (Himedia, India)	15.0

4. Motility Indole Urease Agar

Ingredients	Amount (g/L)	
NaCl (Sigma)	5	Prepare up to 900ml for **autoclave**
Agar (Himedia, India)	4	
KH_2PO_4 (Fisher Chemical, USA)	2	
Peptone (Himedia, India)	30	
Phenol Red (0.25%) (Sigma, India)	2 ml/L	
Urea (Amresco, USA)	20	Prepare up to 100ml for **filter sterilization**

5. Simmon's Citrate Agar (Oxoid, England)

Ingredients	Amount (g/L)
Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	0.2
Sodium ammonium phosphate	0.8
Sodium citrate, tribasic	2.0
Sodium chloride	5.0
Bromothymol blue	0.08
Agar	15.0

6. Triple Sugar Iron Agar (Difco™)

Ingredients	Amount (g/L)
Beef Extract	3.0
Yeast Extract	3.0
Pancreatic Digest of Casein	15.0
Proteose Peptone No. 3	5.0
Dextrose	1.0
Lactose	10.0
Sucrose	10.0
Ferrous Sulfate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Agar	12.0
Phenol Red	0.024

APPENDIX-II

Instruments

Name	Manufacturer
Autoclave	WiseClave
Refrigerator	Electra, Samsung (+4°C)- to store bacteria; Vestfrost (+4°C)- to store bacterial medium;
Freeze	Vestfrost (-20°C) to store stock antibiotics; ESCO (-80°C) to store stock bacteria.
Incubator	Memmert
Shaking Incubator	WiseCube
Oven	WiseVen
Water bath	WiseBath
Micropipette	(2-20µl)- Gilson and Costar® (20-200µl)- Gilson and Costar® (200-1000µl)- Gilson
Bio-Safety Cabinet	ESCO Class-II Type-A2 Labculture® Biological Safety Cabinet
Vortex Mixture Machine	WiseMix
Weighing Machine	OHAUS®
Weighing Paper	Fisherbrand®
Spectrophotometer	Eon™ BioTek®
Take 3 plate	Bio-Tek
Thermal Cycler	INFINIGEN
Centrifuge Machine	Thermo SCIENTIFIC
Light Microscope	OLYMPUS CX41
Antibiotic disks	Oxoid
Gel documentation machine	Bio-Rad
Electronic balance machine	Mega