MOLECULAR AND PHENOTYPIC INVESTIGATIONSOF ENZYME MEDIATED RESISTANCE PROFILES AGAINST AMINOGLYCOSIDE ANTIBIOTICS IN GRAM-NEGATIVE CLINICAL ISOLATES IN DHAKA, BANGLADESH

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THESIS DISSERTATION SUBMITTED TO THE DEPARTMENT OF MATHEMATICS AND NATURAL SCIENCES, BRAC UNIVERSITY

IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF

MS IN BIOTECHNOLOGY

CERTIFICATION OF AUTHENTICITY

I, the undersigned, Saad Hassan Hasib, declare that this dissertation titled "**MOLECULAR AND PHENOTYPIC INVESTIGATIONS OF ENZYME MEDIATED RESISTANCE PROFILES AGAINST AMINOGLYCOSIDE ANTIBIOTICS IN GRAM-NEGATIVE CLINICAL ISOLATES IN DHAKA, BANGLADESH**"is my original work, gathered and utilized especially to fulfill the purposes and objectives of this study and has not been previously submitted to any other university for a higher degree. I also declare that the publications cited in this work are authentic sources, responsibly acquired. It is further proclaimed that this work has been carried out under the joint supervision of Dr. Md. Kaiissar Mannoor, Senior Scientist and Head at the institute for developing Science and Health initiatives (ideSHi), Mohakhali, Dhaka and Prof. Firdausi Qadri, Senior Scientist and Executive Director at ideSHi and Acting Senior Director of the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b); as well as Prof. Naiyyum Choudhury, Former Professor and Coordinator within the Department of Mathematics and Natural Sciences of BRAC University, Mohakhali and Chairman of the Bangladesh Atomic Energy Regulatory Authority- serving as external and internal supervisors, respectively.

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Author,

Saad Hassan Hasib (September, 2018)

ABSTRACT

In recent decades, focus has shifted from notorious Gram-positive pathogens to Gram-negative threats, the majority of which comprise members of the Enterobacteriaceae and Pseudomonadaceae families. These, often commensal bacteria, co-inhabit our environment and within us, however have the propensity to develop opportunistic infections in immunocompromised individuals, especially in intensive care units (ICUs) of hospitals. Aminoglycoside antibiotics are often reserved for severe bacterial infections. Therefore, we conducted surveillance on their resistance phenotypes by antibiotic susceptibility testing (AST) and have found a total of 83.3% of strains to be multidrug resistant (MDR) forms. Among a range of clinical isolates as collected from three major hospitals in Dhaka, ETEC; *Klebsiella pneumoniae*; *Pantoea agglomerans*; *Pseudomonas spp*. and *Aeromonas spp*. were interrogated against five commercially popular aminoglycoside antibiotics, finding phenotypic resistance towards streptomycin (50%); tobramycin (50%); gentamicin (44.4%); amikacin (33.3%), and netilmicin (28%). Molecular methods were then employed, where three aminoglycoside resistance enzyme (AME) encoding genes were prominent: *aac(6')-Ib* (27.8%), *aac(3')-II* (16.7%) and *ant(3")-Ia* (5.6%) among a total of seven genes investigated. Phylogenetic analysis revealed these genes had previous origins in Australia, France and China respectively. These comprised identifical clones which have dispersed world-wide, demonstrating the high possibility for dissemination via horizontal gene transfer using mobile genetic elements. We therefore recommend sparse, cycled use of these drugs in pragmatic combinations with other antibiotic classes for synergistic effects, and report the greatest efficacy remaining towards amikacin and netilmicin amongstthe aminoglycosides.

Key Words:

Gram-negative, Aminoglycosides, Resistance, Enzymes, Bangladesh, Clinical Isolates

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LIST OF ABBREVIATIONS

- 2-DOS: 2-deoxystreptamine
- A (site): Acceptor site
- A: Adenine nucleotide
- AAC: Aminoglycoside acetyltransferase
- ADH: Arginine dihydrolase
- AK: Amikacin
- AME: Aminoglycoside modifying enzyme
- AMP: Adenosine monophosphate
- AMY: Amygdalin
- ANT: Aminoglycoside nucleotidyl transferase
- APH: Aminoglycoside phosphotransferase
- ARA: Arabinose
- ARI: Acute Respiratory Infections
- AST: Antimicrobial Susceptibility Test
- ATP: Adenosine triphosphate
- AZI: Azithtomycin
- BIC: Bayesian Information Criterion
- BLAST: Basic Local Alignment Search Tool
- BLASTp: protein Basic Local Alignment Search Tool
- BMRC: Bangladesh Medical Research Council
- C: Cytosine
- CAR: Carbenicillin
- CAT: Chloramphenicol acetyltransferase
- CAZ: Ceftazidime
- CF: Cystic Fibrosis
- CFM: Cefixime
- CFTR: Cystic Fibrosis Transmembrane Conductance Regulator
- CHAT: Choline acetyltransferase
- CIP: Ciprofloxacin
- CIT: Citrate
- CLSI: Clinical Laboratory Standards Institute
- CN: Gentamicin
- CO_2: Carbon dioxide
- CRO: Ceftriaxone
- CT: Colistin sulphate
- DHLA: Dihydrolipoamide acetyltransferase
- DMCH: Dhaka Medical College and Hospital
- DMD: Duchenne Muscular Dystrophy
- DNA: Deoxyribonucleic acid
- DO: Doxycycline
- E: Erythormycin
- EAggEC: Enteroaggregative *Escherichia coli*
- EPEC: Enteropathogenic Escherichia coli
- ESBL: Extended spectrum beta-lactamase

- ETEC: Enterotoxigenic *Escherichia coli*
- Erm: Erythromycin methyltranferase
- F: Forward primer
- G-C: Guanine-Cytosine
- G: Guanosine
- GB: Gigabytes
- GEL: Gelatin
- GLU: Glucose
- GNAT: CCN5 related *N*-acetyltransferase
- H_20: Water
- H_20_2: Hydrogen peroxide
- H_2S: Hydrogen sulfide
- HGT: Horizontal gene transfer
- HIV: Human immunodeficiency virus
- I: Intermediate resistance (to antibiotic)
- ICU: Intensive Care Unit
- IEDCR: Institute of Epidemiology, Disease Control and Research
- IMCI: Integrated Management of Childhood Illness
- IND: Indole
- INO: Inositol
- IPH: Institute of Public Health
- IPM: Imipenem
- LB: Luria broth
- LDC: Lysine decarboxylase
- M: Molar
- MAN: Mannose
- MDR: Multi-drug resistant
- MEGA: Molecular Evolutionary Genetics Analysis
- MEL: Melibiose
- MEM-Meropenem
- MIU: Motility-Indole-Urease
- ML-Maximum Likelihood
- MLST: Multilocus sequence typing
- MPS I: Mucopolysaccharidosis type I
- MSA: Multiple sequence alignment
- MUSCLE: Multiple Sequence Comparison by Log-Expectation
- $MgCl_2$: Magnesium Chloride
- N.F. H20: Nuclease free water
- NA: Nalidixic acid
- NCBI: National centre for biotechnology information
- NDM: New Delhi Beta lactamase
- NET: Netilmicin
- NOR: Norfloxacin
- NaCl: Sodium chloride
- ODC: Ornithine decarboxylase
- ONPG: *O*-nitrophenyl-b-D-galactopyranoside
- ORF: Open reading frame
- PB: Polymixin B
- PCR: Polymerase Chain Reaction
- PFGE: Pulse field gel electrophoresis
- PMF: Proton motive force
- R: Full resistance (to antibiotic)
- R: Reverse (primer)
- RAM: Random access memory
- RHA: Rhamnose
- RMTase: Ribosomal RNA methyltransferase
- RNA: Ribonucleic acid

- RPM: Revolutions per minute
- S- Streptomycin
- SAC: Sucrose
- SAM: *S*-adenosyl-L-methionine
- SOR: Sorbitol
- SRGA: Swedish Reference Group for Antibiotics
- SSMCH: Shaheed Suhrawardy Medical College and Hospital
- SXT: Trimethoprim-sulfamethoxazole
- Stp-Heat stable toxin
- T: Thymine
- TBE: Tris-Boric acid-EDTA
- TE: Tris-EDTA
- TE: Tetracycline
- TOB: Tobramycin
- TSGG: Tryptone Soya Glucose Glycerol
- TSI: Triple-Sugar-Iron
- TZP: Pipercillin+Tazobactam
- UK: United Kingdom
- URE: Urease
- US: United States (of America)
- USA: United States of America
- USD: United States Dollars
- UTI: Urinary Tract Infection
- S: Sensitive (to antibiotic)

- VP: Vogues-Proskauer test
- WGS: Whole genome sequencing
- WHO: World Health Organization
- Bp: Base pair
- cm: Centimeter
- dNTP: Deoxynucleotide triphosphate
- ddNTP: Dideoxy nucleotide triphosphate
- g: Gram
- icddr, b: International Centre for Diarrhoeal Disease Research, Bangladesh
- ideSHi: Institute for developing Science and Health initiatives
- kb: Kilobase pairs
- mM: miliMolar
- mRNA: Messenger ribonucleic acid
- ml: Mililitre
- mm: Milimeter
- ng: Nanogram
- psi: Pounds per square inch
- r.t.p: Room temperature
- rRNA: Ribosomal ribonucleic acid
- s: Seconds
- tRNA: Transfer ribonucleic acid
- °C: Degrees in Celsius
- µg: Microgram
- µl: Microlitre

Chapter 1

INTRODUCTION

1. INTRODUCTION

1.1 GENERAL BACKGROUND OF STUDY

Multidrug resistant strains of pathogenic bacteria are a common epidemic faced all over the world. Of all patients, it is the immunocompromised population that is most vulnerable to aggravated infections and most urgently requires prophylactic treatment using a broad-spectrum of antibiotics (Popoca et al., 2011; Vasoo et al., 2015). Geriatric patients, postoperative patients, HIV sufferers, under-5 children, cancer patients and those suffering from other extraneous complex infections face a manifold of life-threatening infectious assaults due to opportunistic infections by commensal and ubiquitous bacteria; from bacteriemia, enteric infections, respiratory infections, wound infections and much more, where timely administration of the correct treatment can make all the difference between a positive or negative outcome. However, respiratory and diarrhoeal diseases already comprise the highest incidences of disease in the country regardless of immune status (Faiz and Basher, 2011). Open wounds are also regularly faced in health-care. Common etiological antagonists comprising members of Gram-negative, bacilli of the Enterobacteriaceae and Pseudomonadaceae families are regularly reported in clinical settings. Most often, these encounters include members of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, A. baumannii, P. aeruginosa,* and *Enterobacter*) group that are implicated as the major source of hospital-acquired infections, all of which are swiftly developing resistance to multifarious antibiotic agents, including aminoglycosides (Ramirez, Nikolaidis and Tolmasky, 2013). Gram-positive bacteria, *staphylococci* and aerobic, Gram-negative bacteria comprise the mainstay targets by aminoglycoside antibiotics (Garneau-Tsodikova and Labby, 2016, Ramirez and Tolmasky, 2010; Price et al., 1981). And while resistant Gram-positive pathogens have usually been the focus, such as for infections related to methicillin and penicillin resistant *Staphylococcus aureus*, the fast-paced emergence of resistant Gram-negative pathogens have overtaken the research focus as the primary contemporary concern (Vasoo et al., 2015). Semi-synthetic varieties of aminoglycosides, amikacin, netilmicin and dibekacin were designed in the 1970's to allow clinicians to solve increasing resistance towards older varieties such as gentamicin, kanamycin and tobramycin for management of serious infections. As these antibiotics are globally less frequently used, it has been expected that resistance against them would be less pervasive (Gad, Mohamed and Ashour, 2011). However, the discovery of an increasing range of resistance enzymes that imbue resistance to almost all clinically important aminoglycosides has generated a renewed trepidation, requiring more surveillance data and epidemiological studies, especially in developing countries such as Bangladesh where antibiotic usage is least regulated and

resistance reports portray rapid loss of drug efficacies against common pathogens (Islam et al., 2012). Understanding the patterns and profiles of the existing state of bacterial resistance in Bangladesh would thus go a long way in aiding clinicians to mount adequate, timely treatment measures; especially in the case of critically ill patients with immunocompromised status. Although investigations on aminoglycosides occur across the world, there is a sparsity of data on aminoglycoside resistance rates and mechanisms of resistance agents in Bangladesh, especially by employment of molecular characterization. It is for this reason that this study applied the use of PCR-based gene amplifications for genotyping analysis, and also including phylogenetics to aid future predictions of resistance profiles, and susceptible substrates. In order to do this, our study aimed at collecting several species of Gram-negative bacilli from samples ranging from wound swabs, nasal swabs to diarrhoeal stool, as acquired from three of the main hospitals in Dhaka, Dhaka Medical College and Hospital (DMCH); Shaheed Suhrawardy Medical College and Hospital (SSMCH) and the International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) clinic for resistance determination and genotyping analysis of common resistance enzymes, especially those with a proclivity for dispersion via horizontal gene transfer mechanisms.

1.2 ANTIMICROBIAL AGENTS

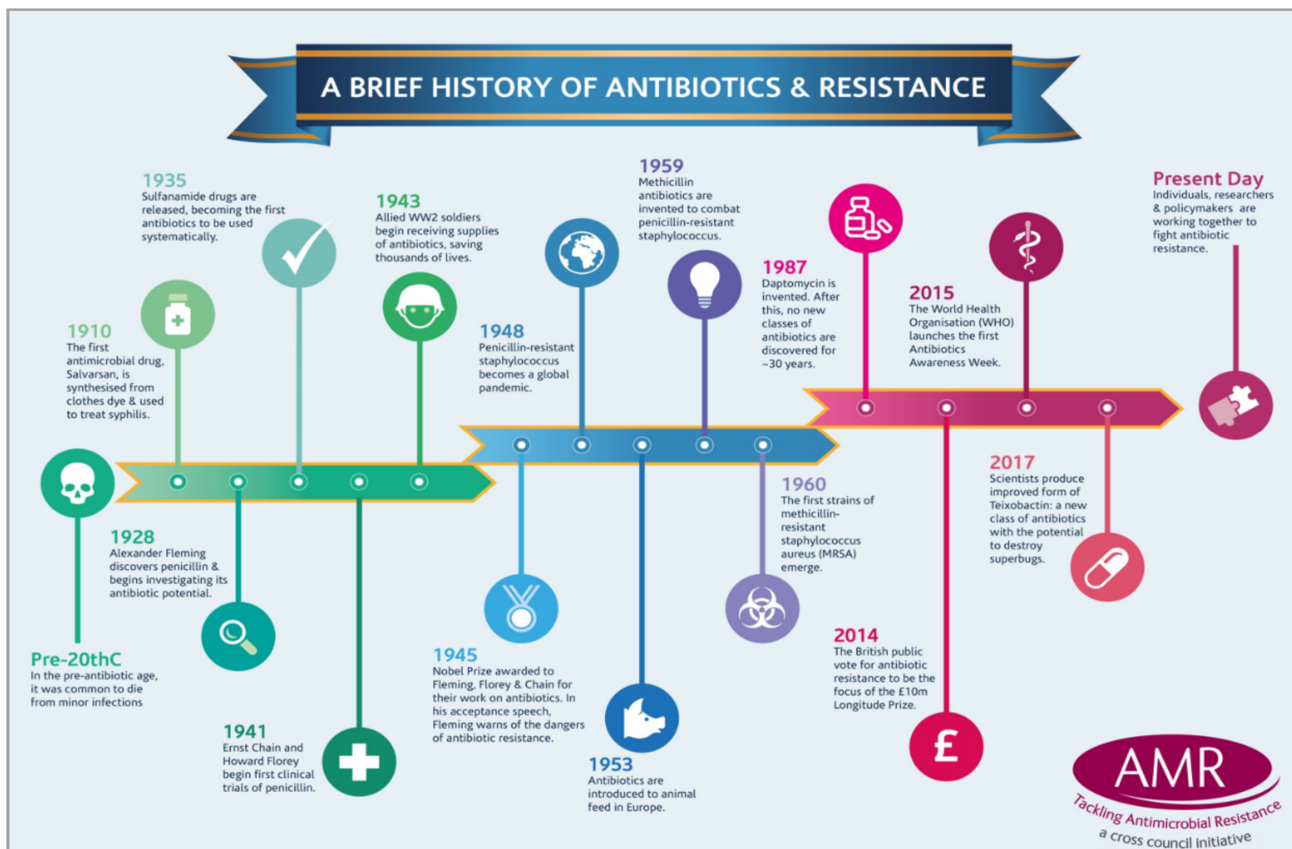

Fig. 1.1 Historical relation between antibiotic use and the development of resistance (Ukri.org, 2018).

1.2.1 A Brief History

Human usages of antimicrobial agents that include antibiotics can be traced much further into the past than most people would consider. Most biologists are privy to the knowledge that the revolutionary "Antibiotic Era" was jump-started by Alexander Fleming and Paul Ehrlich before him. Ehrlich with the help of his team pioneered the very first systemized drug screening program back in 1904 that led to his discovery of the small-molecule that would be marketed as Salvarsan- capable of effectively treating the intractable and rampant Syphilis that plagued his time-period. Rather, Alexander Fleming serendipitously discovered in 1923 the wonder-drug penicillin, where by working with bacterial lawns, he observed how *Penicillium* mold contamination inhibited their growth in the media as observed through visible zones of inhibition. This particular drug would later be extracted and mass-produced thanks to the purification protocol established by Howard Florey's team at Oxford University, thus cementing our reliance on antibiotics in medicine then onwards, as delineated in Figure 1.1 (Aminov, 2010;

Ukri.org, 2018). The work and discovery of both these illustrious scientists paved the way for modern pharmaceuticals to develop programs to screen, develop and mass-produce antibiotics which are ubiquitously used today for every conceivable medical application, from treatment for common infections to use as surgical aides and for cancer treatment (Bengtsson-Palme et al., 2014; Aminov, 2010).

However, it is important to note, evidence pointing to human exposure to antibiotics go back as far as 350-550 CE to the societies of the Sudanese Nubians and Egyptians of the Dakhleh Oasis, whose bones reveal accumulation of tetracycline under fluorescent labelling analysis, suggesting purposeful contact (Aminov, 2010). Other ancient cultures including the Chinese have used drugs such as the qinghaosu, a recently established anti-malarial drug extracted from *Artemisia* plants for thousands of years to treat a number of distinct ailments (Cui and Su, 2009). Therefore, it appears that the actual impact of, and parallel evolution of our relationship with these remarkable compounds transcend commonly assumed timelines.

With the sudden commercial introduction of these “miracle-drugs” in the modern era, starting in the 1940s, it finally became possible to not only combat syphilis with mass effect using penicillin G, but also infant meningitis and pneumococcal pneumonia, while drugs like streptomycin enabled doctors to cure tuberculosis. With their help, surgeries became a much safer option and cancer patients could be protected during the crucial low-leucocyte count phase of chemotherapy enabling better practice of medicine overall (Lee et al., 2011; Bengtsson-Palme et al., 2014).

The golden age of discovery of novel antibiotic classes spanned between the 1950s and 1970s and ended rather abruptly however, after which no new classes have been found. Since this period, the only development strategy regularly employed by pharmaceuticals to counter the constant flux of resistance against antibiotics have been to continuously modify existing structures of familiar compounds (Aminov, 2010).

1.3 ANTIBIOTIC RESISTANCE

1.3.1 General Background

Antibacterial resistance has been predicted since the very beginning of the antibiotic era by Alexander Fleming himself who warned of their unscrupulous use (Aminov, 2010). This phenomenon ties in with the fact that humans have been manipulating antimicrobials for millennia, throughout ancient cultures as discussed previously. However, it was not until more

recent times that widespread resistance has degenerated into a state of international crisis-one that is costing the European Union alone over 1.5 billion Euros every year in health-care expenses as a direct result of multi-drug resistant strain emergences, a staggering 55 billion per annum in the USA, and considerably more on a global scale (Vasoo et al., 2015; Bengtsson-Palme et al., 2014; Aminov, 2010). Over 63,000 patients die every year in the United States as a result of hospital acquired infections, where only 6 species of antibiotic resistant species necessitated additional treatment costs to soar by 1.3 billion USD in 1992, highlighting not just the mortality, but the critical economic burden of the problem (Aminov, 2010). Even in-patient hospitalization times increase, which in the US requires 9.7 additional days of stay (Vasoo et al., 2015).

Antimicrobial resistance is increasingly being observed against virtually all classes of antibiotics in clinical use. For example, resistance to antibiotics have increasingly been observed in community-acquired infections as demonstrated by 3rd generation cephalosporin resistance by both *K. pneumoniae* and *E. coli*, as well as to penicillin G by enterococci (Lee et al., 2011). Additionally, vancomycin resistant enterococci have become a prominent threat, especially as it has often been considered the last line of defense, although it has now materialized that these bacteria show high level resistance against β-lactams, glycopeptides, macrolides and aminoglycosides in recent decades (Emaneini, Amigholi and Aminshahi, 2008). Resistance against essential macrolides such as azithromycin, that are the mainstay treatment against *Shigella spp*., *Salmonella spp*. and infections by other members of the *Enterobacteriaceae*, are also becoming far less reliable as they keep appearing (Gomes et al., 2016). The alarming rates at which resistance is being acquired can be emphasized by the growing frequencies of *M. pneumoniae* in Japan, where resistance strains were surveyed at being at 5.0% in 2003, sharply rising to a startling 89.5% by 2011 due to continued use (Liu et al., 2014). Other resistance genes present in a large number of *Enterobacteriaceae* members including *K. pneumoniae* such as *aac(6')ib-cr* have even been identified that can diffuse not just aminoglycosides but also reduce the effectiveness of quinolones (Tsukamoto et al., 2014; Kocis and Szabo, 2013; Kim et al., 2009). However, the major threat facing mankind is against resistance accumulation in nosocomial settings. Of these agents, the most fearsome encounters are by those pathogens classified as multidrug resistant strains- which are typically defined as bacteria possessing simultaneous resistance to at least three different antimicrobial agent classes (Nie et al., 2014; Lee et al., 2011; Faiz and Basher, 2011). Examples of this include serious Gram-negative pathogens including MDR *A. baumannii* which has recently become resistant to fluoroquinolones, β-lactams, carbapenems and even aminoglycosides (Moniri et al., 2010) and *Burkholderia pseudomallei* reported to be resistant to aminoglycosides, macrolides, β-lactams and polymyxins (Moore et al., 2009).

1.3.2 Origins and Evolution of Antibiotic Resistance

Resistance within pathogenic organisms is not thought to be a result of their clinical evolution as a response to medical administration of antibiotics, but rather one that has its origins rooted deeply within the environment (Becker and Cooper, 2012; Aminov, 2010). Environmental sources such as the soil, aquatic environments including sewage are a cesspool of various resistance genes that are casually disseminated among and within endemic strains via mobile genetic elements such as plasmids, transposons and integrons (Rodriquez, 2016) (Aliakbarzade et al., 2014; Bengtsson-Palme et al., 2014; Nemec et al., 2004). Soil-dwelling bacteria such as *Mycobacterium abscessus* is a prime example as shown in Fig. 1.2, obtained from Luthra, Rominski and Sander, 2018. Resistance can additionally arise through mutations of normal cellular genes induced by antimicrobials or uptake of resistance genes on mobile genetic elements from local habitats and subsequent horizontal gene transfer, for example via plasmid mediated transfer conjugation, transposition or by stimulation of the stress response systems (Rabbee et al., 2016; Shaw et al., 1993). These, often hostile and complex environments teeming with free-living organisms capable of both production of antibiotics and breaking them down have been under intense evolutionary pressures that have generated resistance genes and kept them in positive selection. There is still a considerable lack of understanding as to why these genes evolve within such environments with hypotheses' ranging from a need for innate protection for producer organisms, to attaining competitive advantage against other microbes (Becker and Cooper, 2012). However, much of the evidence points to unknown functions due to the low concentrations often generated by organisms within a meta-community that suggests non-lethal, possibly communicative functional roles (Aminov, 2010; Clardy, Fischbach and Currie, 2009). Somehow commonly encountered pathogens in clinical settings must have come into contact with members within these reservoirs thus empowering them with the forms of resistance observed (Bengtsson-Palme et al., 2014). Other studies on resistance origins suggest mutations of normally occurring cellular genes, or the over-expression of normally latent genes as underlying factors (Shaw et al., 1993).

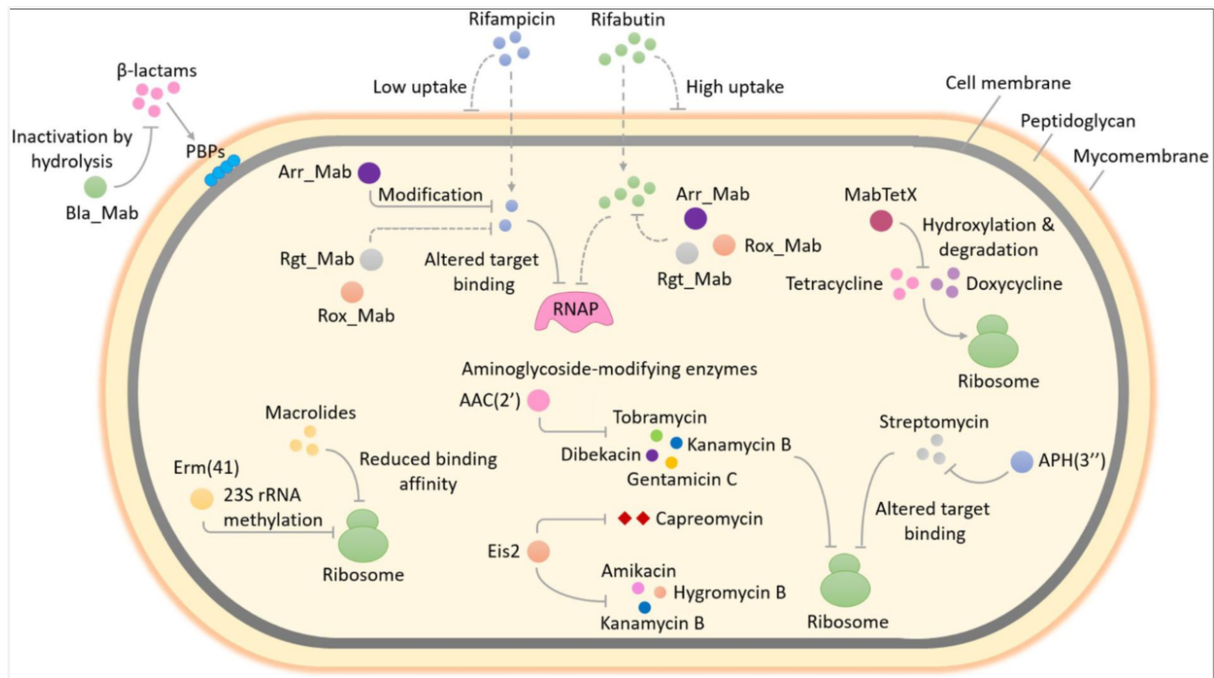

Figure 1.2. Enzyme mediated Multi-Drug Resistance in soil-dwelling pathogen.
An illustration of various mechanisms of enzyme mediated resistance capabilities against multiple drug classes including macrolides, aminoglycosides, tetracyclines and β -lactams in the saprophytic, soil-dwelling bacterium, *Mycobacterium abscessus*. This bacterium is also a common Gram-positive clinical pathogen.

This natural resistance gene reservoir has an innate free-flowing nature, transcending natural ecosystems as it casually interacts with normal commensals or even pathogens within human and animal microbiota. A key example of this is the recent emergence of resistance against synthetic derivatives of quinolones, as acquired through aquatic bacteria carrying the *qnr* genes shuttled via horizontal gene transfer vehicles such as plasmids (Aminov, 2010). That being said, the frequencies of antibiotic resistance dissemination under clinical settings appear to be directly correlated with human activities (Teixeira et al., 2016; Rabbee et al., 2016; Rodriquez, 2016; Faiz and Basher, 2011). While environmental reservoirs explain the existence of these genes, their exaggerated dispersal and selection has been linked to the many complex socioeconomic factors that result in misregulation and overuse of antibiotics which bacteria are quickly able to adapt resistance towards. These include low quality of manufacture, the lack of regulation in production and dissemination, truncated therapy, inequitable access to effective medication (Rabbee et al., 2016; Faiz and Basher, 2011).

1.4 AMINOGLYCOSIDES

1.4.1 An Introduction

Aminoglycosides are a major class of broad-spectrum antibiotics prominent in medical settings due to their efficacy in antimicrobial therapy, particularly against serious aerobic Gram-negative bacterial infections that are particularly difficult to treat, occurring in nosocomial settings (Garneau-Tsodikova and Labby, 2016; Gad, Mohamed and Ashour, 2011; Lee et al., 2011; Becker and Cooper, 2012). This is in spite of the development of newer fluoroquinolone and β-lactam antibiotics. Moreover, for multidrug-resistant strains such as MDR *Mycobacterium tuberculosis* infections, they are on reserve as a crucial auxiliary therapeutic option (Garneau-Tsodikova and Labby, 2016; Aliakbarzade et al., 2014). To obtain synergistic bactericidal activity, they are also often prescribed in combination with cell-wall synthesis inhibiting antibiotics such as β-lactams and penicillins (Nie et al., 2014; Soleimani et al., 2014; Hermann, 2007; Schmitz et al., 1999; Gad, Mohamed and Ashour, 2011).

The very first aminoglycosides were derived from *Micromonospora* and *Streptomyces* species of soil-inhabiting bacteria (Becker and Cooper, 2012; Schatz, Bugie and Waksman, 1944). It was initially isolated from *Streptomyces griseus* in 1943 for the treatment of tuberculosis, and by the mid 1940s was widely available for commercial distribution in clinics. Soon after, development of newer drugs of this class (including gentamicin, tobramycin, neomycin and sisomicin) staved off development of noticeable drug resistance levels, and were found to be highly effective against most Gram-negative infections- including those of *Pseudomonas spp.* as well as certain Gram-positive bacterial infections, such as by *staphylococcus spp.* (Garneau-Tsodikova and Labby, 2016; Schmitz et al., 1999; Becker and Cooper, 2012; Schmitz et al., 1999). However, in only a matter of time, widespread adoption within medical settings finally revealed significant accumulation of resistance, boding ill-news for the advancement of aminoglycosides; especially with the increasing observation of nephrotoxicity and ototoxicity in patients (toxicities of the kidneys and inner ear, respectively) which has as a result, also stifled their widespread clinical use in earlier years (Hansberg et al., 2012; Becker and Cooper, 2012; Gad, Mohamed and Ashour, 2011; Hermann, 2007; Galimand, Courvalin and Lambert, 2003). This led to the maturity of second generation synthetic derivatives that included amikacin, arbekacin, netilmicin, dibekacin and isepamicin during the 1970s after which no further viable iterations have been released in the market (Becker and Cooper, 2012).

Aminoglycosides are characteristically bactericidal, in a concentration dependant manner ie. Their potency increases with increasingly larger doses. Additionally, due to their irreversible binding to their 30S ribosomal RNA target they also include a post-antibiotic effect which makes

them highly potent effectors. As antibiotics, aminoglycosides display a two-step mode of action: beginning with uptake into the bacteria past the cell wall and membrane, followed by binding to ribosomes to inhibit proper protein synthesis (Aliakbarzade et al., 2014). Although members of this antibiotic class are more frequently used as an alternative option when MDR strains are encountered such as in cases of Gram-negative *Acinetobacter baumannii* infections opportunistically taking hold in immunocompromised patients (Aliakbarzade et al., 2014; Lee et al., 2011; Moniri et al., 2010; Nemec et al., 2004; Seward, Lambert and Towner, 1998); resistance against them is suddenly becoming increasingly ubiquitous and spreading rapidly, thus posing a major contemporary public health concern regardless of national borders (Hu et al., 2013; Lee et al., 2011).

1.4.2 Clinical Significance

In the 1980's, the popular use of aminoglycosides as the primary therapeutic agent against cases of bacterial sepsis were quickly being substituted by fluoroquinolones, carbapenems and cephalosporins. This was because of these other antibiotic classes were proven to be less toxic and demonstrated a much broader range of spectral activity against pathogens (Hansberg et al., 2012). As toxicity is primarily due to a renal cortex uptake with a specific threshold, transiently elevated concentrations of the antibiotic increases bactericidal activity without increasing toxicity. As such one-time a day dosage is preferable. Furthermore, their irreversible binding ensures sustained activity even as the peak concentration levels off. And as their positive charges mean poorer oral absorption, aminoglycoside administration requires parenteral injection (Hermann, 2007; Galimand, Courvalin and Lambert, 2003).

Aminoglycosides however, have remained an important part of the treatment regimen for hospital-acquired infections and especially as an alternative therapy for infections due to MDR strains (Nemec et al., 2004). Aminoglycosides possess a broad antibacterial spectrum, with staunch activity against a breadth of bacteria spanning *Acinetobacter spp*., *Enterobacteriaceae*, *Pseudomonas* and *staphylococci* (Teixeira et al., 2016; Garneau-Tsodikova and Labby, 2016; Hansberg et al., 2012; Hermann, 2007; Morita, Tomida and Kawamura, 2012). Traditionally, aminoglycosides are the drug of choice for particular infections for diseases including brucellosis, tuberculosis, tularemia, plague and endocarditis as a result of enterococci (Ramirez, Nikolaidis and Tolmasky, 2013). In general, Tobramycin is particularly effective against *P. aeruginosa* infections, while Amikacin is a more prudent therapeutic approach towards infections by *M. tuberculosis* and *E. coli* strains with observed resistance against ciprofloxacin,

cefotaxime, piperacillin-tazobactam antibiotics, especially when tobramycin or gentamicin are deemed ineffective (Hansberg et al., 2012).

Prominently used against prolific pathogens like *Acinetobacter baumannii* which is the number one risk factor against immunocompromised patients in intensive care unit (ICU) wards due to their capacity to survive on dry surfaces for over four months, aminoglycosides are still rigorously used for such serious bacterial infections (Aliakbarzade et al., 2014; Lee et al., 2011; Moniri et al., 2010; Nemec et al., 2004; Seward, Lambert and Towner, 1998). When it comes to Streptococcal, Enterococcal and even *Acinetobacter* species however, they exhibit only suboptimal effectiveness due to the intrinsic low-grade resistance against this class of drugs as a result of their cell wall structure properties. This can be overcome by using the synergistic effect achieved through combining β-lactam antibiotics alongside the intended aminoglycoside (Hansberg et al., 2012), such as for the treatment of viridans streptococci and *Enterococcus faecalis* infections. The effectiveness of this particular approach arises from the mechanism of β-lactam activity ie. They break down bacterial cell walls, thus allowing for greater penetration of aminoglycosides to their intended target inside the cell's nucleus (Hermann, 2007). This strategy is used in our modern era for cases such as bacterial sepsis or septic shock, where it provides a potent effect against a broader range of Gram-negative pathogens as well as a more rapid therapeutic outcome (Hansberg et al., 2012; Herman, 2007). This is observed in instances of late onset neonatal sepsis due to coagulase-negative staphylococci where they are administered in concert with β-lactamase-stable penicillin (Klingenberg et al., 2004; Schmitz et al., 1999). It should be noted, that for illness pertaining to *Burkholderia cepacia*, *Stenotrophomonas maltophilia* or *Pasteurella multocida* infections, the intrinsic activity of aminoglycosides are insufficient for therapy. For anaerobic bacteria as a whole, this antibiotic class is once again deemed inadequate due to their decreased effect at low pH and the requirement of an oxygen-rich aerobic milieu for membrane permeance mediated by membrane-bound electron chain transporters (Ramirez, Nikolaidis and Tolmasky, 2013; Hansberg et al., 2012; Herman, 2007; Galimand, Courvalin and Lambert, 2003).

More recently, aminoglycosides have been considered for the treatment of Leishmaniasis parasitic infections, fungal infections and also a plethora of genetic diseases that are a result of prematurely terminating codons that include Rett's syndrome, Duchenne muscular dystrophy (DMD) and Cystic fibrosis (CF) (Garneau-Tsodikova and Labby, 2016; Ramirez, Nikolaidis and Tolmasky, 2013; Hermann, 2007). In the case of CF and DMM, up to 10% of suffers are affected by nonsense mutations while with other diseases like mucopolysaccharidosis type I (MPS I) possess as much as 70% of such mutations, that may be treated using decoding-site binding aminoglycosides. These drugs can induce read-through over stop codons in eukaryotic cells that

in statistically large cell populations may be able to demonstrate phenotypical repair as shown in CFTR affected cell cultures using gentamicin and geneticin, especially in the case of autosomal recessive disorders (Hermann, 2007). Moreover, aminoglycosides have even been speculated for use in the treatment of AIDS, as they have been observed to act as inhibitors to the replication of the HIV virus (Ramirez, Nikolaidis and Tolmasky, 2013).

1.4.3 Chemical Structures

Aminoglycosides fall into the category of polycationic molecules, allowing them to interact with the negatively charges of nucleic acids in ribosomal rRNA molecules (Galimand, Courvalin and Lambert, 2003). Not all aminoglycosides found in nature have antimicrobial properties, however, among the ones that do, they can be systematically categorized on the basis of their structural architecture. This chemical blueprint is directly associated to their propensity for susceptibility towards aminoglycoside modifying enzymes (AMEs) which are ubiquitously employed as resistance agents by numerous prokaryotes (Becker and Cooper, 2012). In simplicity, this observation implies that the vulnerability of specific aminoglycosides towards bacteria harboring these enzymes is directly correlated with the chemical structure of these compounds. And although there are several classes of these molecules, the three main sub-classifications that are germane in clinical applications have similar structures, with some variations among them, as in Figure 1.3 from Garneau-Tsodikova and Labby, 2016.

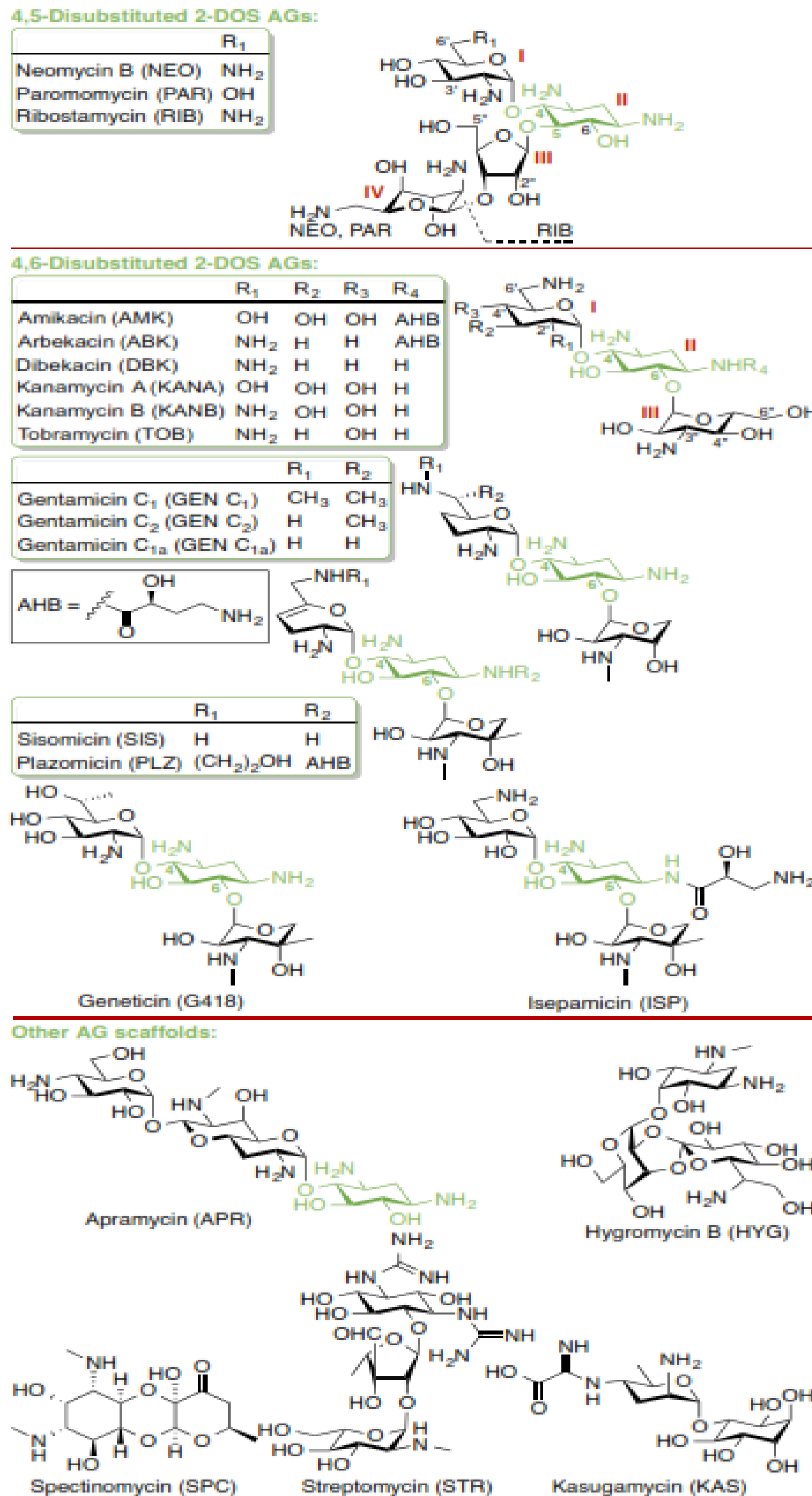

Fig. 1.3. Structures of Aminoglycosides.
Basic structure of some common aminoglycoside antibiotics, according to their chemical compositions, chemical groups, positions and general scaffolding (highlighted in green) as described in this section.

In any case, their efforts to disrupt protein translation via binding to the 30S subunit of ribosomal RNA are facilitated by the many free hydroxyl groups (Becker and Cooper, 2012), usually at least two aminosugars linked by glycosidic bonds as well as other free substituents in

certain cases. Naturally derived aminoglycoside antibiotics are characterized as possessing a non-sugar, 2-deoxystreptamine (2-DOS) scaffold as the key pharmacophore which connects to two or more aminosugars at the 4-, 5 and 6- positions, which can be described as the first class. This paromamine derived moiety is essential for correct docking of the molecules to the RNA target and includes the likes of gentamicins, neomycins and kanamycins (Becker and Cooper, 2012; Garneau-Tsodikova and Labby, 2016; Hermann, 2007; Galimand, Courvalin and Lambert, 2003). Kamaycins are made up of 4,6-substituted 2-DOS derivatives which contain 2-amino or 2,6-diamino-glucose as the B ring while ring C is usually a 3-amino-glucose. Gentamycins also contain the 4,6-substituted 2-DOS as well as two hexose sugars with some additional side-chains or an unsaturated B ring. Neomycins have up to two hexoses containing the amine groups as well as one furanose in a 4,5 substituted conformation on the 2-DOS core (Galimand, Courvalin and Lambert, 2003). The most important of these are the 4,5-(neomycin B) and 4,6-(amikacin, tobramycin and gentamicin) disubstituted 2-DOS derivatives (Hermann, 2007; Galimand, Courvalin and Lambert, 2003), the latter of which are particularly effective agents against most serious Gram-negative borne infections (Hermann, 2007).
The least common variety of aminoglycosides of which there are but a select few in existence (as exemplified by streptomycin), are composite of a single disaccharide unit connected to a guanidinylated streptamine at the 4-position, and make up the second class. Both streptomycin and spectinomycin possess the non-sugar, streptidine scaffold instead of the 2-DOS derivatives, as characterized by a hydroxyl group at the 2- position. And while most aminoglycosides carry the 2-DOS ring as the core moiety (Piepersberg et al., 2007); apramycin, which has applications in veterinary medicine, represents the stand-alone third-class of aminoglycosides with a 4-monosubstituted 2-DOS nucleus, and contains a bicyclic pyrano-pyranose sugar moiety. The 2-DOS derivative aminoglycosides, streptidine aminoglycosides as well as streptomycin are all collectively being referred to as aminocyclitols (Hermann, 2007).

Although a significant proportion of aminoglycosides and synthetic varieties used clinically or are paromamine derived, non-paromamine derived aminoglycoside antibiotics include apramycins, hygromycins and also various pseudodisaccharides. The first two retain the 2-DOS core unit with substitutions appearing at the 4- position for apramycins and 5- for hygromycins. Other than spectinomycin, which much like the others have an extra hexose at the 4- or 5-position and an inositol unit (which in this case is an actinamine), the various pseudodisaccharides have no actual application in human medicine (Becker and Cooper, 2012).

1.4.4 Mechanisms of Action

Our understanding of the general mechanisms evolved by the aminoglycoside antibiotics come from the 4,5- and 4,6-distributed 2-DOS derivatives and their binding to bacterial ribosomes that control mRNA translation which ultimately leads to the manufacture of defective proteins. In normal functioning cells, the fidelity of protein translation accuracy in which the messenger RNA (mRNA) code is deciphered into peptide products by additive linking of amino acids, is hampered at a rate of only $1.4x10^{-4}$ per nucleotide in standard *E.coli* models (Becker and Cooper, 2012). Aminoglycosides essentially disrupt this highly accurate natural process by generating dysfunctional or truncated protein products, thus throwing the essential biophysical processes of translation and translocation of the host cell in disarray (Garneau-Tsodikova and Labby, 2016; Ramirez and Tolmasky, 2010; Galimand, Courvalin and Lambert, 2003; Becker and Cooper, 2012; Hu et al., 2013). Two key factors determine this process: i.) The codon-anticodon recognition between mRNA and transfer RNA (tRNA) stem loop and ii.) Recognition by the decoding site of the ribosomal Adenine residues that determine a tight fit (Becker and Cooper, 2012).

The effectiveness of these drugs rely upon their binding to an internal loop within the highly conserved 16S rRNA portion within the 30S subunit which functions as the ribosomal decoding site, containing three unpaired adenine residues acting as sensors (Ramirez and Tolmasky, 2010; Galimand, Courvalin and Lambert, 2003; Becker and Cooper, 2012; Hermann, 2007). This occurs at the Acceptor (A) site of the 30S rRNA in which the A1408 position Adenine occurs on one strand while the other two, A1492 and A1493 Adenines occur on the complementary strand and are flanked by a couple of G-C pairs in prokaryotic cells, as shown in Figure 1.4 below, taken from Hermann, 2007 (Becker and Cooper, 2012; Hermann, 2007; Galimand, Courvalin and Lambert, 2003).

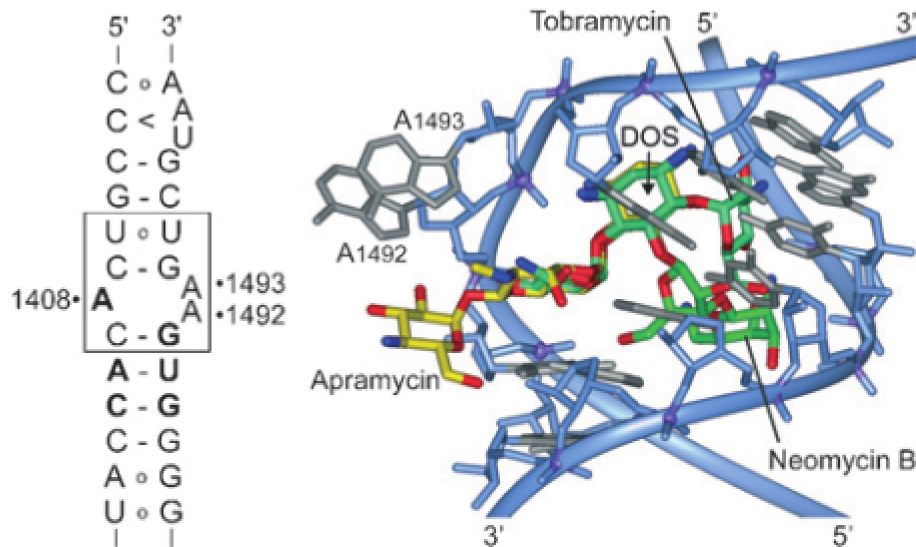

Fig. 1.4. 16S rRNA Decoding centre.
The secondary structure of the decoding site within the 16S ribosomal RNA where the aminoglycoside binding site is marked by a box, and the flexible Adenine residues are clearly labelled (Left). The superpositioning of three aminoglycoside antibiotics in complex with this region is shown by their crystal structure, underscoring the essential nature of the 2-DOS scaffold for docking (Right).

It should be noted at this point, that the ribosomal binding site of aminoglycosides is differentiated in eukaryotes by a Guanosine where in prokaryotes it is an Adenosine at p1408 (*E. coli* numbering) (Garneau-Tsodikova and Labby, 2016, Hermann, 2007; Galimand, Courvalin and Lambert, 2003). Additionally, an Adenosine also exists at position 1491 in eukaryotic rRNA, and it is these two key residual differences between prokaryotic and eukaryotic rRNAs which enables use towards human therapeutic applications (Hermann, 2007). These nuances (highlighted in Figure 1.5 below), along with the fact that eukaryotic cells are recalcitrant to permeance of any molecules positively charged at physiological pH disallow aminoglycoside uptake, whereas bacterial systems allow entry of these drugs as mediated by membrane-bound electron transporters of the respiratory chain via a proton motive force (PMF) (Galimand, Courvalin and Lambert, 2003).

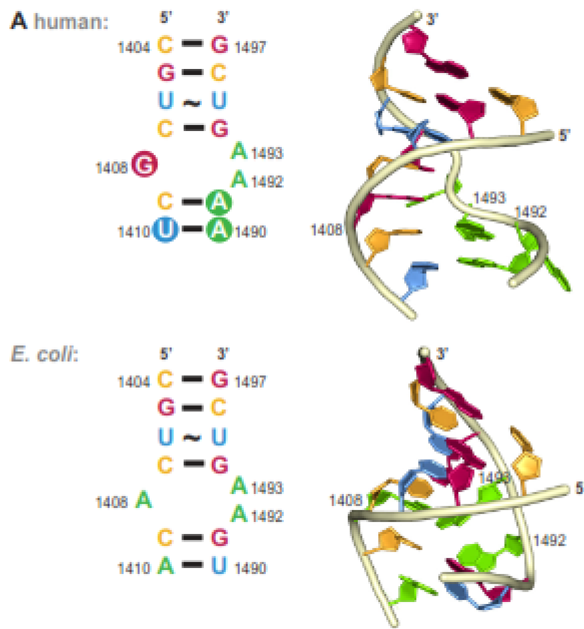

Fig. 1.5. Differences between Human and *E. coli* Ribosomal A sites.

Binary conformation states of the A site demarcate the events that lead up to precise docking of cognate or complementary tRNAs for each codon which determines a specific amino-acid attachment. In the first stage, the A site on the 30S rRNA is in its dynamic, resting, or "off" state, and the docking of a cognate tRNA follows a rapid initial identification step where a major rearrangement in the A site flips out the A1492 and A1493 residues within the internal loop. The second, slower step involves several further conformational changes that ensure a tight, precise fit for the tRNA within the A-site, which is the decoding or "on" state. How, aminoglycosides manipulate this process is by the way in which they bind to the A site, inducing the flipping of the essential A1492 and A1493 residues in the internal loop that mimics the "on" state which allows for non-cognate tRNAs to be misrecognized as valid tRNAs allowing continued translation despite the obvious errors (Becker and Cooper, 2012; Hermann, 2007; Galimand, Courvalin and Lambert, 2003). Essentially, aminoglycosides reduce the energy barrier between the conformational states of the sensor amino acid residues through the process of preparing the decoding centre for accommodating mRNA-tRNA hybridisation, allowing non-valid tRNAs to tether on (Hermann, 2007). This disruption of accurate mRNA reading by rRNA generates faulty or in some instances truncated protein products (Becker and Cooper, 2012; Hermann, 2007; Galimand, Courvalin and Lambert, 2003).

The specific cause of cell death resulting from the use of aminoglycoside antibiotics however, is not necessarily simply tied into the attenuation of mRNA reading fidelity as is often described in literature, but is postulated through several other possible scenarios: Either via destabilization

of the host cell integrity due to assimilation of misinterpreted protein products into the cellular membrane, formation of superoxides and toxic hydroxyl radicals disrupting cellular respiration and metabolism, or via deregulation of Cpx envelope stress-response and redox-responsive Arc systems that are responsible for maintaining membrane constitution and integrity (Becker and Cooper, 2012).

Not all aminoglycoside substrates bind to the same sites on the 16S rRNA (Ramirez, Nikolaidis and Tolmasky, 2013). While most aminoglycosides carry the 2-DOS nucleus with conserved interactions with the rRNA A site, each aminoglycoside antibiotic has their own distinct dynamic binding interaction and this often determines their individual potencies and effective use. For instance, both streptomycin and Hygromycin B bind close to the decoding centre at specific sites of the 16S rRNA of bacterial ribosomes but in the case of the latter, it binds in a region that overlaps with the decoding site loop using nucleotides that are conserved in both eukaryotes and bacteria thus precluding its use as a selective agent. Spectinomycin with its tricylical structure on the other hand targets a “head” site on the 16S rRNA that is distal from the binding sites of other aminoglycosides. As a result, compared to other 4,5- and 4,6- disubstituted-2-DOS aminoglycosides; spectinomycin, hygromycin B and streptomycin differ in their mechanism of action (Hermann, 2007).

1.5 ENZYMATIC RESISTANCE

1.5.1 Enzyme Mediated Aminoglycoside Resistance

Numerous options are availed by bacterial systems to limit or completely evade the effect of aminoglycoside antibiotics which include: innate permeability reduction via membrane charges of either the outer or inner membranes; limitation of inner membrane transport; active efflux pump-based export; sequestering; mutations or methylation of the 16S ribosomal rRNA target leading to reduced affinity; extracellular shielding of DNA in biofilm structures and last but not least, enzymatic modifications of either the drug or the drug target (Garneau-Tsodikova and Labby, 2016; Teixeira et al., 2016; Liang et al., 2015; Ramirez, Nikolaidis and Tolmasky, 2013 ;Liang et al., 2015; Kocis and Szabo, 2013; Galimand, Courvalin and Lambert, 2003; Gad, Mohamed and Ashour, 2011; MacLeod et al., 2000). Four of the major mechanisms are graphically defined in Figure 1.6 (Garneau-Tsodikova and Labby, 2016).

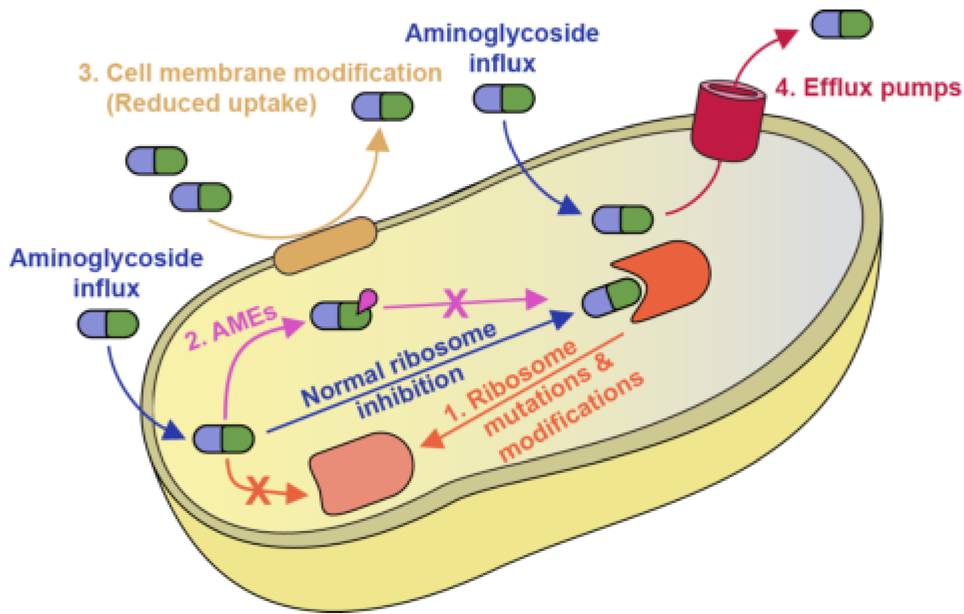

Fig. 1.6. Mechanisms of aminoglycoside resistance availed by bacterial cells.

Although several methods may be utilised, the major mechanism of resistance is reported to be enzyme mediated, particularly via deactivation of aminoglycoside antibiotics by AMEs encoded on mobile genetic elements (Emaneini, Aligholi and Aminshahi, 2008; El Badawy et al., 2017; Moniri et al., 2010; Gad, Mohamed and Ashour, 2011; Lee et al., 2011). Global frequencies of aminoglycoside resistance are increasing at an alarming rate, especially for important clinical pathogens such as *P. aeruginosa* and methicillin-resistant *Staphylococcus epidermidis* and *Staphylococcus aureus*, among other Gram-negative bacteria (Hansberg et al., 2012; Ida et al., 2001; Schmitz et al., 1999). For treatment of hospital-acquired infections caused by *Pseudomonas aeruginosa* the aminoglycosides gentamicin, amikacin and tobramycin have been most regularly used. To enhance the desired bactericidal effect in such cases, treatment involves a combination of various antimicrobials which rather than reduce, has instead increased the overall levels of resistance against them, as a result of resistance enzymes becoming more frequent (Teixeira et al., 2016; Morita, Tomida and Kawamura, 2012; Poonsuk, Tribuddharat and Chuanchuen, 2013). In fact, inactivation of aminoglycosides as a result of enzyme mediated modifications is the most prevalent mechanism of resistance in Gram-negative bacilli (Teixeira et al., 2016). This usually occurs through modification of the hydroxyl or amino groups of aminoglycoside antibiotics, thus reducing affinity for the intended target region of the 16S rRNA binding site by either Gram-negative or Gram-positive bacteria, inhibiting their phase II energy-dependant activation imbuing resistance (Gad, Mohamed and Ashour, 2011; Ramirez and Tolmasky, 2010; Schmitz et al., 1999).

The majority of these are aminoglycoside modifying enzymes which are classed into three categories based on their specific mode of chemical modification on an aminoglycoside substrate: AACs- Aminoglycoside *N*-acetyltransferase, APHs- Aminoglycoside *O*-phosphotransferases and ANTs- Aminoglycoside *O*-nucleotidyl transferases which will be further elucidated upon below (Garneau-Tsodikova and Labby, 2016; Nie et al., 2014; Poonsuk, Tribuddharat and Chuanchuen, 2013; Nemec et al., 2004; Schmitz et al., 1999; Galimand, Courvalin and Lambert, 2003; Schmitz et al., 1999; Seward, Lambert and Towner, 1998). There have been over 100 variations identified and characterised thus far and it is believed their functional origins like in normal cellular metabolic pathways which have diverted their functions via evolution towards acting on aminoglycosides, probably as a result of direct exposure (Garneau-Tsodikova and Labby, 2016; Ho et al., 2010; Kim et al., 2012). This phenomenon is illustrated by the phylogenetic relationship represented in Fig 1.7 from Shaw et al., 1993, clearly showing a relationship between all such AMEs with many other (including non-AME) types of acetyltransferases commonly found in cells, as well as non acetylating AMEs. Another, distinct group of enzymes that also confer resistance, act via methylation of the 16S A-site ribosome target- these are known as 16S rRNA methyltransferases (RMTases). This special class of enzymes are endemically produced by *actinomycetes* and by some Gram-negative bacilli in order to protect their own ribosomes from antibiotic inhibition, which they are able to do using *S*-adenosyl-L-methionine (SAM) as a co-substrate (Galimand, Courvalin and Lambert, 2003; Garneau-Tsodikova and Labby, 2016; Galimand, Courvalin and Lambert, 2003).

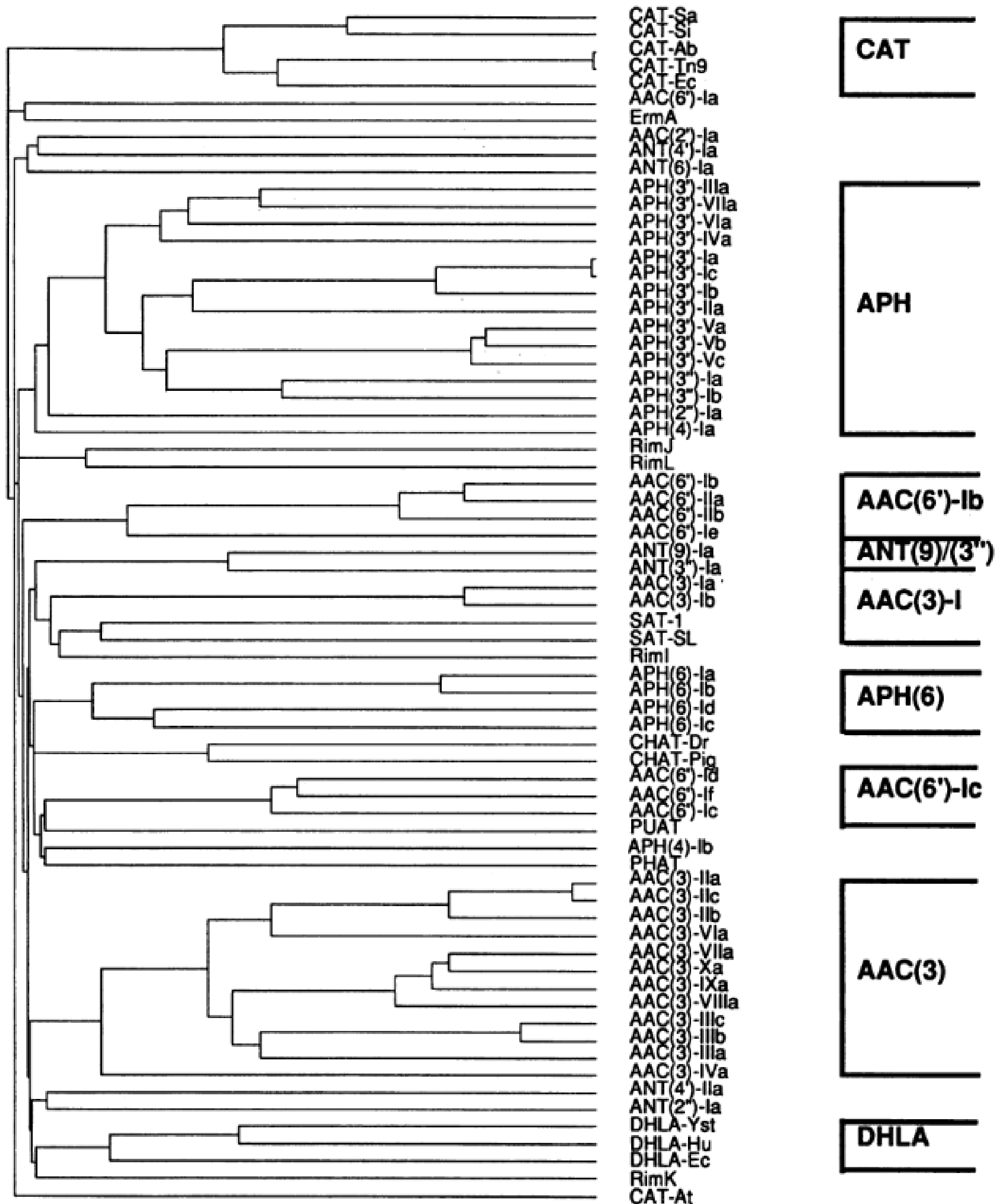

Fig 1.7. Relationship between aminoglycoside modifying enzymes and other acetyltransferases. The abbreviations in the dendogram are as follows: DHLA (dihydrolipoamide acetyltransferase), CAT (chloramphenicol acetyltransferase), AAC (aminoglycoside acetyltransferase), APH (aminoglycoside phosphotransferase), ANT (aminoglycoside adenyltransferase), SAT (Streptothricin acetyltransferase), Erm (Erythromycin methyltransferase) and Rim (acetylates 30S ribosomal subunit), CHAT (choline acetyltransferase).

1.5.2 Horizontal Gene Transfer

Aminoglycosides have long been used in both traditional treatment of hospitalized patients but also as an alternative therapy used against multi-drug-resistant strains. However, in clinical settings an undeniable fact of life has become that resistance against these drugs is becoming more and more common around the world. Cross-infection has largely been attributed to this rapid increase (Hansberg et al., 2012; Lee et al., 2011; Nemac et al., 2004; Weinstein et al., 1980; Seward, Lambert and Towner, 1998). The primary arsenal employed against them by Gram-negative resistant bacteria has been the various aminoglycoside modifying enzymes which inactivates these chemical agents, rendering them ineffective. AMEs as well as genes for RMTases are both known to be predominantly found on mobile genetic elements (although may also exist chromosomally) (Kim et al., 2012; Schmitz et al., 1999) such as plasmids, integrons, transposons as well as other transposable elements, allowing for rapid inter- and intra-species dissemination (Figure 1.8) (Garneau-Tsodikova and Labby, 2016; Bengtsson-Palme et al., 2014; Chiou et al., 2014; Ramirez and Tolmasky, 2010; Nemec et al., 2004; Ida et al., 2001; Shaw et al., 1993; Seward, Lambert and Towner, 1998).

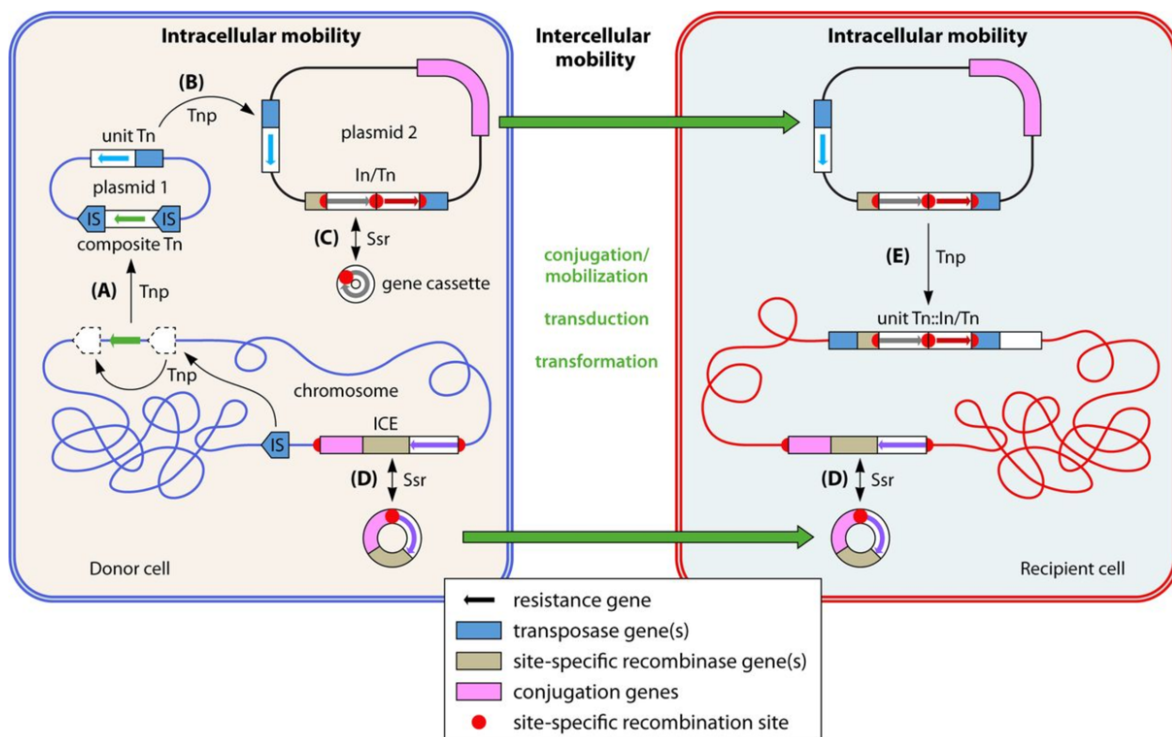

Fig. 1.8. Mechanism of mobile genetic element-mediated gene transfers.
Such elements are implicated in horizontal transfer of resistance genes via elements that include transposons and conjugative plasmids (Patridge et al., 2018).

Moreover, it appears additional resistance determinants may also be picked up along the way, exacerbated by possible co-selection of various resistance gene types on the same mobile

genetic element via co-conjugation events and occurrences of genomic islands, for example as seen for fluoroquinolone antibiotics enabling transformations into MDR strains (Chiou et al., 2014; Bengtsson-Palme et al., 2014; Ramirez and Tolmasky, 2010). This has allowed for MDR strains to develop through rapid accumulation of resistance elements and create multiple resistance loci. These can circulate across vast geographic locations, and for particularly dominant varieties, to take over as the dominant infectious strains (Klemm and Dougan, 2016). Emaneini and colleagues in 2008 has reported the increasing levels of resistance to aminoglycosides via AME possession in enterococci which also carry other genetic determinants for resistance against other antibiotic classes. *P.aeruginosa* also represents an important archetype of dangerous human pathogens capable of pan-resistance to aminoglycoside antibiotics by harbouring numerous AME classes on mobile elements, removing the limitations of individual substrate-specificity of each enzyme (Morita, Tomida and Kawamura, 2012; Poole, 2005). Genetic exchanges of such genes mediated by mobile genetic elements can occur either via intra or inter taxa dissemination, with positive selection favoured by the perpetual use of these antimicrobials in and out of clinical settings, although originally these are sourced from the environmental ecology and persist in anthropological-derived cesspools such as pharmaceutical and chemical manufacturing plant discharge sites (Garneau-Tsodikova and Labby, 2016; Teixeira et al., 2016; Bengtsson-Palme et al., 2014; Clardy, Fischbach and Currie, 2009).

1.5.3 Aminoglycoside Resistance Gene Nomenclature and Classification

Chemical modification of aminoglycosides via AMEs remain the most prolific method of resistance by bacteria (Garneau-Tsodikova and Labby, 2016; Liang et al., 2015). Aminoglycoside resistance gene nomenclature can be classified according to the type of enzymatic modification employed, the site of modification, unique resistance profiles and unique protein designations in order. The type of enzymatic modification activity can be either acetyltransferase (Aac), phosphotransferase (Aph) or nucleotidyltransferase/adenylyltransferase (Ant). The site of modification is denoted as (1), (3), (6), (9), (2’), (3’), (4’), (6’), (2”) or (3”). Each unique profile is identified through roman numerals such as I, II, III, IV, V and onwards whereas each unique protein is labeled using lowercase English alphabets such as a, b, c, d, etc (Becker and Cooper, 2012; Ramirez and Tolmasky, 2010; Shaw et al., 1993). As an example, *aac(3’)-Ia* is an enzyme gene that modifies an aminoglycoside antibiotic at the 3’ hydroxyl group position via transfer of an acetyl group, having a unique resistance profile against several drugs denoted by the “I” and is a protein identified uniquely as “a”, and should not be confused by another enzyme of a similar name such as *aac(3’)-Ib* as coding the same protein, although they may be of the same

class, possess the same site of modification and the same substrate specificities. Although the characterization delineated above will be used throughout this study, it is important to note that a second nomenclature standard also exists, which operates by assigning type of activity via a three letter, italicized code (eg. *aac*, *aad* or *aph*) followed by a capitalized letter that denotes the specific modification site and finally a number at the end to identify each unique gene. These systems are used interchangeably in literature as there is no common consensus (Becker and Copper, 2012; Ramirez and Tolmasky, 2010).

Regarding RMTases, these possess a less defined nomenclature system but are classified into two specific families according to whether they methylate at the N7 position of G1405, or the N1 position of A1408 nucleotides respectively. The first family which includes ArmA, RmtA, RmtB, RmtC, RmtD1, RmtD2 RmtE, RmtF, RmtG and RmtH grant resistance against the 4,6-disubstituted 2-DOS aminoglycosides (Kanamycin A, amikacin, tobramycin and gentamicin) but has no effect against any of the 4,5-disubstituted 2-DOS aminoglycosides such as neomycin, nor against apramycin. The second family of RMTases include NpmA and enable resistance to both 4,5- and 4,6-disubstituted 2-DOS species and apramycin (Garneau-Tsodikova and Labby, 2016).

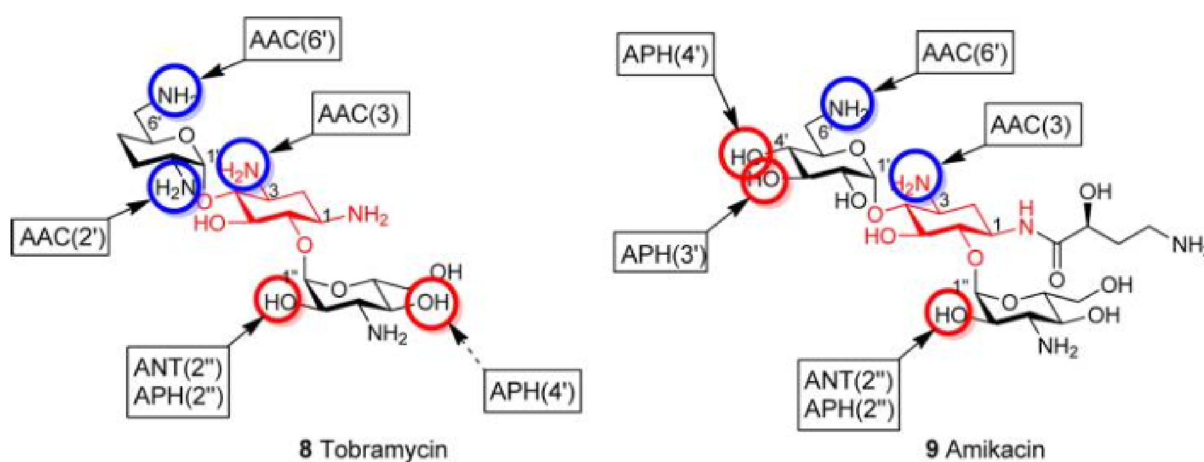

Fig. 1.9. Aminoglycoside modification sites.
Sites of modification on two typical aminoglycoside antibiotics: Tobramycin on the left and Amikacin on the right by various AAC, APH and ANT sub-groups.

1.5.3.1 Aminoglycoside *N*-Acetyltransferases (Aacs)

Aminoglycoside acetyltransferases are aptly named due to their ability to attach acetyl groups to the amino groups within aminoglycoside structures. Collectively, these enzymes fall into the GCN5-related *N*-acetyl transferases (GNAT) protein superfamily that includes over 10,000 proteins from a gamut of different organisms, all catalyzing primary amine group acetylation using acetyl CoA donor substrate (Ramirez, Nikolaidis and Tolmasky, 2013; Kocis and Szabo, 2013; Becker and Cooper, 2012). Modification by acetylation can occur at one of four possible

amino groups: 1, 2’, 3 and 6’ positions thus possess the potential for deactivating the entire range of clinically seminal antibiotics including amikacin, netilmicin, tobramycin and gentamicin (Poole, 2015; Shaw et al., 1993). These four classes can be further sub-categorized accordingly. AAC(1) is the only class to have no subclasses; AAC(2’) possesses only one subclass, namely AAC(2’)-I; AAC(3) has a total of 9 subclasses while AAC(6’) has two, AAC(6’)-I and AAC(6’)-II (Becker and Cooper, 2012; Shaw et al., 1993).

AAC(1) is the least nefarious variety, with a weak potential for for antibiotic inhibition, and as such are rarely observed in clinical isolates (Becker and Cooper, 2012). This class is characterized by resistance against paromomycin, ribostamycin, lividomycin and apramycin (Shaw et al., 1993). Members of the AAC(2’) group however, imparts resistance to numerous important aminoglycosides spanning the classes of neomycins, gentamycins and kanamycins, being found most often within Gram-negative bacteria as well as *Mycobacterium* isolates. AAC(3) species are currently only found in Gram-negative bacteria and widespread among *Enterobacteriaceae*, with the most clinically major subclasses being AAC(3)-II enzymes which are associated with numerous bacterial genera (Becker and Cooper, 2012; Ramirez and Tolmasky, 2010; Shaw et al., 1993). Among the remaining eight subclasses, four are rarely observed while the remaining members comprising AAC(3)-VII to X are only found in actinomycete species. AAC(6’) members comprise the most widespread class of these enzymes, spanning both Gram-negative and Gram-positive bacteria; thus making them the most common of all AMEs. Moreover, as the 6’ amino group holds a major role in rRNA binding, modification of this group leads to a sweeping resistance to a large category of medically useful antibiotics Not all aminoglycoside substrates bind to the same sites on the 16S rRNA (Ramirez, Nikolaidis and Tolmasky, 2013; Becker and Cooper, 2012). The AAC(6’) family endows resistance to kanamycin, tobramycin, netilmicin, and depending on whether it is composite of the I or II subfamily, they can also modify amikacin or gentamicin, respectively (Poole, 2005). More dangerously, AAC(6’) proteins can also exist as fusion proteins such as AAC(3’)-Ii/AAC(6’)-IId or AAC(6’)-Ie/APH(2”)-Ia bifunctional enzymes which can increase the breadth of resistance conferred by possessing a single enzyme in the organisms which harbour them (Becker and Cooper, 2012; Schmitz et al., 1999; Shaw et al., 1993). The modificational deactivation of aminoglycosides by AAC(2’), AAC(3) and AAC(6’) classes are illustrated above (Fig 1.9) using the example of tobramycin and amikacin aminoglycosides.

1.5.3.2 Aminoglycoside *O*-Phosphotransferases (Aphs)

Aminoglycoside phosphotransferases inactivate aminoglycosides by phosphorylating the hydroxyl (OH) group of the targeted antibiotics (Ramirez and Tolmasky, 2010; Poole, 2005). This modification affects the ability of the aminoglycoside drug to bind to the ribosomal A-site, due to the negative charge introduced into the molecule. These enzymes are stratified into classes and subclasses accordingly as: APH(2")-I to IV, APH(3')-I, APH(3")-I, APH(4)-I, APH(6)-I, APH(7")-I and APH(9)-I (Becker and Cooper, 2012; Ramirez and Tolmasky, 2010; Shaw et al., 1993).

APH(2")- enzymes are nefarious in their ability to provide resistance against gentamicin in Gram-positive bacteria, and can also exhibit broad-spectrum activity against other aminoglycosides in the bifunctional form produced as AAC(6')-Ie/APH(2")-Ia. APH(3') enzymes collectively consist of the largest family within the class, phosphorylating at the 3'-OH of the B ring in numerous aminoglycosides, being found across the spectrum of Gram-negative bacteria. Interestingly, *aph(3')-I* and *aph(3')-II* genes are also utilized as cloning vectors and vehicles. APH(3") enzymes on the other hand are less important clinically, as they only provide resistance against streptomycin (Becker and Cooper, 2012). The APH(4) class are also less clinically relevant as they only inhibit the action of hygromycin (Becker and Cooper, 2012; Shaw et al., 1993). The activities of APH(2"), APH(3") and APH(4) are exemplified by their activity on the model aminoglycosides tobramycin and amikacin in Figure 1.9, above. APH(6) is important in two respects, one being its activity against streptomycin, but also as a component of the Tn5 transposon, as relevant in molecular genetics applications. APH(7") and APH(9) enzymes are less clinically important as they only confer resistance to hygromycin B and spectinomycin, respectively. APH genes are commonly carried in transposons, integrons and also R plasmids of MDR strains. This is particularly problematic for infections caused by enterococcal and staphylococcal infections (Becker and Cooper, 2012; Ramirez and Tolmasky, 2010).

1.5.3.3 Aminoglycoside *O*-Nucleotidyltransferase (Ants)

Aminoglycoside nucleotidyltransferases modify aminoglycosides by attachment of an adenosine monophosphate (AMP) nucleotide group from an ATP molecule onto a hydroxyl group. As the smallest family of AMES, they include ANT(2"), ANT(3"), ANT(4') with subclasses I and II, ANT(6) and ANT(9) (Becker and Cooper, 2012; Ramirez and Tolmasky, 2010; Shaw et al., 1993).

The ANT(2") class is immensely important as it confers resistance to amikacin, tobramycin and gentamicin. The ANT(2")-Ia gene is particularly well distributed among non-fermentative Gram-negative bacilli and enterobacteria occurring within plasmids and transposons, and conferring

resistance to a wide range of kanamycin class aminoglycosides (Becker and Cooper, 2012). Its activity is used as an example above in Figure 1.9 to demonstrate the various sites of modifications accessible to AME classes. ANT(3”) is the class that is most commonly reported however, endowing resistance against both streptomycin and spectinomycin. ANT(3”) genes are casually found ubiquitously in all Gram-negative bacteria as gene cassettes, plasmids, transposons and integrons and can even exist as fusion genes, enhancing their resistance range (Becker and Cooper, 2012; Ramirez and Tolmasky, 2010; Shaw et al., 1993). ANT(4’) is special as it can in certain instances modify the 4”- position as well. The ANT(4’)-I subclass is disseminated within plasmids of *Enterococci*, *Bacillus* species and *Staphylococci* (Becker and Cooper, 2012; Schmitz et al., 1999; Shaw et al., 1993) while the ANT(4’)-II subclass is distributed among several Gram-negative species. ANT(6) confers resistance to streptomycin and exists more commonly in Gram-positive species. The final class, ANT(9) is found only in some *Enterococci* and *Staphylococci* and modifies spectinomycin (Becker and Cooper, 2012; Shaw et al., 1993).

1.5.3.4 Ribosomal RNA (rRNA) Methyltransferases (RMTases)

Methylation is a mechanism often used in prokaryotes to regulate processes that include transcription, transposition, replication, and mismatch repair (Becker and Cooper, 2012). Direct methylation of prokaryotic ribosomal RNA instead of aminoglycoside antibiotics themselves, by bacterial enzymes is one of the most effective and efficient methods of resistance. This modification essentially reduces binding efficacy of the drugs leading to high level resistance to aminoglycosides such as amikacin, kanamycin and arbekacin (Liang et al., 2015). This relatively new evolutionary mechanism of protection at even lower copy numbers is able to modify every copy of the 16S rRNA molecule, imparting much higher levels of resistance to aminoglycoside antibiotics currently used in therapy, with the single exception of streptomycin (Galimand, Courvalin and Lambert, 2003). Naturally produced by *actinomycetes* group of bacteria, the natural function of RMTases involves a form of self-defense against the action of aminoglycosides they prolifically produce themselves (Garneau-Tsodikova and Labby, 2016). Their mode of action involves methylation of amino acids into their corresponding 7-methyl derivatives within the A site target of the 16S ribosomal RNA where aminoglycoside antibiotics bind, by using the *S*-adenosyl-L-methionine (SAM) co-substrate- this joint association is shown by the crystal structure-donor substrate combinations of RMTases in Figure 1.10 (Garneau-Tsodikova and Labby, 2016; Becker and Cooper, 2012; Galimand, Courvalin and Lambert, 2003). These ribosomal methyltransferases have the propensity for mass dissemination, and have currently been found in species ranging the likes of *Escherichia coli, Klebsiella pneumoniae,*

Pseudomonas aeruginosa, A. baumannii, Serratia marcescens and *Proteus mirabilis* (Becker and Cooper, 2012). These RTMases are often carried in plasmids and can be easily acquired by other bacterial species via HGT, and are officially distinguished by two distinct families (Garneau-Tsodikova and Labby, 2016; Becker and Cooper, 2012; Hu et al., 2013). If the methylation occurs at the N7 portion of the G1405 nucleotide they fall into the first class which includes RmtA, RmtB, RmtC, RmtD1, RmtD2, RmtE, RmtF, RmtG, RmtH and ArmA. These enzymes can only act on 4,6-disubstituted 2-DOS aminoglycosides such as kanamycin, tobramycin, gentamicin and amikacin, but not 4,5-disubstituted varieties. In the case of the second class, the methylation instead occurs on the N1 position of the A1408 residue, which includes NpmA. NpmA is a particularly special enzyme as it confers resistance to 4,5- and 4,6- disubstituted varieties to include neomycin and apramycin. As such, it should be noted that neither of these classes can confer resistance to non A-site binding aminoglycosides such as spectinomycin and streptomycin. Genes encoding 16S rRNA methyltransferase enzymes such as *armA* and *rmtB* are therefore capable of offering protection. As ArmA and RmtB which both occur from the first family serve as the prevailing RMTases, spreading wide into the world particularly into human pathogens and bacteria found in livestock, these have been the focus of this study (Garneau-Tsodikova and Labby, 2016; Hu et al., 2013). While both ArmA and RmtB, like other ribosomal methyltransferases provide a almost pan-resistance to most aminoglycosides; it has currently been hypothesized that as ArmA contains a modification to the G1405 residue, it has an inhibiting propensity specifically towards 4,6-linked 2-DOS varieties of aminoglycosides (Galimand, Courvalin and Lambert, 2003).

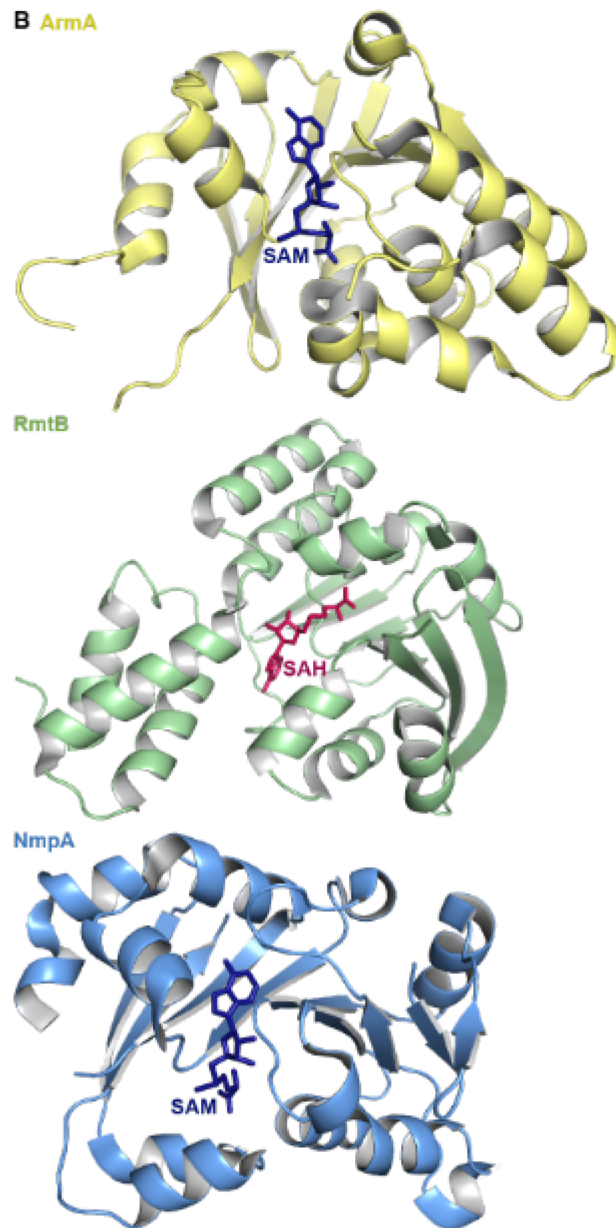

Fig. 1.10. Crystal structures of the RMTases and donor molecules.
ArmA, RmtB and NmpA are shown, along with their modifying group donor molecules, SAM; SAH and SAM, respectively.

1.5.3.5 Extended Spectrum Beta-Lactamases (ESBLs)

Members of the *Enterobacteriaceae* family are nefarious harbourers of extended spectrum beta (β) lactamase (ESBL) resistance genes which include among them *CTX-M*, *TEM-1*, *TEM-2*, *SHV*, *NDM-1* and many more (Aliakbarzade et al., 2014; Kocis and Szabo, 2013; Arpin et al., 2003). They account for a class of group A β-lactamase drugs capable of hydrolysing first to third generation cephalosporins. These genes are notorious for spreading through plasmid based transmission and therefore compromise treatment options, especially considering they may

persist even in discharged patients (Arpin et al., 2003; Aliakbarzade et al., 2014). Moreover, many resistance genes for other antibiotics including aminoglycosides, specifically as per the *aac(6')-Ib* gene which disseminates as mediated through plasmids, may also co-inactivate β-lactamases (Kim et al., 2009) The use of broad-spectrum antimicrobials increases their selective persistence as they are able to interact with the natural gut flora, expounding the problem as they disperse into the wider community (Rodriguez, 2016; Arpin et al., 2003). This threat must be accounted for when discussing aminoglycoside resistance considering the majority of serious infections require dualistic administration for optimal effects, having become common practice (Liang et al., 2015; Aliakbarzade et al., 2014). Moreover, many RMTases are often observed occuring in *Enterobacteriaceae* strains containing the nefarious New Delhi β-lactamase (NDM) conferring additional pan-resistance to aminoglycosides (Hansberg et al., 2012).

1.6 GRAM-NEGATIVE BACTERIAL PATHOGENS

1.6.1 General Epidemiology

Gram-negative organisms, characterized by their inability to retain the Gram-stain are often the most notorious of all pathogenic bacteria. Members of the Enterobacteriaceae and *Pseudomonadaceae* families have been particularly relevant clinical pathogens, and often must be differentiated by their distinctive biochemical characteristics, as defined in Figure 1.11.

The largest cause of childhood mortality is due to acute respiratory illnesses (Qadri et al., 2005), which according to the WHO cause between 1.9 and 2.2 million childhood deaths worldwide (Bhuyan et al., 2017). These deaths are prolifically attributed to opportunistic hospital-acquired pathogens such as *K. pneumoniae*, which is responsible for cases of pneumonia in addition to pyogenic liver abscess as well as urinary tract infections (Chung, 2016) as well as *Haemophilus influenzae* among other non-Gram negative bacteria, viruses and fungi (Bhuiyan et al., 2017). In respect to the etiology of respiratory infections, particularly of the lower respiratory tract such as in nosocomial pneumoniae, Gram-negative, aerobic bacilli such as *Escherichia coli*, *Enterobacter* and *Pseudomonas aeruginosa* species among several others are also regularly reported as primary antagonists (Teixeira et al., 2016; Qadri et al., 2005; Weinstein et al., 2005). Moreover, certain prolific Gram-negative pathogens such as diarrheagenic *E. coli* forms, *Shigella spp*. and *V. cholerae* make up the major etiological antagonists for enteric infections, causing acute infectious diarrhoea, which in developing countries serves as the second largest mortality burden in children, accounting for a total of 1/5th of total deaths. Among the six diarrheagenic *E.* coli variations, ETEC sub-species

dominates in the developing states as the primary infectious agent responsible for mild to acute diarrhoea, and prominently, Traveller's diarrhoea which also affects visitors arriving from the Western world among other international territories (Qadri et al., 2005; Youssef et al., 2000).

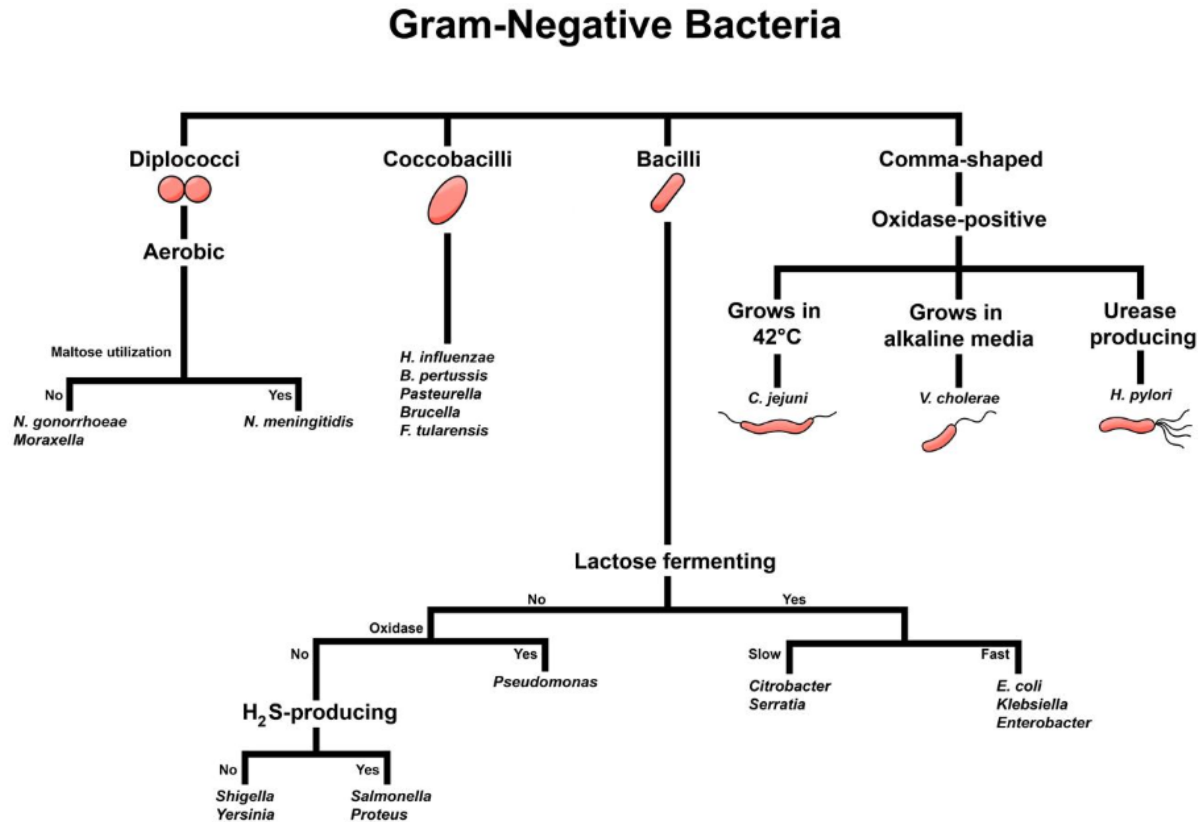

Fig. 1.11 Overview of Gram-negative bacteria and their defining biochemical attributes (Dominguez, 2018).

A National Nosocomial Infection Survey by Weinstein et al. reported in 2005 the prominence of 6 species in ICU related infections comprising pneumonia, bloodstream infections, surgical site infections and urinary tract infections over a period of 18 years. According to the findings of this study, the most prevalent Gram-negative isolates are *E. coli*, *K. pneumoniae*, *Enterobacter spp.*, *P. aeruginosa*, *Acinetobacter spp.* and *Serratia marcescens*. Urinary tract infections are regularly caused by *E. coli* and *P. aeruginosa* but also *A. baumannii* and *K. pneumoniae*. In these same settings, surgical site infections are frequented by *Acinetobacter* species, but also *Enterobacter* species and *E. coli*, while incidences of bloodstream infections are populated most frequently by *E. coli* (Weinstein et al., 2005). Moreover, these species can rapidly acquire resistance to antibiotics and disseminate world-wide. As such, Gram-negative resistant pathogens are a current global phenomenon and can become rapidly ubiquitous as epitomized by the sporadic case of carbapenamese-resistant *K. pneumoniae* from a single point of origin, illustrated in Fig.

1.12 by McKenna, 2013. In acquisition of novel resistance determinants, their infections become an increasing challenge for clinicians and patients alike- marking the importance of surveillance and epidemiological studies.

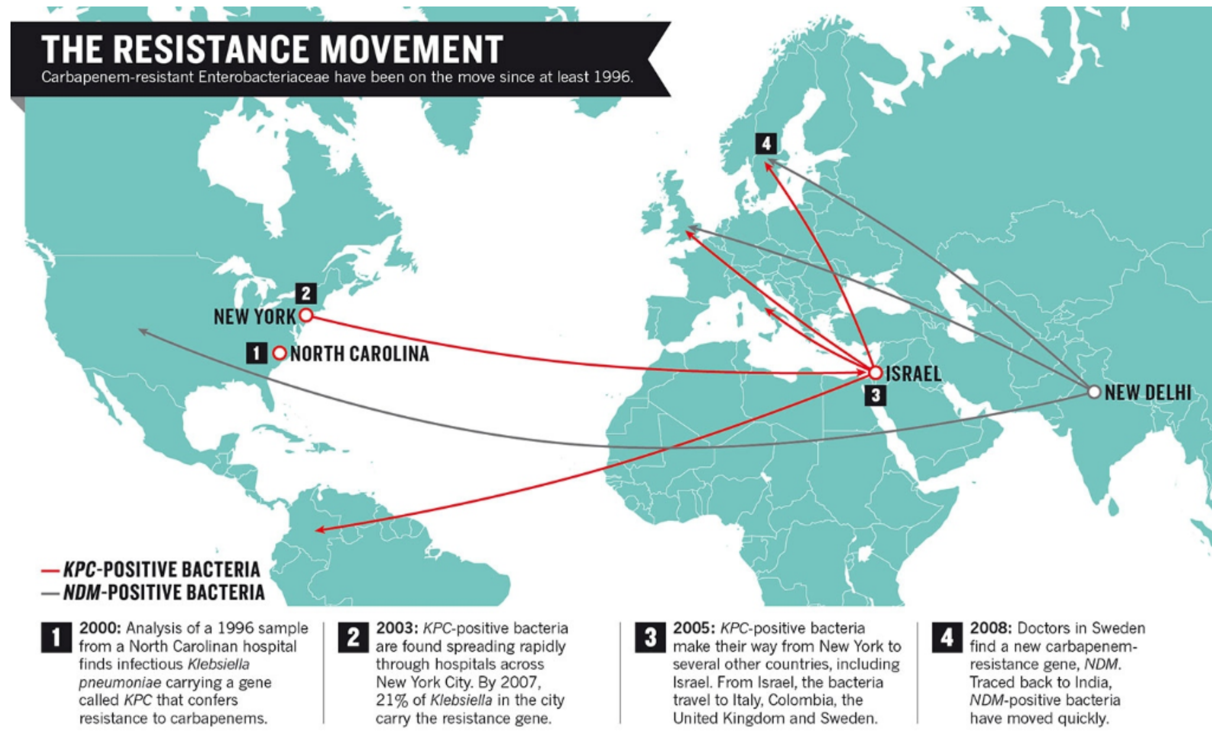

Fig. 1.12. Epidemiology of carbapenem resistant Enterobacteriaceae during 2000-2008.

1.6.2 Enterobacteriaceae

The defining observable characteristics for *Enterobacteriaceae* are a bacillus shape, length of approximately 1-3 µm and Gram-negative status. Other biochemical differentiations between species are elaborated in Figure 1.11. Physiologically, they are facultative aerobes having natural hosts in the form of humans and animals wherein they reside primarily in the intestines; fulfilling their niche as largely benign, commensal microflora comprising the likes of *Escherichia coli*, *P. agglomerans, Enterobacter spp.*, *Klebsiella spp.*, *Providentia spp.*, *Proteus spp.*, *Serratia spp.*, *and Morganella spp.*. However, this is not the case for all members of this family, as many including *Yersinia spp.*, *Shigella spp.* and *Salmonella spp.* are in fact, notable obligate pathogens in humans. These species are capable of causing infections of the respiratory tract, urinary tract, and bloodstream as well as in wounds as opportunistic pathogens in nosocomial settings where patients present immunocompromised states (Thenmozhi et al., 2014; Kocis and Szabo, 2013; Büyükcama et al., 2018; Dele´toile et al., 2009). Other species such as *E. coli*, *K. pneumoniae* and *Proteus spp*. have also been implicated as causative pathogens of such infections (Akhi et al.,

2016; Rabbe et al., 2016). More specifically, this family of bacteria account for one-third of all cases of ICU related urinary tract infections, pneumonia, and about 10-15% of bloodstream infections (Thenmozhi et al., 2014). Normally these must be treated using various antibiotics such as β-lactams, fluoroquinolones and also aminoglycosides (Kocis and Szabo, 2013). However, they are prone to carrying defense mechanisms against this chemical arsenal, with many species including *E. coli*, *Klebsiella* and *Enterobacter* species generating β-lactamase enzymes against penicillins and 1st to 3rd generation cephalosporins and recently, even extended spectrum cephalosporins. Moreover they are frequently being seen to harbour genes that combat quinolones, cotrimoxazole, ESBLs and even most aminoglycosides, often as MDR organisms (Thenmozhi et al., 2014). Members of the *Enterobacteriaceae* family are most commonly encountered in in the form of outbreaks within Intensive Care Units of hospitals making them particularly interesting from a clinical standpoint (Arpin et al., 2013).

1.6.3 Pseudomonadaceae

Pseudomonadaceae are a family of Gram-negative bacteria that include the likes of *Pseudomonas spp*. and *Aeromonas species*. The genus *Aeromonas* and *Pseudomonas*, in particular the species *pseudomonas aeruginosa* are reportedly well documented infectious agents. *Aeromonas* species are described as being Gram-negative, bacillus shaped, facultative anaerobic, non-spore forming bacteria (Igbinosa et al., 2012). Like the Enterobacteriaceae, they must be differentiated by various other biochemical and physical attributes. *Aeromonas* species are prolific pathogens in fish, humans and many other warm and cold blooded animals and are naturally endemic to aquatic environments. Although *A. aquariorum*, *A. veronii* and *A. caviae* have clinical presentations in gastroenteritis, pneumonia, wound and soft tissue infections in countries like Australia, Taiwan and Malaysia; they are also capable of forming beneficial, commensal associations in a number of animals (Joerg Graf, 2015; Igbinosa et al., 2012). Pertinently, they are causative agents in gastrointestinal infections causing loose stools in children, the elderly and immunocompromised individuals, though they can also induce short-term severe diarrhea, particularly observed as Traveler's diarrhea. Moreover, in case of traumatic injuries occuring in aquatic environments, they can cause wound infections and cellulitis. Aeromonads have also been implicated as causing septicemia in underlying conditions that include urinary tract infections, wound infections, peritonitis, endocarditis, meningitis, cirrhosis, cancers like leukemia and more (Igbinosa et al., 2012). *Pseudomonas aeruginosa* happens to be the prevailing etiological agent for severe hospital-acquired infections (Morita, Tomida and Kawamura, 2012; Emaneini, Amigholi and Aminshahi, 2008; Espararragon et al., 1999;

Livermore, 2002). This is especially problematic due to respiratory infections in Cystic Fibrosis patients, where aerosol-based aminoglycoside treatment is required for adequate penetration into the lungs, although the bacteria is also known to be active in wound infections (Poonsuk, Tribuddharat and Chuanchuen, 2013; MacLeod et al., 2000; Morita, Tomida and Kawamura, 2012; Weinstein et al., 1980). It has been repeatedly reported to express high level resistance against fluoroquinolones, β-lactams and even aminoglycosides carrying resistance elements against these drugs both within plasmids and chromosomally- which constitute the common treatment regimen against associated infections (Vasoo et al., 2015) (Morita, Tomida and Kawamura, 2012; Emaneini, Amigholi and Aminshahi, 2008; Espararragon *et al.*, 1999; Livermore, 2002). It is said that even strains susceptible to antibiotics possess a formidable arsenal of defences such as OprF porins and MexAB-OprM efflux-pumps that exclude multiple classes of antibiotics ranging from tetracyclines, sulfonamides, macrolides, fluoroquinolones, β-lactams, macrolides and even some dyes and detergents, as well as an inducible AmpC β-lactamases (Livermore, 2002).

1.6.4 Typical Pathologies & Epidemiology of Enterobacteriaceae & Pseudomonadaceae Species

1.6.4.1 ETEC and Other Pathogens of Diarrhoeal Diseases

For diarrhoeal diseases the occurrence is routinely due to enteric pathogens, not limited to bacterial species. For example in developing countries like Jordan, Rotavirus is the main antagonist; however, *E. coli* strains, particularly diarrohenic species, are particularly common. Their frequencies are cited to be 12.8% Enteropathogenic *E. coli* (EPEC), 10.2% Enteroaggregative *E. coli* (EAggEC) and 5.7% ETEC in order of prevalence, according to one study (Youssef et al., 2000). On a global scale, ETEC is undoubtedly the most common cause of bacteria-induced gastrointestinal pathologies as routinely observed in Traveller's diarrhoea. Traveller's diarrhoea is most frequent in North Americans and Europeans visiting developing countries due to contact with contaminated food and water, especially in India (45%), Kenya (51%) and Jamaica (58%). The most menacing quality of ETEC strains is that it is a highly promiscuous and travel-adept species, making it a pathogen of transnational concern; especially considering that it is a particularly difficult sub-species to detect through traditional assays or microbiological methods as compared to other notable diarrhoeal agents such as *Shigella spp.*, rotavirus and *V. cholerae*.

1.6.4.2 *Klebsiella Pneumoniae* and *Pantoea Agglomerans* in Respiratory Infections

Klebsiella pneumoniae comprises the second most frequent bacterial pathogen implicated in acute respiratory infections (ARI). Consistently implicated in respiratory disorders regardless of geographic or ethnic boundaries, MDR forms of *K. pneumoniae* is particularly disconcerting due to the presence of hypervirulent strains which due to their production of a thick, polysaccharide capsule, can evade a range of host defence systems and enable formation of communal biofilms with other bacterial species. This attributes to its prolonged survival and enhanced dispersal of resistance elements, making their infections especially difficult to treat. Emerging clones have already been observed in Spain that are resistant to ESBLs, carbapenems, quinolones and aminoglycosides (Chung, 2016).

While *K. pneumoniae* poses a constant looming threat, *Pantoea agglomerans* is more often associated as an opportunistic pathogen in complex respiratory infections of immunocompromised patients such as those suffering from tuberculosis due to *Mycobacterium tuberculosis*, pneumonia due to *Pseudomonas aeruginosa* and human immunodeficiency virus (HIV). This Gram-negative bacilli, formerly known as *Enterobacter agglomerans,* is ubiquitously found in environmental habitats such as soil and water, but also in a host of living species spanning plants, animals and even human beings (Dele´toile et al., 2009; Dutkiewicz et al., 2016; Büyükcama et al., 2018). It is medically seminal particularly as it has a proclivity towards the lungs of an immunocompromised host for growth, and is regularly associated in co-infections (Pococa et al., 2011). Less frequently, it may cause human infection after being pierced by a piece of colonised plant material, or indirectly elicit a hyperimmune allergic response due to its specific antigens; although, the majority of pathological cases occur in hospital settings, often as a result of contact with contaminated intravenous fluids, catheters or other material, having been isolated from a wide range of clinical samples (Büyükcama et al., 2018; Dutkiewicz et al., 2016).

1.6.4.3 *Aeromonas* and *Pseudomonas* Species in Wound Infections

Infections due to *Aeromonas* species are by far the least reported and are infrequent in humans, particularly as it is endemic to aquatic environments and are better known as pathogens of fish (Rutteman et al., 2017; Joerg Graf, 2015; Valley et al., 2004). As such infection onset requires exposure to contaminated water or food or by handling of fish but more often as a result of contact with lacerations, abrasions or any wounds. Infections due to these species occur most frequently in warmer climates, and can include acute or chronic gastrointestinal illnesses or septicaemia in patients with attenuated immune systems. Pathogenicity of these species is attributed to the production numerous extracellular toxins that can lead to rapid infection

progression and possible sequelae, especially in the case of immunocompromised patients (Igbinosa et al., 2012; Vally et al., 2004). Standard wound infection treatments are directed towards typical skin-colonising bacteria such as *Staphylococcus aureus* or *Streptococcus pyogenes*, which require amoxicillin-clavulanic acid administration (Rutteman et al., 2017). *A. hydrophila* is most commonly observed in this regard, and is a dangerous concern considering related-wounds are recalcitrant to traditional empirical antibiotic treatments (Rutteman et al., 2017; Vally et al., 2004). They have been observed to have acquired resistance to a multitude of drug classes including penicillins, carbapenems and colistins (Vally et al., 2004), Moreover, they are intrinsically resistant to β -lactam based classes of antimicrobials, as they possess intrinsic, chromosomally encoded, inducible β -lactamase enzymes (Vally et al., 2004; Jones and Wilcox, 1995).

Pseudomonas species on the other hand, in particular *P. aeruginosa,* are ubiquitous in the environment and on medical equipment of hospitals (Nagoba et al., 2013; Vasoo et al., 2015). As such it is also one of the most universal nosocomial infectious agents (Poonsuk, Tribuddharat and Chuanchuen, 2013; Morita, Tomida and Kawamura, 2012; Esparragon et al., 1999), associated with respiratory infections (particularly CF), ear, throat, urine and wounds and soft tissue infections (Kim et al., 2015; Gad, Mohamed and Ashour, 2011; Gad, Mohamed and Ashour, 2011; Faiz and Basher, 2011; MacLeod et al., 2000). As a result, it is also implicated in high mortality rates (Poonsuk, Tribuddharat and Chuanchuen, 2013; Morita, Tomida and Kawamura, 2012). This species can easily penetrate through the skin, and is responsive to the presence of open wounds due to a sensitive quorum sensing capability (Kim et al., 2015). The pathogenic nature of *P. aeruginosa* arises from its opportunistic nature, most widespread around South East Asia, southern Europe and Turkey (Livermore, 2002). The genus is often responsible for infecting traumatised, exposed tissue, as complementary to patients in burn victims and ICUs. One third of all burn victim infections are reported to be directly correlated to *P. aeruginosa* (Nagoba et al., 2013). Aminoglycosides are regularly cited to be a potent form of therapy against them, often in conjunction with β-lactam (Poonsuk, Tribuddharat and Chuanchuen, 2013; Morita, Tomida and Kawamura, 2012).Unfortunately they are also intrinsically resistant to a multitude of antimicrobials agents due to membrane impermeability, and active efflux-pumps such as the MexAB-OprM system; as well as rapid mutational acquisitions and plasmid-mediated resistance genes, all combining to confer resistance to common antiseptics, disinfectants and a range of antibiotics (Nagoba et al., 2013; Poonsuk, Tribuddharat and Chuanchuen, 2013; Morita, Tomida and Kawamura, 2012; Gad, Mohamed and Ashour, 2011; Livermore, 2002; Gad, Mohamed and Ashour, 2011). The efflux-system is the most common means of antibiotic resistance, followed by resistance gene elements (Poonsuk, Tribuddharat and Chuanchuen, 2013; Livermore, 2002). Moreover they also encode β-lactamase elements

similar to Aeromonads and also possess AMEs (Livermore, 2002). So far they have demonstrated resistance towards aminoglycosides, β-lactams, chloramphenicol, fluoroquinolones, macrolides, sulfonamides, tetracyclines and a host of other antimicrobial agents (Vasoo et al., 2015; Livermore, 2002). Their broad-spectrum of resistance makes them particularly difficult to treat (Nagoba et al., 2013).

1.7 CURRENT STATE OF HEALTH CARE & ANTIBIOTIC USE IN BANGLADESH

1.7.1 Usage of Antibiotics

The resistance rates of various antibiotics usually reflect the proportional use of those antibiotics in any given country (Miller et al., 1997). Antibiotics make up the most popular treatment option in Bangladesh for virtually all diseases (Faiz and Basher, 2011), moreover, they are the most prescribed of all drug options for acute watery diarrhoea and respiratory illnesses. In fact, 72.5% of patients are actually treated prior to hospitalization, among which 46.3% were provided antibiotics. Cephalosporins accounted for 31.8% of total antibiotic prescriptions in the south-west of Bangladesh, in particular cefixime, ceftriaxone and cefuroxime for respiratory as well as other illnesses. In Bangladesh, the most commonly prescribed drugs are amoxicillin, ampicillin, cloxacillin, ceftriaxone and gentamicin. (Rashid et al., 2017). Another study found that ceftriaxone, cefixime and amoxicillin as the most commonly prescribed, in order. Cephalosporins account for 55% of the total antibiotics used, demonstrating a particular preference of clinicians towards 3rd generation Cephalosporins (Faiz and Basher, 2011). However, the rise of ESBL producing pathogens are fast-reducing the utility of 3rd gen cephalosporins in Bangladesh, implicated by frequently observed incidences of CTXM-1 and AmpC resistance conferring enzymes, as we have found in a separate study (Rabbe et al., 2016). Furthermore, other than the widespread application of the aminoglycoside gentamicin; amikacin is also administered in various combinations with other drugs, usually of the penicillin or cephalosporin classes (Rashid et al., 2017).

1.7.2 State of Health-Care in Bangladesh as Implicated in Antibiotic Resistance

The underlying problems facing Bangladesh are a host of factors that may be summarised through underuse, overuse, mismanagement, poor availability of affordable drugs with expected qualities, poor lack of accurate information and unethical practices. To develop on this, the following malpractices are notable at various levels of hierarchy. In terms of hospitals and clinics, many if not most are managed inefficiently; lack proper facilities; are understaffed;

maintain poor hygiene and employ unqualified, inexperienced or poorly qualified physicians. They have even been known to prescribe antibiotics for unnecessary illnesses such as for viral fevers (Rashid et al., 2017). Many qualified doctors are also prone to overprescribing of certain antibiotics due to personal preferences (Rashid et al., 2007) or to placate ill-educated customer demands (Rabbe et al., 2017). Other faults lie in the proclivity towards prescribing antibiotics prior to the arrival of empirical evidence, such as blood cultures, microbiological and sensitivity tests. Many clinicians even appear to appeal best-guess estimations for diagnoses, rather than simply wait for diagnostic results to come through (Faiz and Basher, 2011; Rashid et al., 2017).

Aiming at patients next, there appears to be a lack of compliance, tendencies towards self-medication, truncating prescribed therapy dosages and reliance on informal health-practitioners. A large part of this problem stems from poverty, as antibiotics are readily available over the counter, and quite often the pharmacy staff themselves write prescriptions serving as informal-pundits. Doctor visits are bypassed by a large subset of the population due to the cost burden and lack of a positive customer service experience (Faiz and Basher, 2011).

Both the faults by clinicians and patients are intimately tied to pharmaceutical manufacturers who employ questionable marketing tactics (Faiz and Basher, 2011; Rashid et al., 2017). There is a dearth of accessible information towards the public, for which the middle-man that fills the gap are pharmaceutical corporations that are prone to spreading false information with the intention of increasing sales. These unethical practices extend onto physicians who are coaxed into promoting certain antibiotics over others. Unregulated production and manufacture by pharmaceuticals exacerbates the problem. It has been found that 92% of prescriptions are dispensed by pharmaceuticals without any prescriptions (Faiz and Basher, 2011). Moreover, these companies have time and time again, been found to manufacture drugs that are of dubious quality, and do not fulfil the promised dosages inscribed (Rashid et al., 2017; Rabbe et al., 2017; Faiz and Basher, 2011).

Disconcerting, there is no single authorisation body that has been entrusted by physicians or the public that would monitor and governs these practices in order to remedy this systemic failure. Thus, all of these facets collectively contribute towards rapid resistance accumulation and dissemination towards a wide-range of antimicrobials, expediting multidrug resistant emergences, and also compromising patient health-care due increasing side-effect manifestations; whilst also increasing the economic burden due to longer duration of patient hospitalization periods which invariably increases the total cost of treatments (Rabbe et al., 2017).

As an example, a case study for pneumonia exemplifies the underlying problem. The WHO revised a program known as the Integrated Management of Childhood Illness (IMCI) in 2013, to aid health-care practitioners and hospital staff from developing countries to assess, diagnose,

manage and prescribe antibiotic regimens that would mitigate childhood pneumonia, and ensure ease of adherence. This program was remarkably successful where applied, reducing overall child mortality from 2.3 million in 1990 to just 90, 000 by 2015 worldwide. These guidelines were however found to be neglected by several nations according to numerous reports that included Bangladesh among these offenders (Rashid et al., 2017). Faiz and Basher, 2011 had noted that in only 57.1% and 67.8% of cases were appropriate treatments provided for, in cases of pneumonia and diarrhoea, respectively. Moreover, only 26.7% of cases with children younger than 5 years of age with diarrhoea were handled in a manner consistent with WHO recommendations.

1.8 AIMS AND OBJECTIVES OF THE STUDY

The objectives of the study can be summarised as below:

1. Re-evaluation and characterization of the five Gram-negative, bacillus species of 18 bacterial isolates selected for study from 3 hospitals across Bangladesh.
2. Determination of each of their resistance phenotypes against some of the most clinically relevant antibiotics, with a focus on aminoglycoside antibiotics.
3. Investigation of the presence of aminoglycoside modifying and rRNA modifying enzyme genes using a set of seven gene-specific primers by molecular analysis.
4. Construction of phylogenetic analysis using sequenced gene data for inferences on gene dissemination patterns and evolution.
5. Evaluate results in order to suggest pragmatic and up-to-date recommendations for clinicians.

Chapter 2

Materials & Methods

2. MATERIALS & METHODS

2.1 PLACE OF STUDY

The study was conducted at the Institute for Developing Science and Health Initiatives (ideSHi), Infectious Disease Laboratory (2nd floor), Institute for Public Health, Mohakhali.

2.2 ETHICAL APPROVAL

The study was approved by the Ethical Review Committee of Bangladesh Medical Research Council (BMRC). Samples were collected with written consent, obtained from the patients, patients' parents or legal guardians as the eligible participants of the study comprised of children as young as five years of age and below. The risks and benefits had been clearly stated in the consent form, and were unequivocally agreed upon.

2.3 STRAIN COLLECTION, STORAGE AND SELECTION FOR STUDY

A total of 46 bacterial strains were cultured from various patient samples, collected from three hospitals spread out across Dhaka, Bangladesh:

- Dhaka Medical College and Hospital (DMCH)
- Shaheed Suhrawardy Medical College and Hospital (SSMCH)
- International Centre for Diarrhoeal Disease Research, Bangladesh (icddr, b).

The collected specimens were cultured, characterized and preserved in a -70°C freezer in skim-milk, tryptone, glucose, glycerin (STGG) media prior to the start of this study, of which 18 pre-characterized strains were selected for this study. The natures of these samples are fully described in Table 2.1 below.

Table. 2.1. Clinical specimens in study.

Isolated Bacterial Species	Patient Pathology	Sample Origin	Number of Resistance Strains	Number of Sensitive Strains
Enterotoxigenic Escherichia coli	Diarrhoeal Disease	Stool	8	4
Klebsiella pneumonia	Respiratory Illness	Nasal Swab	1	1
Pantoea agglomerans	Respiratory Illness	Nasal Swab	1	1
Pseudomonas spp.	Wound	Wound Swab	1	0
Aeromonas spp.	Wound	Wound Swab	1	0

2.4 MICROBIAL CULTURE

The following isolate species were included in this study: Enterotoxigenic *Escherichia coli* (ETEC), *Pantoea agglomerans, Klebsiella pneumonia*, *Pseudomonas* species and *Aeromonas* species as shown in Table 1 below. Viability and validation of stored strains was confirmed by first thawing the samples and sub-culturing on MacConkey Agar plates (BD Difco™ ; Cat no: 212123) for 24 hours at 37°C, subject to microaerophilic conditions, followed by standard streaking methods using aseptic techniques for subculture. MacConkey agar medium was used as it was specifically developed for the identification of non-fastidious Gram-negative rods which were a focus of this study. The selective media contains crystal violet and bile salts to inhibit Gram-positive bacterial proliferation and contains a pH indicator (Neutral Red) that sways between red and colourless hues depending on which side of pH 6.8 is ambient during growth.

2.4.1 Culture Media Preparation

MacConkey agar media was the general differential media used for preliminary subculture of all strains from their frozen stocks, while Mueller-Hinton agar was used for Antimicrobial Susceptibility Testing (AST).

2.4.1.1 MacConkey Agar Plate Preparation

MacConkey agar media is a differential media that only selects for Gram-negative bacteria isolation and culture, while also allowing for lactose fermenting colonies to be distinguished. To prepare 1 litre of this media, 50g of MacConkey agar powder (BD Difco™ ; Cat no: 212123) was measured using an electron balance, combined, and thoroughly mixed using a magnetic stirrer for 30 minutes until homogenous with 1 litre of deionized water, measured till the meniscus line of the 1 litre Screw-cap flask. This was autoclaved at 12°C for 15 minutes at 15 psi (standard autoclave operating conditions) and then cooled to 40°C before pouring 15 ml into each sterile petri dish, ensuring horizontal surface level and uniform depth. Once cooled to room temperature (r.t.p), these were stored in a 4°C fridge until needed.

2.4.1.2 Mueller-Hinton Agar Plate Preparation

MacConkey agar media is the preferred media for conducting AST testing according to the CLSI guidelines due to its basic constitution devoid of antibacterial inhibitors, consistency and opacity, as compared to other types of media. As such, to prepare 1 litre of this media, 38g of Mueller-Hinton agar powder (BD Difco™) was measured on an electron balance, combined, and thoroughly mixed using a magnetic stirrer for 30 minutes until homogenous with 1 litre of deionized water, measured till the meniscus line of the 1 litre Screw-cap flask. This was autoclaved at 121°C for 15 minutes at 15 psi and then cooled to 40°C before pouring 15 ml into each Petri dish, ensuring horizontal surface level and uniform depth. Once cooled to r.t.p, these were stored in a 4°C fridge until needed.

2.5 PREPARATION OF GRAM STAIN AND MORPHOLOGICAL IDENTIFICATION

Differential staining was used to characterize each of the bacterial isolates taken from MacConkey plates, as being either Gram positive or Gram negative.

1) For each strain, 20 µl of normal saline solution was dropped onto the centre of a microscopic glass slide.
2) A flamed, platinum loop was used to pick up and mix into the saline, up to three bacterial colonies, depending on their size.
3) The resulting smear was heat-fixed by successive, rapid movement over a Bunsen flame, and the slides were placed onto a staining tray.
4) The glass slide was first flooded with Crystal Violet for 1 minute.
5) This was washed off with deionized water before Gram's Iodine solution was added for another minute (The Iodine forms a complex with the Crystal Violet, which binds on to the bacterial cell wall).
6) After another swift wash with deionized water and blotting with tissue paper, 95% Ethanol was then applied over the slide for approximately 30 seconds (This acts as a decolorizing agent- it dehydrates the cell wall trapping the purple Crystal Violet stain in the thick, shrinking peptidoglycan layer found in Gram positive bacteria. As Gram negative bacteria possess a much thinner layer of peptidoglycan, they are unable to retain the dye through this process).
7) After washing once again with deionized water; a counterstain, Safranin was then applied for 45 seconds which paints Gram negative bacteria a red/pink colour.
8) This was washed and blotted with deionized water for a final time and allowed to air dry.
9) Examination of the slides took place under a light microscope (Olympus, CX41, Japan) using 100x magnification under oil immersion.

2.6 BIOCHEMICAL TESTS

Biochemical tests were used to differentiate between closely related bacterial species or genus. These tests were conducted according to the standard specifications described and applied in the Microbiology Laboratory at ideSHi. Negative controls were used for all experiments (not shown) which typically reveal themselves as a lack of visual change of reagents/media despite any growth observed (in certain cases) upon inoculation and subsequent incubation. The biochemical tests used were as follows:

- Simmons Citrate utilization test
- Motility Indole Urease (MIU) test
- Triple Sugar Iron (TSI) test.
- Oxidase Test
- Catalase Test
- Analytical Profile Index (API 20E)

2.6.1 Simmons Citrate Utilization Test

2.6.1.1 Brief: Simmons Citrate utilization test was employed as part of a series of standardized tests used for identifying members of the *Enterobacteriaceae.* This test aids in differentiating on the basis of differential metabolite production, between environmental and faecal coliforms. This test is used to distinguish between strains according to their ability to utilize Citrate as the sole carbon source.

2.6.1.2 Media Preparation: To prepare the media, 2.3g of Simmons Citrate Agar powder (Oxoid) was suspended in 100 ml of deionized water, mixed until homogenous and heated with rapid agitation, then boiled for 1 minute for complete dissolution of the powder. The resulting solution was dispensed into glass, screw-cap tubes and autoclaved at standard operating conditions for 15 minutes.

2.6.1.3 Procedure: A single, isolated colony of each strain was inoculated into Simmons citrate agar using a sterilized straight needle to stab through the centre point, halfway the depth from the top then streaking over the surface of the agar slant. This was then incubated overnight at 37°C. Abundant growth on the slant and a coloration change of the media from forest green to Prussian blue indicated a positive test.

2.6.1.4 Interpretation: A positive reaction is indicated by growth of organisms, and the formation of an intense blue colour in the slant portion. A negative reaction however produces no colour change, and remains forest-green.

2.6.2 Motility Indole Urease (MIU) Test

2.6.2.1 Brief: Motility indole urease (MIU) media is a mainstay test for the identification of Gram-negative bacteria of the Enterobacteriaceae family such as *E. coli* and *Pantoea agglomerans*. It is a single medium which incorporates three separate tests that allows assessment of bacterial motility, indole production and expression of urease enzyme.

2.6.2.2. Media Composition: The media contains tryptone, a pancreatic digest of casein- a milk protein rich in amino acids and nitrogen. This hydrolysate contains a high proportion of tryptophan and is used to test for indole production. A small amount of agar is included to generate a semi-solid media, where sodium chloride maintains the osmotic balance. Potassium dihydrogen phosphate buffers the medium, while phenol red is a pH indicator. The semi-solid nature of the media allows for observation of bacterial motility by eye. Growth of non-motile organisms occur only in the stab-line, moderately motile organisms diffuse from this line, while highly motile ones diffuse throughout the entire media.

2.6.2.3 Procedure: To conduct the test, a sterile straight needle was used to pick up a distinct, isolated colony from a freshly cultured plate and used to inoculate the test tube by indenting a single stab through the centre of the surface of the media to approximately half the depth. An indole paper treated with Kovac's reagent was also attached to the very top of the very top of the tube. The media tube was subsequently incubated overnight at 37°C to develop.

2.6.2.4 Interpretation: In case of growth of a motile organism, a diffused, hazy growth disseminated through the media appears to give an opaque visual indicative of flagella-based movement. In case of indole production by the growing organism, the paper attached changes from yellow to pink through breakdown of the amino acid tryptophan by endogenous tryptophanase enzyme. Moreover, if the organism is found to be positive for urease activity, urea will be converted to ammonia which reacts with water to generate ammonium hydroxide altering the yellow media to red or pink.

2.6.3 Triple Sugar Iron Agar (TSI) Test

2.6.3.1 Brief: The TSI test is utilized for differentiation of Gram-negative enteric bacilli based on a) carbohydrate utilization and b) production of hydrogen sulfide gas. The TSI examines the ability of organisms to ferment either of three sugars: Lactose, Sucrose and Glucose added in the ratios of 10:10:1 (10 parts of 1% Lactose, 10 parts of 1% Sucrose and 1 part of 0.1% Glucose).

The media also contains Iron (ferrous sulfate) serving as an indicator for the production of hydrogen sulfate, Peptone as a Nitrogen source and Phenol red indicator which is red in acidic conditions and yellow in basic conditions. To aid detection of organisms with the ability to metabolize dextrose only, it is used in 1/10th of the concentration of either sucrose or lactose. Dextrose fermentation is detected by the production of a small amount of acid that alters the pH in the slant that rapidly oxidises, reverting the colour to the original red (alkaline). In case of the butt however, the acid reaction produces a colour change to yellow (alkaline), due to the low oxygen tension in the region. Upon dextrose depletion, organisms will then begin to utilize either the sucrose or lactose.

2.6.3.2 TSI Media Preparation: TSI media was prepared by suspending 6.5g of TSI Agar Powder (Difco), in 100 ml of deionized water, mixed till homogenous, then heated with regular agitation until boiling for approximately 1 minute to allow for complete dissolution. The resulting solution was dispensed into glass, screw-cap tubes and autoclaved under standard operating conditions for 15 minutes. These were allowed to cool at a slant to allow deep butts to form.

2.6.3.3 Procedure: To conduct the assay, a sterile straight needle was used to pick up a well isolated colony from freshly cultured media and used to inoculate the media by indenting a single stab through the centre of the surface of the media to approximately half the depth then streaking across the slanted surface. The inoculated TSI agar was then incubated at 34°C under aerophilic conditions for 18-24 hours, ensuring the flask cap is slightly loosened.

2.6.3.4 Results Interpretation: The resulting assays were compared with negative controls, expected to generate no alterations. The following observations may be established:

- **A.** In case of carbohydrate utilization, the medium will change from red to yellow due to acidic pH conditions.
- **B.** A yellow colour of the slant and butt indicates the ability of the organism to utilize dextrose as well as either lactose or glucose, or both.
- **C.** Red colouration of the slant and butt indicates that the organism cannot ferment any of the substrates.
- **D.** Hydrogen gas production results in a black precipitate in the butt region.
- **E.** Fermentative gas production is observed by visible cracks in the media.

2.6.4 Kovac's Oxidase Test

2.6.4.1 Brief: The oxidase test investigates the ability of an organism to utilize oxygen as the terminal electron transfer agent in the bacterial electron transport chain via cytochrome c oxidase, as typical of most aerobic organisms, but is characteristically absent in Enterobacteriaceae but present in Pseudomonadaceae members.

2.6.4.2 Procedure: A filter paper was cut out and soaked with fresh tetramethyl-p-phenylenediamine dihydrochloride (Kovac's oxidase reagent), which serves as the substrate reagent for the test. The soaked portion was inoculated with a portion of bacterial colony using a wooden applicator stick, once freshly subcultured. In case of a positive result, there will be a clearly visible colour change from light blue to deep blue or purple within 10 seconds of application. In case of a negative result, no colour change is expected. To be noted, this test must invariably be conducted prior to the analytical profile index strip test.

2.6.5 Catalase Test

2.6.5.1 Brief: This test differentiates strains by whether they possess the enzyme, catalase. This enzyme aids in breaking down toxic oxygen derivatives such as hydrogen peroxide (H_2O_2) into the harmless metabolites, carbon dioxide (CO_2) and water (H_2O), typical of many bacterial species which are aerobic and require neutralization of toxic oxides.

2.6.5.2 Procedure: The bacterial strain to be tested was inoculated onto the surface of a clean, grease-free microscopic glass slide, onto which an approximately 20 µl drop of 3% H_2O_2 was mixed in using a platinum loop.

2.6.5.3 Interpretation: Rapid effervescence as observed by the evolution of CO_2 bubbles indicated a positive result, and the lack of any observable reaction after 10 seconds indicated a negative test.

2.6.6 Analytical Profile Index 20E (API 20E)

2.6.6.1 Brief: The API 20E is a commercially available biochemical panel designed for identification and differentiation of bacterial members from the Enterobacteriaceae family up to the species level. It consists of a plastic strip with a series of 20 mini-test chambers containing dehydrated media as shown in Figure 2.1 below, with chemically-defined constitutions for each

individual test. Each media constitutes substrates for enzymatic activity or fermentation of sugars by the inoculated organism. Colour changes are produced after inoculation spontaneously, or upon addition of other reagents. Colour changes are determined through indicators which detects pH alterations. Assimilation tests demonstrate a positive result by the growth of the organism if they are able to assimilate, or utilize the corresponding substrates. This latter test is inoculated with a minimal medium (API AUX medium).

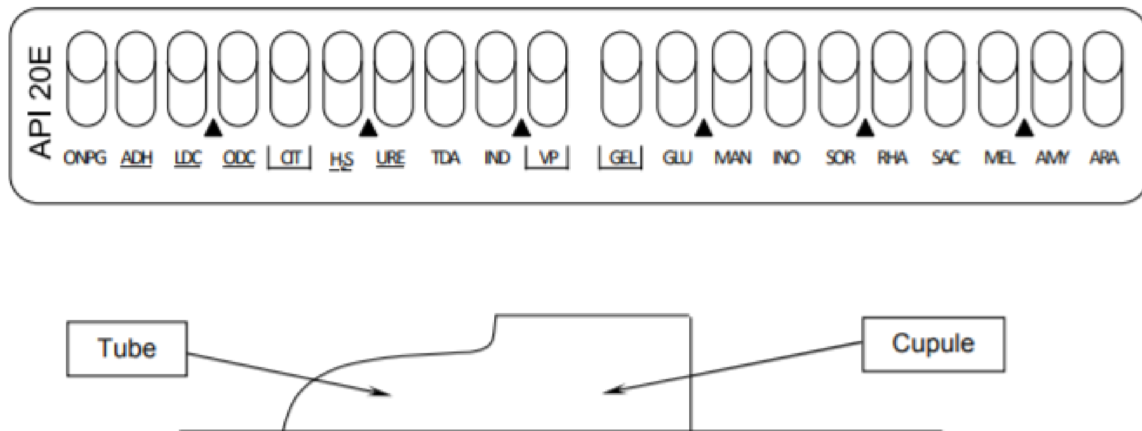

Fig. 2.1. Schematic Representation of API 20E Strip and Associated Tube/Cupule.

2.6.6.2 Assays: The tests in order (from left to right on the series) are as follows:

1. ONPG: test for β-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside
2. ADH: decarboxylation of the amino acid arginine by arginine dihydrolase
3. LDC: decarboxylations of the amino acid lysine by lysine decarboxylase
4. ODC: decarboxylations of the amino acid ornithine by ornithine decarboxylase
5. CIT: utilization of citrate as only carbon source
6. H2S: production of hydrogen sulfide
7. URE: test for the enzyme urease
8. TDA (Tryptophan deaminase): detection of the enzyme tryptophan deaminase: Reagent to put- Ferric Chloride.
9. IND: Indole Test-production of indole from tryptophan by the enzyme tryptophanase. Reagent- *Indole is detected by addition of Kovac's reagent.*
10. VP: the Voges-Proskauer test for the detection of acetoin (acetyl methyl carbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway
11. GEL: test for the production of the enzyme gelatinase which liquefies gelatine

12. GLU: fermentation of glucose (hexose sugar)
13. MAN: fermentation of mannose (hexose sugar)
14. INO: fermentation of inositol (cyclic polyalcohol)
15. SOR: fermentation of sorbitol (alcohol sugar)
16. RHA: fermentation of rhamnose (methyl pentose sugar)
17. SAC: fermentation of sucrose (disaccharide)
18. MEL: fermentation of melibiose (disaccharide)
19. AMY: fermentation of amygdalin (glycoside)
20. ARA: fermentation of arabinose (pentose sugar)

2.6.6.3 Methodology: The process of this identification kit requires preparation of the strips prior to inoculation and the actual preparation of the bacterial cultures before beginning.

2.6.6.3.1 Preparation of Strip:

1) An incubation box containing the tray and lid was prepared, and 5 ml of deionized water distributed into each honeycomb well to produce a humid atmosphere.
2) The test strip was then placed into the incubation box.

2.6.6.3.2 Preparation of Inoculum:

1) 5ml of API NaCl 0.85% medium was collected.
2) A well isolated colony was selected from an isolation plate as removed using a pipette, avoiding cultures older than 24 hours.

2.6.6.3.3 Procedure:

1) A pure culture for each bacterial strain to be tested was obtained, from which an isolated, single colony inoculated in distilled water to generate a suspension.
2) Using a pipette, each of these compartments was filled to the brim with the resulting bacterial suspension.
3) Sterile mineral oil was then added into the ADH, LDC, ODC, H_2S and URE test compartments to create an anaerobic environment.
4) Drops of autoclaved deionized water were added into the tray after which the API Test strip was inserted, and the tray closed.
5) The tray was finally incubated at 37°C for 18-24 hours.

6) In case of certain compartments, the colour changes observed enables easy analysis; however for other compartments other reagents must also be added. Next, the following reagents were added to these specific test capsules:

   A. VP: Put one drop of 40% KOH (VP reagent 1) and one drop of VP reagent 2 (α-Naphthol)- waited 10 minutes before negative was called
   B. IND: Put one drop of Kovacs reagent
   C. TD: Put one drop of Ferric Chloride

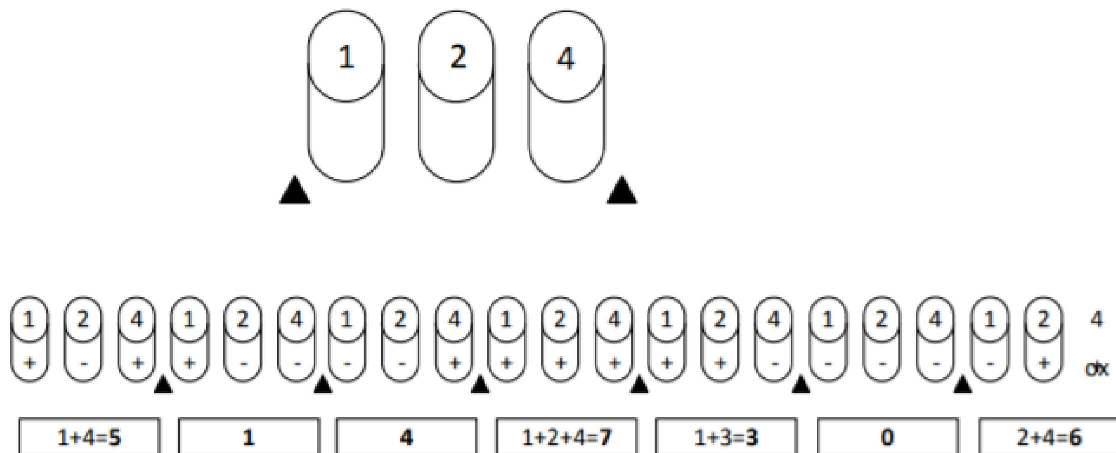

Fig. 2.2. A Sample positive API 20E Strip.

2.6.6.3.4 Interpretation: The strip was read based on the provided Reading Table after the end of the incubation period. Each well is assigned a value of 1, 2 or 4. Compartments were marked as either "positive" or "negative" on the tray lid, and the results scored as triplets. Only positive wells were counted, with a maximum possible score of 7 or a minimum of 0 for each triplet. The oxidase reaction constitutes the 21st test and has a maximum value of 4 if positive. The organism's identity was therefore derived from the seminal API catalogue based on the numeral profile from the list of profiles on the database, which is also available through the apiweb™ portal. An example of this is illustrated above in Figure 2.2.

2.7 ANTIMICROBIAL SENSITIVITY TEST (AST)

2.7.1 Procedure: For determination of antibiotic susceptibility of the test organisms, a modified method of the Kirby Bauer (1966) disk-diffusion assay was conducted, according to

Clinical Laboratory Standards Institute (CLSI) guidelines, 2015 specifications using commercially available antimicrobial disks (Oxoid, Hampshire, United Kingdom) as follows:

1) According to this protocol, the Gram-negative organisms freshly sub-cultured on MacConkey Agar plates were suspended in 1 ml of normal saline solution in Drum vials and incubated for 4 hours until log phase was achieved.
2) The inoculum turbidity were adjusted to 0.5 McFarland standard by either adding more organisms (if too dilute) or more saline solution (if too dense) to achieve an approximate log phase cell density of 1.5×10^8 organisms/ml.
3) A sterile cotton swab was then immersed into the bacterial suspension, rotating the swab with firm pressure against the upper inner side of each tube to remove excess suspension. This swab was used to lawn the entire surface of a Mueller-Hinton agar plate, rotating the plate by 60°C after the first lawn, in order to produce a uniform distribution of cells.
4) After allowing the inoculum to dry on the plate at room temperature with the lid closed, up to 6 antibiotic discs (Oxoid, UK) [selected according to CLSI, 2015 recommendations for each strain] were then placed on each plate using flame sterilized tweezers. They were pressed gently being placed no less than 25mm from each other, and 15 mm from the outer edges of the Petri dish.
5) These were applied within 15 minutes of inoculation and incubated at 37°C under aerophilic conditions in an incubator (Memmert, Germany) for 18 hours overnight for zone development.

2.7.2 Measurement of Zones of Inhibition: At the end of the incubation period, the zones of inhibition for each disc were measured using an electronic slide calliper. Measurements were made from observation of the bottom of each plate, ensuring to ignore satellite colonies or areas of incomplete inhibitions. Moreover, measurements were made by observing the diameter at two or more angles for non-circular clearings, taking the average of each zone of clearing observed.

2.7.3 Interpretation of Inhibition Zones: The measured zones were conferred with the CLSI, 2015 guidelines tables as reference for Enterobacteriaceae and Pseudomonadaceae, according to individual species and antibiotic sensitivities. Different antibiotic disks were used according to the popular antibiotics prescribed against each species (Tables 2.2-2.6), and ensuring to include the top five most common aminoglycosides in the market. Accordingly, for each individual antibiotic disk assessed, they were marked as Susceptible (S), Intermediate Resistance (I) or wholly resistant (R).

Table 2.2. List of antibiotic disks and drug concentrations used for *Pseudomonas* strain.

Strain	***Pseudomonas spp.***												
	Antibiotic Disks												
	CT	MEM	CN	TOB	S	CAZ	CIP	IPM	CAR	TZP	AK	NET	PB
Conc. (ug)	10	10	10	10	10	30	05	10	100	110	30	30	300

Table 2.3. List of antibiotic disks and drug concentrations used for *Aeromonas* strain.

Strain	***Aeromonas spp.***												
	Antibiotic Disks												
	CT	MEM	CN	TOB	S	CAZ	CIP	IPM	CAR	TZP	AK	NET	PB
Conc. (ug)	10	10	10	10	10	30	05	10	100	110	30	30	300

Table 2.4. List of antibiotic disks and drug concentrations used for *Klebsiella pneumoniae* strains.

Strain	***Klebsiella pneumoniae***												
	Antibiotic Disks												
	CRO	MEM	CN	TOB	CFM	S	AZI	CIP	IPM	CAR	TZP	AK	NET
Conc. (ug)	30	10	10	10	05	10	15	05	10	100	110	30	30

Table 2.5. List of antibiotic disks and drug concentrations used for *Pantoea agglomerans* strains.

Strain	***Pantoea agglomerans***													
	Antibiotics Disks													
	CRO	MEM	CN	TOB	CFM	S	AZI	CIP	IPM	CAR	TZP	AK	NET	PB
Conc. (ug)	30	10	10	10	05	10	15	05	10	100	110	30	30	300

Table 2.6. List of antibiotic disks and drug concentrations used for ETEC strains.

Strain	**ETEC**															
	Antibiotic Disks															
	CRO	CN	TOB	AMP	S	SXT	CFM	AZI	CIP	DO	TE	E	NET	AK	NOR	NA
Conc.(ug)	30	10	10	10	10	25	05	15	05	30	30	15	30	30	10	30

2.8 DNA EXTRACTION FOR ETEC IDENTIFICATION: BOILING METHOD

2.8.1 Brief: The boiling method is a simple technique to isolate bacterial DNA by thermogenic break-down of the cell-wall and nuclear structures.

2.8.2 Procedure:

1) Four to six colonies of each *E. coli* strain were transferred into 300 µl of nuclease free water in a DNase/RNase free Eppendorf tube using a sterile pipette tip.
2) The suspension was heated for 95-99°C for 10 minutes in a hot water bath.
3) Next, the Eppendorf tubes were cooled down on ice for 1 minute before being centrifuged for 10 minutes at 13, 000 RPM.
4) Finally the supernatant was transferred into a fresh Eppendorf tube and stored at -20°C until ETEC confirmation PCR was carried out.

2.8.3 ETEC Identification Using Multiplex-PCR

Polymerase chain reaction (PCR) was conducted for the detection of the heat-labile (LT) and heat-stable (STh and STp) enterotoxins that are characteristic to ETEC strains. Six distinct lactose-fermenting colonies with deep pink coloration isolated from MacConkey agar plates were randomly selected using a platinum loop and their DNA extracted, for use as template in a test for *ETEC* determination using multiplex PCR using LT, STh and STp gene specific primers (Table 2.8). The PCR master mix was prepared manually using the following reaction mixture components: 10x PCR buffer (with 15 mM $MgCl_2$), 25 mM $MgCl_2$, 2.5 mM dNTPs, 10 mM forward primers, 10 mM reverse primers, Taq polymerase (Qiagen, US) and 60 µg/µl template DNA, with the final reaction volume added up to 10 µl by addition of nuclease free H_2O. This composition is also shown in Table 2.7 below. Samples were run on a PCR thermocycler (Infinigen, USA) using

optimized temperature conditions as follows: Initial denaturation at 95°C for 5 minutes; 39 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds and an extension at 72°C for 30 seconds; with a final extension at 72°C for 5 minutes at the end of the last cycle.

Table 2.7. Multiplex-PCR master mix composition for ETEC identification.

Reaction Component	Volume (µl)
10x PCR buffer (with 15mM $MgCl_2$)	2.5
dNTP (2.5 mM)	4.0
$MgCl_2$ (25 mM)	0.5
Primer LT mixture (4pm/µl)	4.0
Primer STh mixture (4pm/µl)	0.5
Primer STp mixture (4pm/µl)	2.0
Taq polymerase (5U/µl)	0.15
Nuclease free H_2O	10.35
DNA Template	1.50

Table 2.8. Forward (F) and Reverse (R) primers for ETEC toxin amplifications.

Primer ID	Sequence (5'-->3')	Amplicon Size
ST_h-F	**F:** TTCACCTTTCCCTCAGGATG	300bp
ST_h-R	**R:** CTATTCATGCTTTCAGGACCA	
ST_p-F	**F:** TCTTTCCCCTCTTTTAGTCAG	166bp
ST_p-R	**R:** ACAGGCAGGATTACAACAAAG	
LT-F	**F:** ACGGCGTTACTATCCTCTC	100bp
LT-R	**R:** TGGTCTCGGTCAGATATGT	

2.9 DNA Extraction for Aminoglycoside Resistance Gene PCR Template Preparation [50:49:1 Phenol-Chloroform-Isoamyl Alcohol Method]

1) 1.5 ml of an overnight culture of *E. coli* grown in Luria broth (LB) was transferred to a 1.5 ml Eppendorf tube, and then centrifuged for one minute at maximum speed in order to pellet cells.
2) The supernatant was discarded carefully without disruption of the pellet.
3) The pellet was then resuspended in 600 µl of lysis buffer and vortexed until homogeneity was achieved.
4) The suspension was incubated for 1 hour at 37°C.
5) At this point, a combination of phenol: chloroform and isoamyl alcohol (50:49:1 ratio) was transferred and completely mixed by gentle inversions.
6) This was spun at maximum speed for 5 minutes at r.t.p until three distinct layers were produced.
7) The uppermost phase (aqueous) was carefully transferred to fresh tubes.
8) Steps 5-7 were repeated until no white, protein layer remained.
9) In order to remove the phenol, an equal volume of chloroform was added into the aqueous layer and mixed gently by inversions.
10) The mixture was once again spun at maximum speed for 5 minutes.
11) The aqueous layer was then transferred into more fresh tubes.
12) Finally, 2-3 volumes of cold 200-Proof Ethanol (stored at -20°C) was added and the mixture itself incubated for approximately 30 minutes at -20°C in order to completely precipitate the DNA (which appears as thin, white strands).
13) This was spun for 15 minutes at maximum speed at 4°C.
14) After removing the supernatant, the DNA pellet was washed using 1 ml 70% ethanol.
15) The tubes were then spun for 2 minutes at maximum speed at r.t.p.
16) The supernatant was carefully discarded and the DNA pellet allowed drying by inverting the open tubes on tissue paper.
17) The pellet was resuspended in 50 µl of TE buffer.
18) The DNA was checked for fragmentation by running on a 0.8% agarose gel.
19) Finally the DNA was quantified using NanoDrop (ThermoFisher SCIENTIFIC, USA) and each sample diluted to 60 ng/µl for use as template for subsequent PCRs.

2.9.1 Quantification and Quality Assessment of PCR Products

The DNA concentration of each purified PCR product was quantified using a Nanodrop 2000 (ThermoFisher SCIENTIFIC, USA) spectrophotometer. 2 µl aliquots of each sample measured was transferred onto the platform slide and before obtaining the nucleic acid results under 260 nm wavelength. In each case, the 260/230 and 260/280 ratio results used for quality assessment, where ~1.8 and ~2.2 respectively were the ideal ratios desired.

2.9.2 Template Dilution

After measurement and quality assessment of the DNA, each sample was either used as is (at the appropriate increased volume) or diluted down using nuclease free water, such that all templates added to the PCR master mix would amount to approximately 60 ng/µl in order to standardize reactions. These were then re-quantified using the Nanodrop 2000 (ThermoFisher SCIENTIFIC, USA) spectrophotometer once again.

2.9.3 Aminoglycoside Resistance Gene Primers

Five aminoglycoside modifying enzyme and two ribosomal target modifying enzyme genes that confer resistance to aminoglycoside antibiotics were chosen according to previous assessment of relevance based on literature review. Their forward and reverse primer sequences obtained from various research papers, and ordered from Bioneer (South Korea) as described in Table 2.9 below:

Table 2.9. List of Aminoglycoside resistance gene primers selected for study.

Gene	Primers (5'-->3')	Amplicon Size (bp)	Reference
Aac(3')-II	**F:** ATATCGCGATGCATACGCGG **R:** GACGGCCTCTAACCGGAAGG	877	**Arpin et al., 2003**
Aac(6')-Ib	**F:** TTGCGATGCTCTATGAGTGGCTA **R:** CTCGAATGCCTGGCGTGTTT	472	**Kim et al., 2009**
Aac(6')-II	**F:** CGACCATTTCATGTCC **R:** GAAGGCTTGTCGTGTTT	542	**El-Badawy et al., 2017**
Ant(3")-Ia	**F:** CATCATGAGGGAAGCGGTG **R:** GACTACCTTGGTGATCTCG	787	**El-Badawy et al., 2017**
Aph(3')-VI	**F:** ATGGAATTGCCCAATATTATT **R:** TCAATTCAATTCATCAAGTTT	780	**Hu et al., 2013**
ArmA	**F:** CCGAAATGACAGTTCCTATC **R:** GAAAATGAGTGCCTTGGAGG	846	**Hu et al., 2013**
RmtB	**F:** ATGAACATCAACGATGCCCTC **R:** CCTTCTGATTGGCTTATCCA	769	**Hu et al., 2013**

2.9.4 Aminoglycoside Resistance PCR

Standard PCR was conducted using a on a T100™ Thermal Cycler (Bio-Rad, USA) after optimising conditions for each gene, such that all 18 strains including a negative control could be investigated. The master mix and thermocycler conditions used are described below in Table 2.10.

Table 2.10. Optimized master mix compositions and thermal cycler profiles.

Gene	Master Mix (Single Reaction)	Thermocycler Profile
Aac(3')-II	➢ 10x PCR Buffer (+15mM $MgCl_2$): 1.0 µl ➢ $MgCl_2$ (25 mM): 0.3 µl ➢ F-Primer (10 µM): 0.5 µl ➢ R-Primer (10 uM): 0.5 µl ➢ dNTPs (2.5 mM): 1.0 µl ➢ 5x Q solution: 2.0 µl ➢ Taq Polymerase (5 Units): 0.075 µl ➢ N.F. H_2O: 3.7 µl ➢ Template: 1µl	***94°C initial denaturation for 3 minutes; 35 cycles of: 94°C denaturation for 30 seconds, 60°C annealing for 45 seconds and 72°C extension for 40 seconds; 72°C extension for 10 minutes and 10°C hold for infinity***
Aac(6')-Ib	➢ 10x PCR Buffer (+15mM $MgCl_2$): 1.0 µl ➢ $MgCl_2$ (25 mM): 0.3 µl ➢ F-Primer (10 µM): 0.3 µl ➢ R-Primer (10 µM): 0.3 µl ➢ dNTPs (2.5 mM): 1.0 µl ➢ Taq Polymerase (5 Units): 0.075 µl ➢ N.F. H_2O: 6.1 µl ➢ Template: 1µl	***94°C initial denaturation for 3 minutes; 35 cycles of: 94°C denaturation for 30 seconds, 60°C annealing for 30 seconds and 72°C extension for 30 seconds; 72°C extension for 10 minutes and 10°C hold for infinity***
Aac(6')-II	➢ 10x PCR Buffer (+15mM $MgCl_2$): 1.0 µl ➢ $MgCl_2$ (25 mM): 0.3 µl ➢ F-Primer (10 µM): 0.5 µl ➢ R-Primer (10 µM): 0.5 µl ➢ dNTPs (2.5 mM): 1.0 µl ➢ 5x Q solution: 2.0 µl ➢ Taq Polymerase (5 Units): 0.1 µl ➢ N.F. H_2O: 3.6 µl ➢ Template: 1µl	***95°C initial denaturation for 3 minutes; 35 cycles of: 95°C denaturation for 30 seconds, 58°C annealing for 45 seconds and 72°C extension for 50 seconds; 72°C extension for 10 minutes and 10°C hold for infinity***
Ant(3")-I	➢ 10x PCR Buffer (+15mM $MgCl_2$): 1.0 µl ➢ F-Primer (10 µM): 0.5 µl ➢ R-Primer (10 µM): 0.5 µl ➢ dNTPs (2.5 mM): 1.0 µl ➢ 5x Q solution: 1.0 ul ➢ Taq Polymerase (5 Units): 0.05 µl ➢ N.F. H_2O: 4.0 µl ➢ Template: 2 µl	***94°C initial denaturation for 3 minutes; 35 cycles of: 94°C denaturation for 30 seconds, 60°C annealing for 45 seconds and 72°C extension for 1 minute; 72°C extension for 10 minutes and 10°C hold for infinity***

Aph(3')-VI	➢ 10x PCR Buffer (+15mM $MgCl_2$): 1.0 µl ➢ $MgCl_2$ (25 mM): 0.3 µl ➢ F-Primer (10 µM): 0.3 µl ➢ R-Primer (10 µM): 0.3 µl ➢ dNTPs (2.5 mM): 1.6 µl ➢ 5x Q solution: 2.0 µl ➢ Taq Polymerase (5 Units): 0.1 µl ➢ N.F. H_2O: 3.4 µl ➢ Template: 1 µl	***94°C initial denaturation for 3 minutes; 35 cycles of: 94°C denaturation for 30 seconds, 60°C annealing for 30 seconds and 72°C extension for 1 minute; 72°C final extension and 10°C hold for infinity***
ArmA	➢ 10x PCR Buffer (+15mM $MgCl_2$): 1.0 µl ➢ F-Primer (10 µM): 0.5 µl ➢ R-Primer (10 µM): 0.5 µl ➢ dNTPs (2.5 mM): 1.0 µl ➢ 5x Q solution: 1.0 µl ➢ Taq Polymerase (5 Units): 0.05 µl ➢ N.F. H_2O: 4.0 µl ➢ Template: 2 µl	***94°C initial denaturation for 3 minutes; 35 cycles of: 94°C denaturation for 30 seconds, 56°C annealing for 30 seconds and 72°C extension for 1 minute; 72°C extension for 10 minutes and 10°C hold for infinity***
RmtB	➢ 10x PCR Buffer (+15mM $MgCl_2$): 1.0 µl ➢ F-Primer (10 µM): 0.5 µl ➢ R-Primer (10 µM): 0.5 µl ➢ dNTPs (2.5 mM): 1.0 µl ➢ 5x Q solution: 1.0 µl ➢ Taq Polymerase (5 Units): 0.05 µl ➢ N.F. H_2O: 4.0 µl ➢ Template: 2 µl	***94°C initial denaturation for 3 minutes; 35 cycles of: 94°C denaturation for 30 seconds, 56°C annealing for 30 seconds and 72°C extension for 1 minute; 72°C extension for 10 minutes and 10°C hold for infinity***

2.10 AGAROSE GEL PREPARATION

Amplified PCR products were resolved on 0.8-2% Gels according to their molecular weight. For higher molecular weight products (>500 bp) 0.8-1% gels were used and for lower molecular weight products (<500 bp) 1%-2% gels were applied.

Agarose powder (Ultrapure, Invitrogen, USA) was measured on a standard/analytical electronic balance (Wisd Laboratory Instruments, Ireland) and transferred into a fresh conical flask. An appropriate volume of 1x TBE (Tris-Borate-EDTA, pH 8.1) buffer was added and a paper cover with holes punched-in used to cover the top of the flask. The suspension was microwaved to produce molten agarose. Finally, 1 µl of GelRed (Biotium, Cat no: 41003, USA) was aliquoted into the agarose, and the final mixture cast upon cooling to approximately 40°C, into an appropriately sized, balanced tray with a comb inserted.

2.10.1 Gel Electrophoresis

The cast tray of solidified agarose was submerged after removing the comb, in an electrophoresis machine ensuring the 1x TBE buffer bath rose 3-5mm above the gel surface. 4 µl of PCR product was mixed with 2 µl loading dye (ThermoFisher, code no: R0611) and aliquoted into individual wells. A 1 kb plus gene ladder (Generuler, ThermoFisher, USA) with a range between 75-20,000 bp was used as a reference in each instance. The PCR products were finally resolved at 130 volts/cm and allowed to run for 40-60 minutes until a clear picture was generated. The visualization of the gels was carried out on a Gel Doc™ XR+ (Bio-Rad, USA) molecular imager using supplemented Image Lab™ software to take a picture.

2.11 PCR PRODUCT PURIFICATION

The following procedure is adapted from the manufacturer's instructions, as delineated by the QIAquick PCR Purification Kit protocol (Qiagen, Germany):

1) Five volumes of Buffer PB was added to 1 volume of the PCR sample and mixed.
2) To the Buffer PB, pH indicator I was added prior to use, which should turn the mixture yellow, however if the color of the mixture was observed to be orange or violet, 10 µl of 3 M sodium acetate, pH 5.0 was added and mixed which turned the colour of the mixture yellow.
3) A QIAquick spin column was set in a provided 2 ml collection tube.
4) To bind DNA, the sample was applied to the QIAquick column and centrifuged for 30–60 seconds.
5) Next, the flow-through was discarded, and the QIAquick column transferred back into the same tube.
6) For the wash step, 0.75 ml Buffer PE was added to the QIAquick column and centrifuged for 30–60 seconds.
7) The flow-through was once again discarded and the QIAquick column placed back in the same tube. The column was then centrifuged for an additional 1 minute.
8) The QIAquick column was placed in a clean 1.5 ml microcentrifuge tube.
9) Twenty microlitres of Nuclease free water (pH 7.0–8.5) was then added to the centre of the QIAquick membrane and the column centrifuged for 1 minute in order to elute the DNA.

10) The DNA was quantified using Nanodrop 2000 (ThermoFisher Scientific, USA), ran on a 1% agarose gel and then diluted down to ~10 μl for sequencing.

2.12 DNA SEQUENCING [SANGER SEQUENCING]

2.12.1 Brief: This process is undertaken at the ideSHi affiliated institute: the Institute of Epidemiology, Disease Control and Research (IEDCR). DNA sequencing is a process by which nucleotide bases (Adenine, Thymine, Cytosine and Guanine) of a selected section of DNA are read in their original order. Sanger sequencing is one of oldest and most reliable sequencing technologies available. It has the ability to decipher the code within single stranded DNA templates by addition of dideoxynucleotides (ddNTPs) instead of the usual deoxynucleotides (dNTPs) which are additionally labelled with distinctly coloured fluorescent dyes, unique to each of the four dideoxy bases for easy colorimetric recognition. The additive chain elongation process initiates from an oligonucleotide primer occurring from the 3' end. Extension occurs in a 5' to 3' direction by formation of a phosphodiester bridge between the 3' hydroxyl of the existing deoxynucleotide in the chain, and 5' phosphate groups of the incoming dideoxynucleotide. Once each of the 2', 3'-dideoxynucleotide substrates are added, chain termination occurs due their lack of the 3' hydroxyl group necessary for further elongation. Thus extension products of varying lengths are generated with fluorescently-tagged complementary terminating nucleotides whose wavelengths can be then read by the machine. Therefore, in each cycle, the nucleotide complement of each base added is determined until the whole sequence is read.

2.12.2 Procedure:

1) Calculation of the number of cycles of sequencing was determined according to the measured template concentration. Templates used for the reaction were the purified PCR products of each sample to be confirmed.
2) Tubes containing this template were spun and 10-20 ng/μl (depending on the template concentration) was added to the 8-tube PCR strip.
3) Nuclease free water was then added to each mixture to make up a total volume of 10 μl per reaction.
4) Following this, the PCR tubes were centrifuged at 4000 RPM for 3 minutes before the PCR strip was placed in the Mastercycler gradient (Cat. No. 4095-0015, USA Scientific) Thermal Cycler and subjected to the following thermal cycling profile: initial denaturation at 95°C for 10 minutes, then 25 cycles of denaturation at 95°C for 10

seconds; annealing at 55°C for 5 seconds; and extension at 72°C for 4 minutes followed by a final extension step at 72°C lasting for 6 minutes.

5) Upon completion of the cycle sequencing, the reaction plate was centrifuged at 41,000 RPM for 1 minute.
6) Next, for each 10 µl reaction, 45 µl of SAM solution and 10 µl of X-terminator (Applied Biosystems, USA) were added.
7) Homogenization of each solution was ensured by vortexing for a minimum of 30 seconds, and wide bore micropipette tips being used to aliquot the viscous X-terminator solution to maintain required volumes. Both of these reagents aid the removal of impurities, in particular salts which interfere with the electrokinetic injection as well as excess ddNTPs leftover which may generate dye blobs which contribute to significant background noise in the final read.
8) The reaction plate was then sealed and vortexed for 30 minutes.
9) Finally, the mixture was centrifuged for 2 minutes at 4,100 RCF before being collected for capillary electrophoresis. For this process, 10 µl of supernatant were transferred to fresh sequencing tubes, covered with Septa mat., and then placed into the ABI PRISM 310 capillary electrophoresis machine.
10) Remaining supernatant was stored at 4°C for future use.

2.12.3 Analysis of Sequence Reads

Data obtained through sequencing were assessed and analyzed using Chromas Lite 2.4 software which enabled evaluation of sequencing reads. This program was used to export each AB1 file to FASTA format for further processing.

2.13 VALIDATION OF GENES AND PROTEIN-CODING ORFS

Upon converting the .ab1 chromatogram file format to FASTA format, the GeneMark.hmm program under Heuristics MetaGeneMark (Zhu, Lomsadze and Borodovsky, 2010) parameters was used (available from http://exon.gatech.edu/GeneMark/gmhmmp.cgi) under default settings searching for "Gene nucleotide sequence" under "Output options" to obtain only the protein coding portion. The output sequence was then inserted into the NCBI ORFfinder online tool (available at: https://www.ncbi.nlm.nih.gov/orffinder/) modifying search parameters to include "1. Standard" Genetic code, and allowing for "ATG and alternative initiation codons" under "ORF start codons to use" section with a maximum ORF length of 75 as is the default. The longest output ORF was considered the putative protein ORF for each gene. The translated

version of the ORF provided was analysed using NCBI BLAST to verify the correct gene's sequence was obtained, and the nucleotide sequence was used for gene sequence population.

2.13.1 BLAST (Basic Local Alignment Search Tool) Analysis:

2.13.1.1 Brief: BLAST is an algorithm that can align and compare a query nucleotide or protein sequence with all or selected sequences stored in its database. Through calculation of statistical significance of matches, it is able to both rapidly and sensitively find regions of local similarity between a particular query sequence and the various reference sequences uploaded to the database that match in terms of identity to various degrees of similarity.

2.13.1.2 Procedure: Nucleotide BLAST was selected for the analysis of sequence data to ensure the correct gene was sequenced in each case by using Protein BLAST (BLASTp) tool, as well as populating the top 50 strains nucleotide sequences that matched with the gene ORF sequence previously obtained. This was then used for phylogenetic analysis, for each individual gene with reference data obtained from the non-species specific "nucleotide collection"database as optimized for the program "highly similar sequences" or "megablast" search. This bioinformatics tool is found online at the National Centre for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.14 PHYLOGENETIC TREE CONSTRUCTION USING MEGAX

2.14.1 Brief: MEGA stands for Molecular Evolutionary Genetic Analysis, and is a fully integrated tool that enabled generation of phylogenetic trees for evolutionary relationship estimations. MEGAX is the most recent adaptation of the all-in-one software that enables users to use sequenced data for comparative analysis by populating sequences from NCBI, generating multiple sequence alignments, modifying and re-annotating data and ultimately estimating and testing evolutionary relatedness among various species using either translated or direct nucleotide sequence data. The reliability of this downloadable tool that functions across multiple operating systems and platforms can be underscored by the over one hundred thousand publications that has applied this tool for analysis over the 25 years of its existence in a diverse array of biological fields (Kumar et al., 2018: https://www.megasoftware.net/pdfs/kumar_stecher_2018.pdf). This tool can be acquired from https://www.megasoftware.net/ as a free tool for both students and researchers. The application of this software, particularly the methodology used in this study, has been adapted

from the manuals outlined by Hall, 2013 and Bast and Bast, 2013 which were originally designed to work with MEGA5. However as a more advanced and updated iteration is now available, MEGAX has been used for the purposes of this project as it could be easily adapted from these manuals. The main interface and utility options are elucidated clearly in Figure 2.3 below.

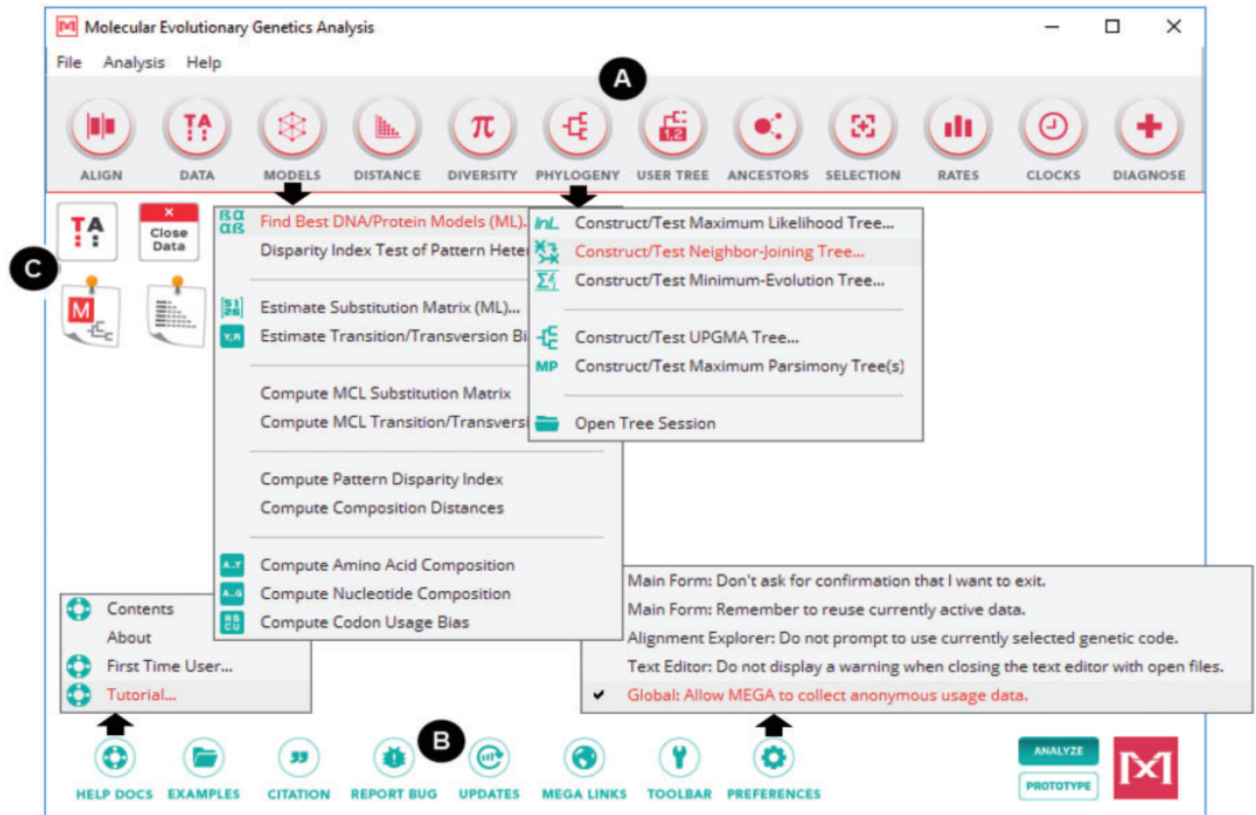

Fig. 2.3. The main elements of the MEGA interface and software utility options.
The tool-bar at the top opens up the main options for data analysis as highlighted by (**A**) which includes access to files, alignment options, model estimations and a range of phylogenetic and sequence manipulation tools. The bottom toolbar options are highlighted by (**B**) - it provides access to several utilities including help section, guides, updates and software usage preferences among other tools. Access to tools in use is pinned to the main interface as shown by (**C**).

2.14.2 Methodology:

2.14.3 Multiple Sequence Alignment (MSA)

Prior to alignment, the ORF sequence for each gene was incorporated into the FASTA file containing the 50 top hit's sequences. This file was uploaded to MEGAX, where any redundant sequences were manually searched for and removed. The final list of nucleotide sequences was used for alignment using the MUSCLE algorithm, aligning by codons to account for accurate

biological significance, considering only the coding sequences were selected for analysis (Sutton, 2008). This resulting alignment was then used for Phylogenetic tree generation.

2.14.4 Phylogenetic Tree Construction

Maximum Likelihood (ML) was used as the method of choice, although Maximum Parsimony is usually suggested for highly similar sequences. ML is not only more robust than other model algorithms in terms of generating the most accurate tree, but due to the computing power available (Intel(R) Core i5, 2.20 GHz processor, 6.00 GB RAM, 64-bit Operating System, Windows 10 laptop) it was also highly feasible being able to produce results within 5-10 minutes per operation. Under Models, "Find best DNA/Protein models (ML)" using default parameters was used to find the best fit model for each gene which were determined to be Tamura (1992), Jukes and Cantor (1969) and Kimura (1980) models for *aac(3')-II*, *aac(6')-Ib* and *ant(3")-Ia* genes respectively, as predicted by the software according to the lowest BIC scores. Maximum Parsimony, another character-based method like ML was used to make the initial tree in each case, using "Uniform Rates" for Rates among Sites" as recommended by the model selection tool, using "Use all sites" for "Gaps/Missing data treatment" as the sequences were all short (under 1 Kb length) bacterial DNA, and the "Bootstrap Method" for "Test of Phylogeny" with a value of 100, leaving all other parameters as default.

2.14.5 Tree Viewing and Manipulation

In this case, a separate tree-viewing software Figtree V.1.4.3 Windows version was used for graphical viewing, modification and generation of "publication-ready" phylogenetic trees as described on their website by Rambaut, 2007. It produces trees with more clarity and greater depth of annotation. All versions of the software are freely available from: http://tree.bio.ed.ac.uk/software/figtree/.

For each tree, default parameters were retained other than for the following exceptions:

- "Tip Labels" were altered by using the "Arial" font, increasing font size to 9.
- "Display" and "Colour" options were optimised to highlight bootstrap values, and the colour, hue and saturation options increased for optimum clarity.
- "Node Shapes" options were manipulated to display a diamond shape with a maximum size of 5, and painted according to the bootstrap values also.
- The" Scale Bar" option was checked.
- The "Legend" was attributed according to bootstrap values in a size 10 font.

- Go to the Trees side-menu and check “Transform Branches”.
- Finally, the study specimen branch was coloured using the toolbar at the top to differentiate the branch by painting it a hue of red.

Chapter 3

RESULTS

3. RESULTS

3.1 RECHARACTERISATION OVERVIEW

The clinical isolates used in this study were previously isolated and characterized as members of the Enterobacteriaceae and Pseudomonadaceae families of bacteria prior to storage at –80°C in Tryptone-Soya-Glucose-Glycerol (TSGG) media. For the purposes of confirming viability of the stock samples and validating identities of the individual species such that the possibility of contamination (either from previous culturing or recent handling) may be eliminated, the strains were subcultured on MacConkey agar plates, and a number of appropriate biochemical tests were conducted.

3.2 COLONY CHARACTERISTICS

As expected, *Escherichia coli*, *Pantoea spp.* and *Klebsiella spp.* turned the media red indicating an acidic environment below pH 6.8 due to their fermentation of lactose within the media, while *Aeromonas spp*. and *Pseudomonas spp*. generated a colourless media indicative of producing a basic environment, demonstrative of non-lactose fermenting species.

As observed on the plates after the 18-24 hour incubation period, *E. coli* colonies in all plates were distinctly rotund, shiny pink and non-mucoidal. *Klebsiella spp.* colonies were moderately large, mucoidal, distinctly round and pale pink. *Pantoea spp.* colonies were large, pink and globular while being enveloped by mucus, particularly around the bordering regions. *Pseudomonas spp*. colonies were sparsely distributed, small and almost translucent in colour. *Aeromonas spp*. colonies were much larger and more densely populated than seen for *Pseudomonas spp.*. These were also oblong and pale white, making them easy to differentiate. No suspicious, anomalous colonies were observed in any of the plates, eliminating possibility of contamination. These observations were made by eye, as shown in Figure 3.1.

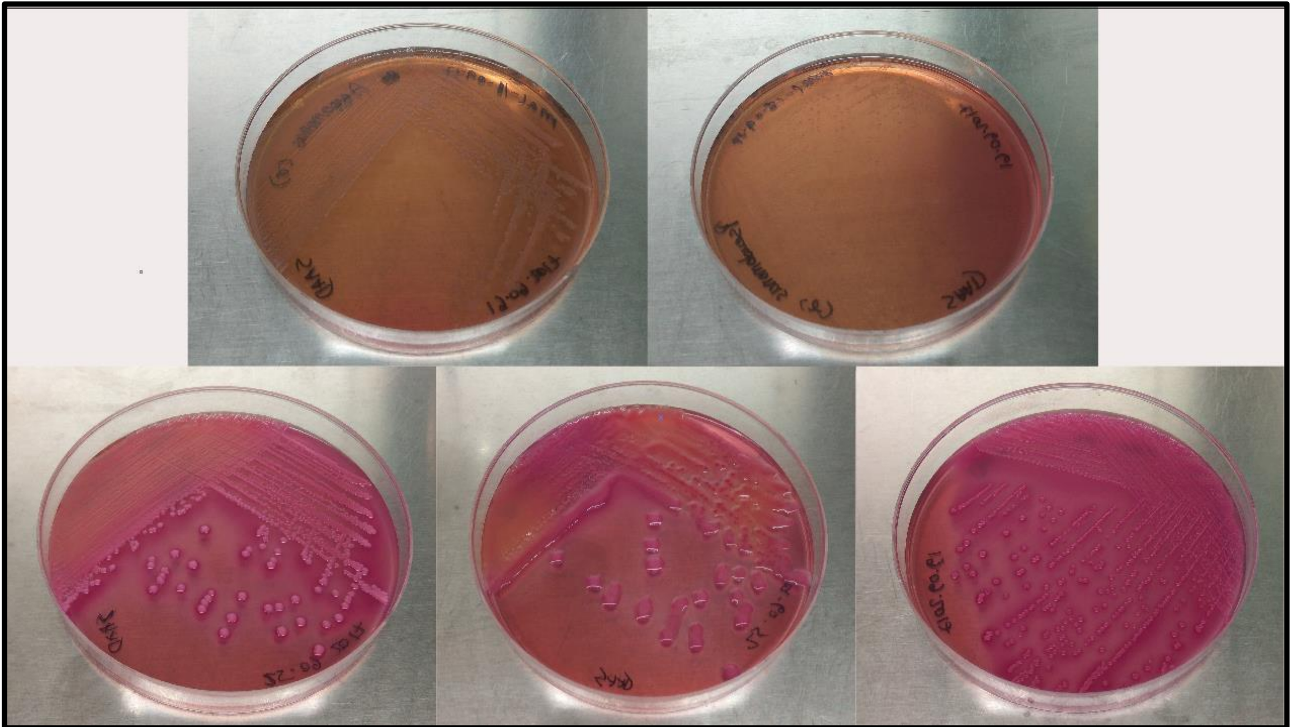

Fig. 3.1. Sub-culture of colonies on MacConkey plates.
In order (top left to bottom right): *Aeromonas spp*., *Pseudomonas spp*., *Klebsiella spp*., *Pantoea spp*., and ETEC grown on MacConkey agar media plates.

3.3 GRAM STAIN AND MORPHOLOGY

At 100x magnification under oil immersion lens, all examined strains were found to be stained colours between pink to deep red due to their ability to retain the counterstain Safranin, and not the Gram-stain itself. Moreover, all the specimens were characteristically rod shaped. Therefore, it was deduced that all the sample strains were in fact, Gram-negative bacilli as determined by the Gram-staining procedure, shown in Figure 3.2.

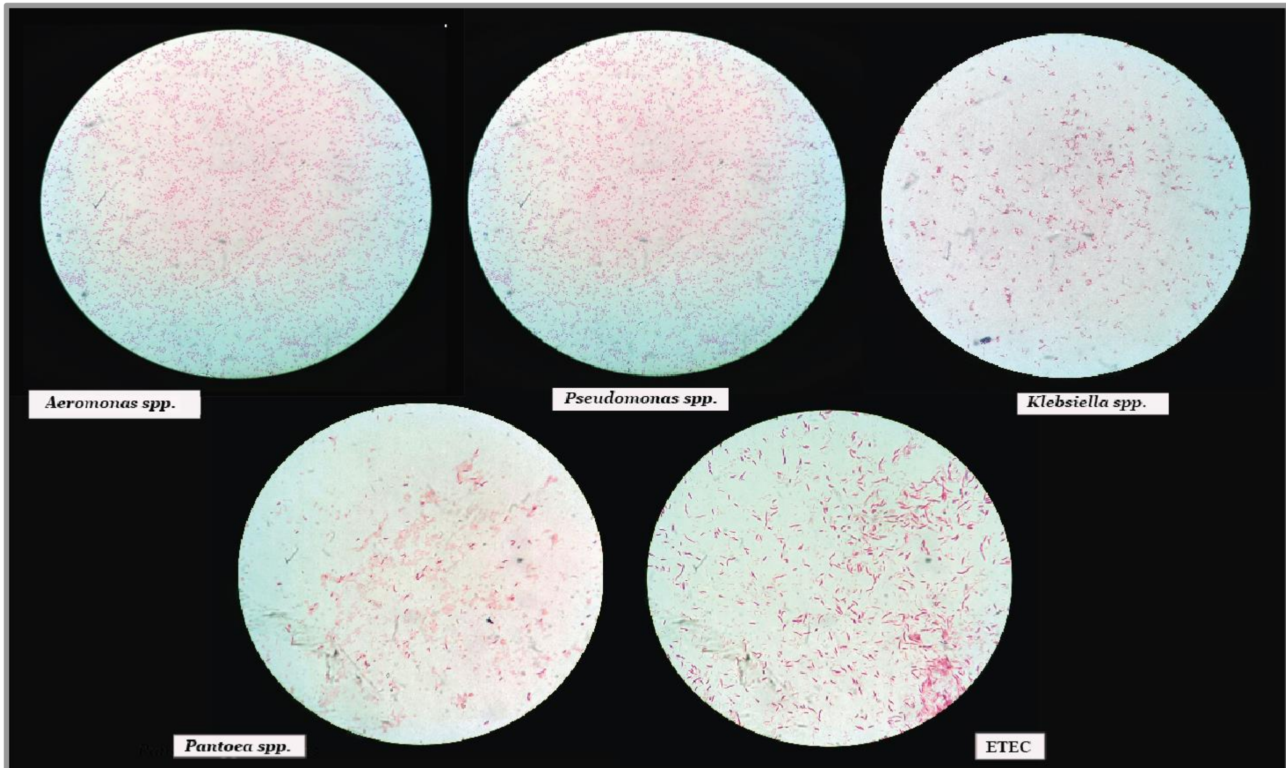

Fig.3.2. Gram-stained morphologies of culture specimens.
Gram-negative bacilli as observed under an electron microscope, stained deep pink and appear as longitudinal rods. The species from top left to bottom right are described as samples of *Aeromonas spp., Pseudomonas spp., Klebsiella spp., Pantoea spp.* and ETEC, as obtained from our clinical samples.

3.4. CHARACTERIZATION OF ETEC STRAINS USING PCR

For characterization of isolated *E. coli* isolates as being of the pathogenic enterotoxigenic subclass, PCR's for the strain specific Heat stable (STh and STp) and heat labile (LT) toxins were conducted. Single-bands for STh (*estA*), STp (*st1*) and LT (*eltB*) toxins were observed as 300 bp, 166 bp and 100 bp amplifications, respectively as shown in Fig 3.3. Heat Stable STp was found in 12 strains (100%), STh was found in 8 strains (66.7%) and LT in just 5 (41.7%). Thus, it could be reported that all *E. coli* specimens used in the study were ETEC strains.

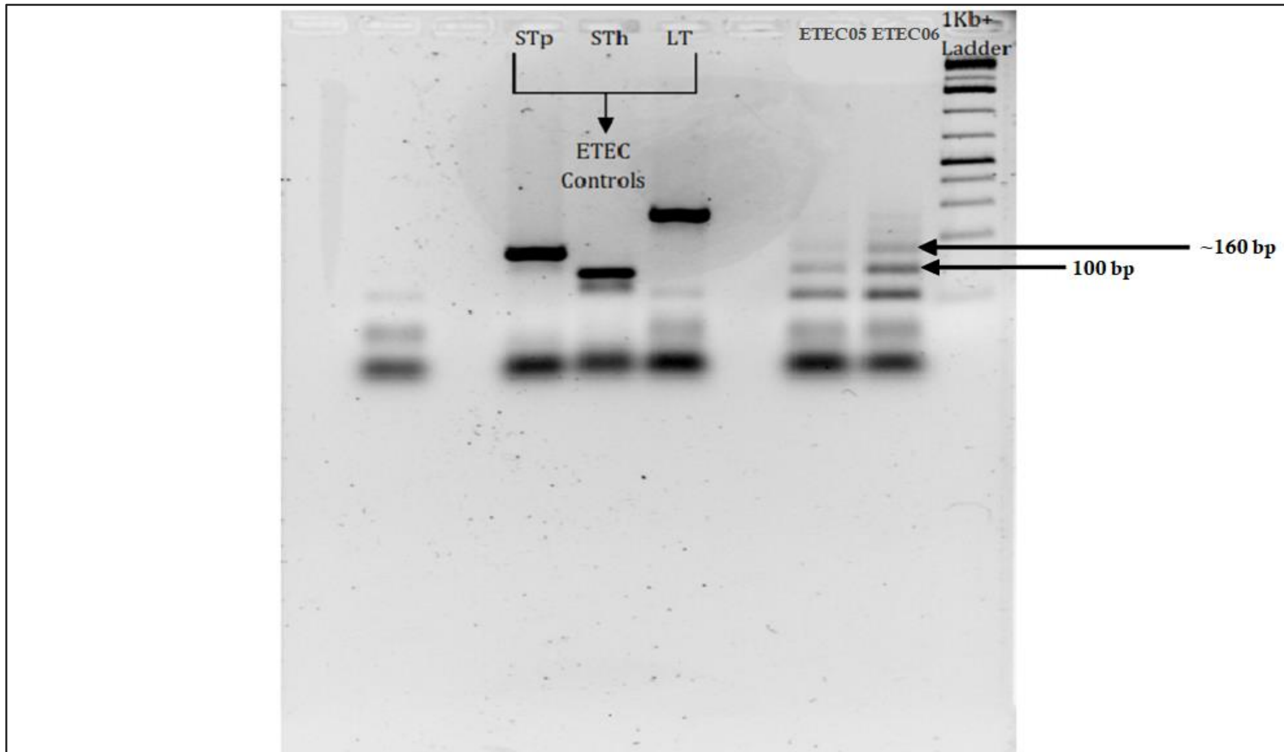

Fig. 3.3. ETEC PCR.

3.5 BIOCHEMICAL TESTS

All species in the study were interrogated using a battery of biochemical tests which are standards for the given strains' identifications. This began with the three media tests: Triple Sugar Iron agar (TSI), Citrate utility test and Motility-Indole-Urease (MIU) test. Observations of tests 3.4.1., 3.4.2 and 3.4.4 are illustrated in Figure 3.4 below.

3.5.1 Triple Iron Agar (TSI) Test

In the case of TSI tests, inoculation of ETEC strains, *Pantoea spp.* and *Klebsiella spp.* revealed acidic slant and butt regions of the agar as observed by a colour change from red to yellow of the phenol red pH indicator in the media. This lends to the fact that these species are fermenters of glucose, lactose and/or sucrose. Moreover, they were found to generate gas as observed through the production of bubbles or cracks in the medium, but not hydrogen sulfide gas via conversion of ferrous sulfate which would have turned the media black. *Pseudomonas spp.* and *Aeromonas spp.* neither generated any colour change in either the slant or butt regions, inferring they do not ferment any of the three sugars supplemented in the media, nor did they produce observable gases or H_2S. These results are shown in Figure 3.4.

3.5.2 Simmons Citrate Test

Within 24 hours of incubation, *Pantoea spp.* and *Klebsiella spp.* isolates tested showed visible growth on the slants, and turned the bromothymol blue indicator in the agar medium a deep Prussian blue from its original Forest Green colour indicating that the pH had increased from neutral to 7.6 or more. This was not the case for ETEC*, neither Aeromonas spp. nor Pseudomonas spp.* as the media as inoculated by all three species remained green with little to no visible growth making it clear that they were unable to utilize Citrate as the only carbon source. These results are displayed in Figure 3.4.

3.5.3 Motility Indole Urease (MIU) Test

All strains observed were urease enzyme negative as they demonstrated no colour change indicating the environment was acidic and not basic, which would have otherwise turned the phenol red indicator within the media pink through breakdown of urea to generate alkaline ammonium carbonate.

Except for ETEC which turned the indole test paper containing Kovac's reagent pink, all other strains were determined to be indole negative as no colour change was observed. Diffused, hazy growth around the stab-line in the semi-solid agar media was observed for ETEC, *Pseudomonas spp*., *Aeromonas spp*, *Pantoea* samples suggesting they were all motile specimens in possession of flagella. *Klebsiella* specimens however were non-motile as the stab-line was relatively clear of diffusion. These results are shown below in Figure 3.4.

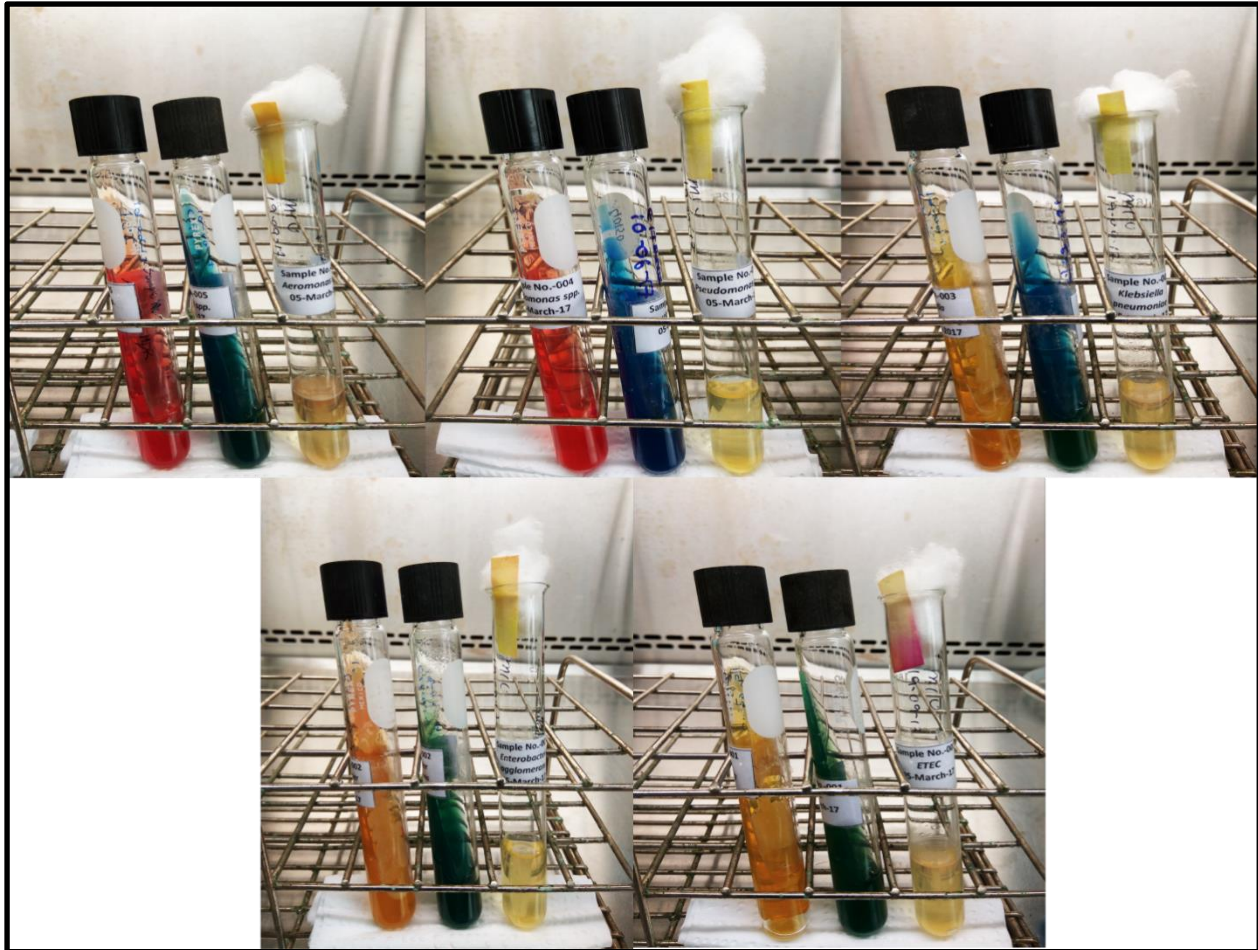

Fig. 3.4. Biochemical tests of cultured specimens.
These were conducted in series of threes (negative controls not shown); for the characterization of each species, in order of *Aeromonas spp*., *Pseudomonas spp*., *Klebsiella spp*., *Pantoea spp*. and ETEC species (from top left to bottom right). In each triad, the tests correspond to (from left to right) TSI, Simmons Citrate and MIU tests.

3.5.4 Catalase and Oxidase Tests

Only the *Aeromonas spp.* and *Pseudomonas spp.* specimens were tested for Catalase and Oxidase activity. 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. Supplementarily, 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. These reactions are shown in Figure 3.5.

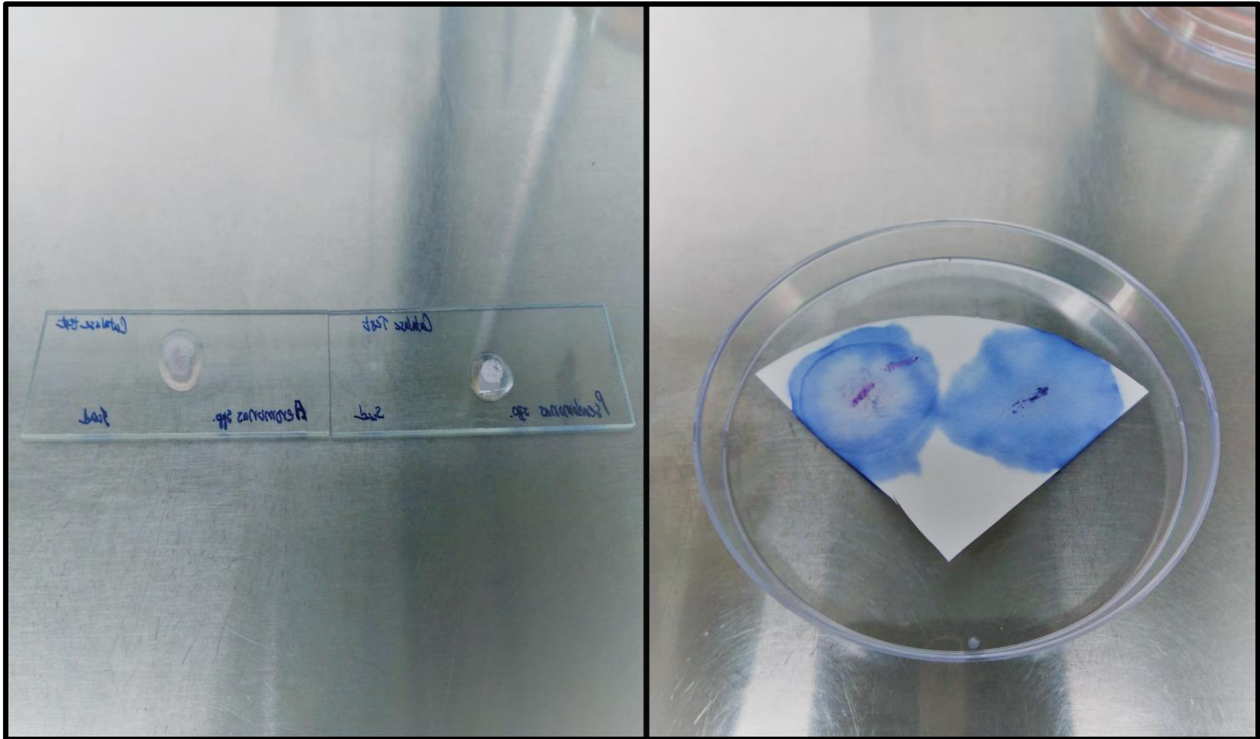

Fig. 3.5. Catalase and Oxidase tests.
Catalase tests (left) and Kovac's Oxidase tests as conducted on *Aeromonas spp.* (left) and *Pseudomonas spp.* (right) in each image.

3.5.5 Analytical Profile Index (API 20E)

The identification of each of the two *Pantoea* and *Klebsiella* strains were obtained after scoring according to the apiweb reference database. This revealed the identification up to a species level, showing that the *Pantoea* strains were in fact, *Pantoea agglomerans* and that the *Klebsiella* strains were of the *Klebsiella pneumoniae* variety. These results for the test series are shown in the Figures 3.6 to 3.9 below.

Fig. 3.6. Reactions of API 20E for *Pantoea agglomerans.*

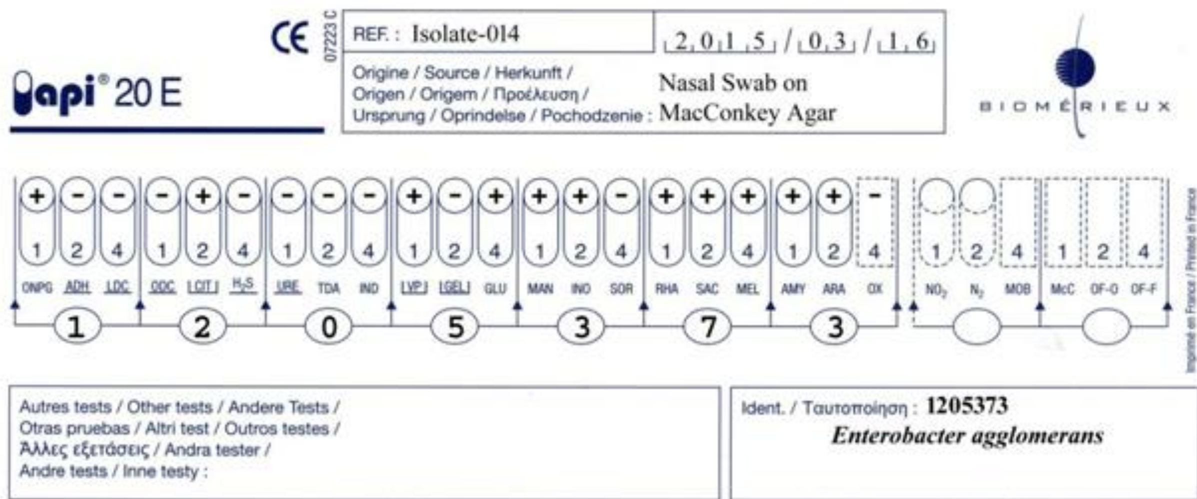

Fig. 3.7. API 20E reference tabulation for the positive identification of *Pantoea agglomerans* (*Enterobacter agglomerans*).

Fig 3.8. Reactions of API 20E for *Klebsiella pneumoniae*.

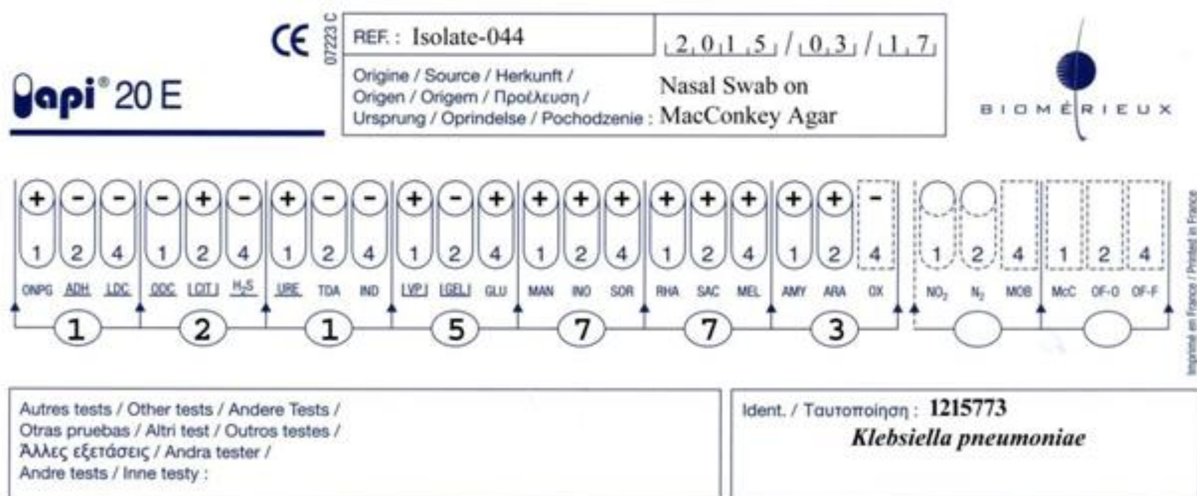

Fig. 3.9. API 20E Reference tabulation for the positive identification of *Klebsiella pneumoniae.*

3.6 ANTIMICROBIAL SENSITIVITY TEST

3.6.1 Summary of Total Resistance Phenotypes

Up to 16 different drugs were tested amongst 10 different drug classes. Due to the large number of strains and antibiotics tested, these results are summarised 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.

Thus, according to the measurements of the zones of inhibition of each antibiotic disk of the disk diffusion tests (as illustrated in Figure 3.10 below), a startling 100% of antibiotics tested were resisted by a single strain of *P. agglomerans*. Additionally, *K. pneumoniae* strains showed resistance to 10 out of 12 antibiotics tested, while *Aeromonas spp*. and *Pseudomonas spp*. were resistant to only 2 out of the 12 tested. All ETEC strains tested were MDR isolates showing resistance to most of the antibiotics across the board.

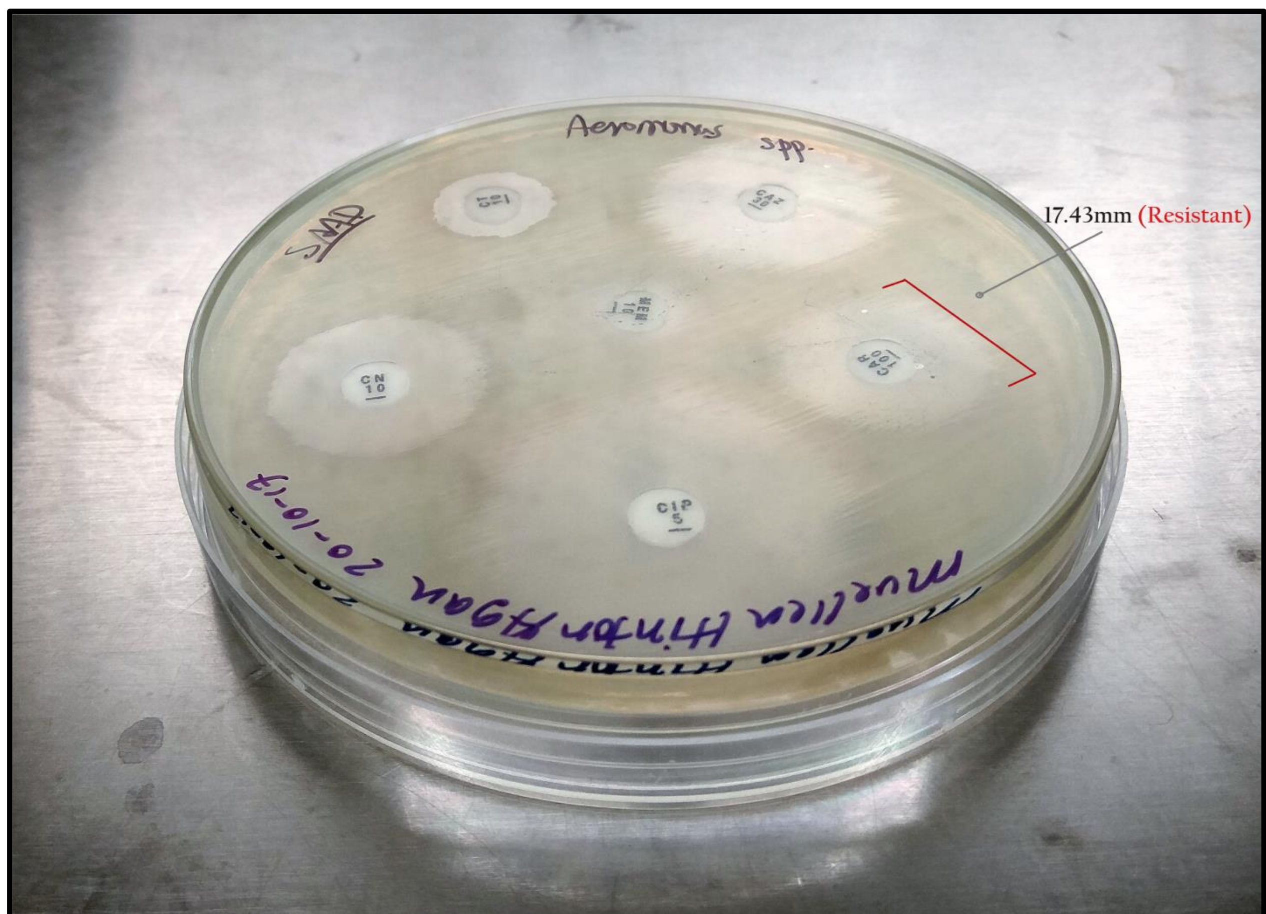

Fig. 3.10. Zone of inhibitions observed and measured from Mueller-Hinton Agar AST plates. As measured by the diameter of the zone, the strain is observed to be within the resistant (R) limit against carbenicillin (CAR).

3.6.2 Phenotypic Results for ETEC Strains

Sixteen drugs were tested against 12 ETEC specimens as shown in Fig. 3.11. On the positive, of the 12 ETEC strains investigated, only 16.7% of the population demonstrated intermediate level resistance phenotypes to the two aminoglycosides amikacin and streptomycin, however for the latter drug, complete resistance was also found in 1/3rd of the remaining strains. The 3rd generation cephalosporins fared far worse, with two-thirds of the strains showing either intermediate or full resistance against ceftriaxone, with 50% exhibiting complete resistance; while cefixime was also completely resisted by a full two-thirds of our ETEC strains. Two-thirds of the strains also showed complete resistance to the second generation fluoroquinolone, ciprofloxacin; while the tetracyclines, doxycycline and tetracycline both were resisted by exactly half of the ETEC populations. The quinolones, norfloxacin and nalidixic acid, were resisted by 41.7% and 75% of the strains respectively. 58.3% of the strains also showed resistance to the combination antibiotic trimethoprim/sulfamethoxazole. The absolute worst responses were observed against the macrolides, azithromycin and erythromycin against both of which, 91.7% complete resistance was observed; while the penicillin, ampicillin was completely ineffective against 100% of strains.

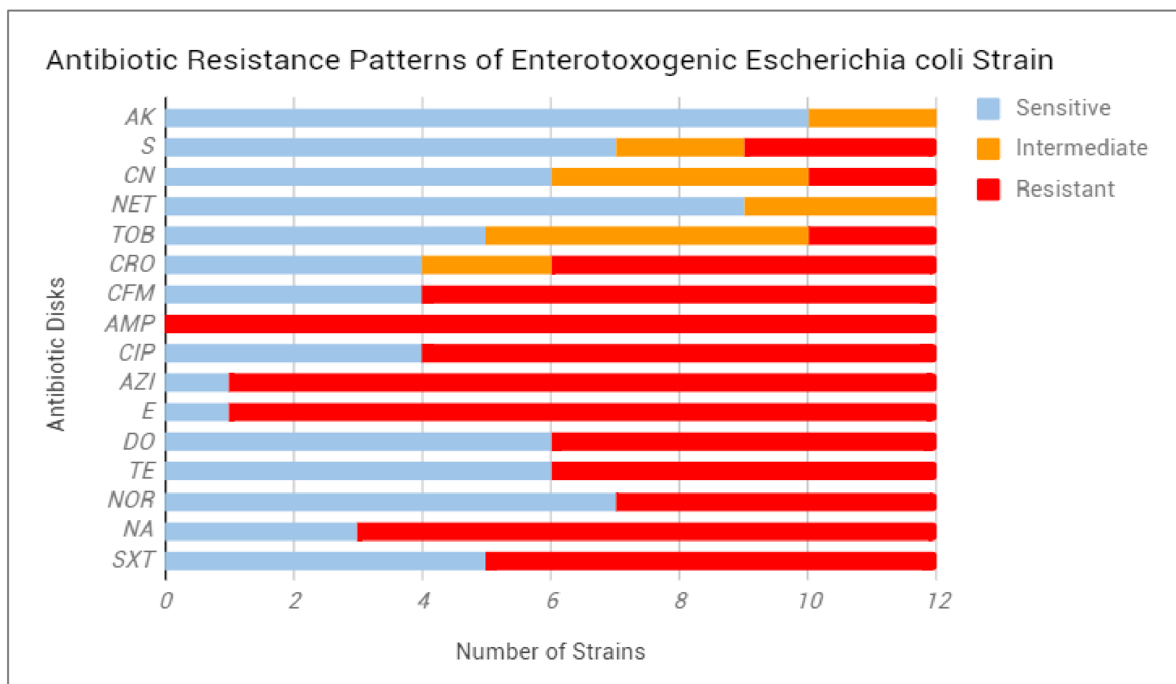

Fig. 3.11. AST resistance profile for ETEC strains (n=12) tested in study.

3.6.3 Phenotypic Results for *P. agglomerans* Strains

A total of 14 drugs were tested against two *P. agglomerans* strains as shown in Figure 3.12. Among them, 100% of strains were shown to be resistant to the carbapenem, imipenem; the macrolide, azithromycin; and the aminoglycosides, amikacin and streptomycin; albeit only at an intermediate (I) level for one of them, in case of the aminoglycosides. One of the strains (50%), was resistant to all fourteen of the antibiotics tested across the spectrum.

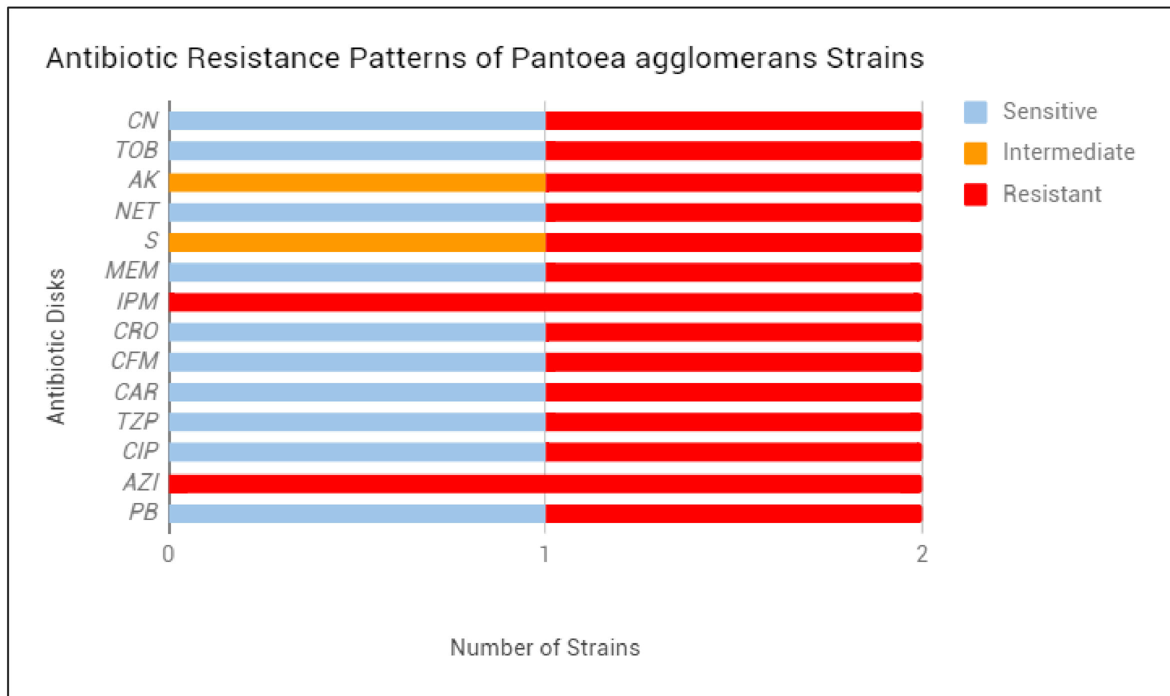

Fig 3.12. AST resistance profile for *P.* agglomerans strains (n=2) tested in study.

3.6.4 Phenotypic Results for *K. pneumoniae* Strains

A total of thirteen drugs were tested against two strains of *K. pneumoniae* as shown in Figure 3.13. Only 50% i.e. one strain was MDR. There was absolutely no resistance towards the carbapenems, imipenem and meropenem in either strain. 100% resistance was however found for the macrolide azithromycin and the aminoglycosides; streptomycin and amikacin, while the other antibiotics across the spectrum were resisted by 50% of our strains. The MDR strain (*K. pneumoniae (2)*, was resistant to all antibiotics but the carbapenems.

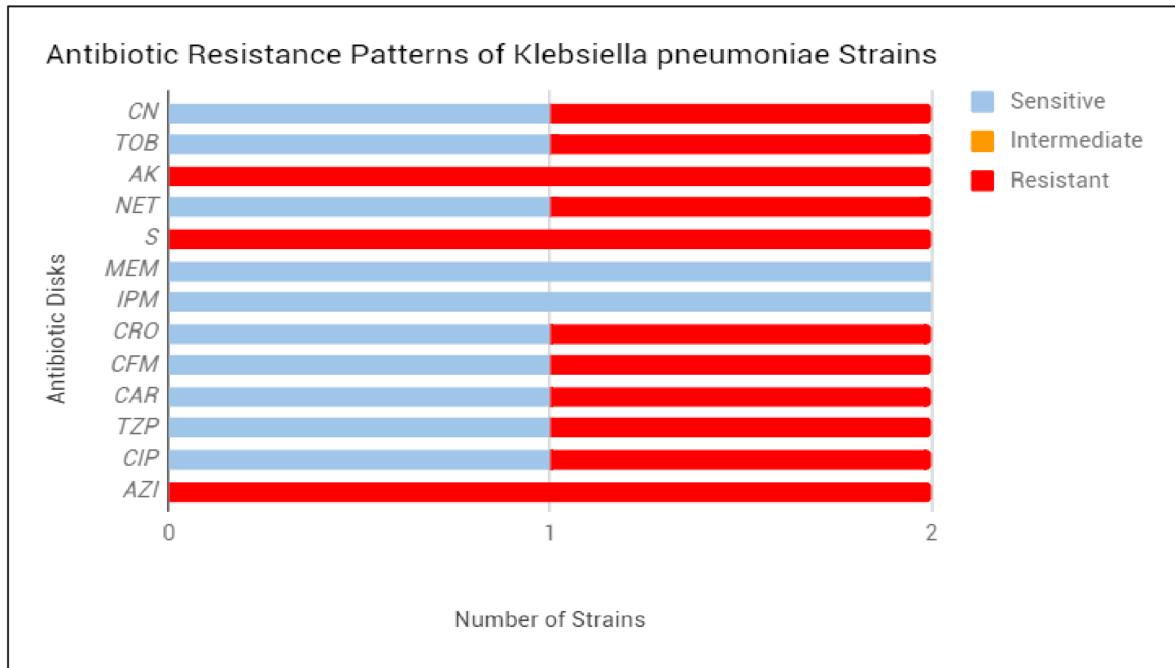

Fig. 3.13. AST resistance profile for *K. pneumoniae* strains (n=2) tested in study.

3.6.5 Phenotypic Results for *Pseudomonas* and *Aeromonas* Species

A total of 13 drugs were tested against single representative strains for *Pseudomonas spp.* and *Aeromonas spp*, each, as shown in Figures 3.14 and 3.15, respectively. Both strains displayed parallel results with full resistance to only two drug classes- the carbapenem, meropenem and the penicillin, carbenicillin among thirteen different drugs inspected.

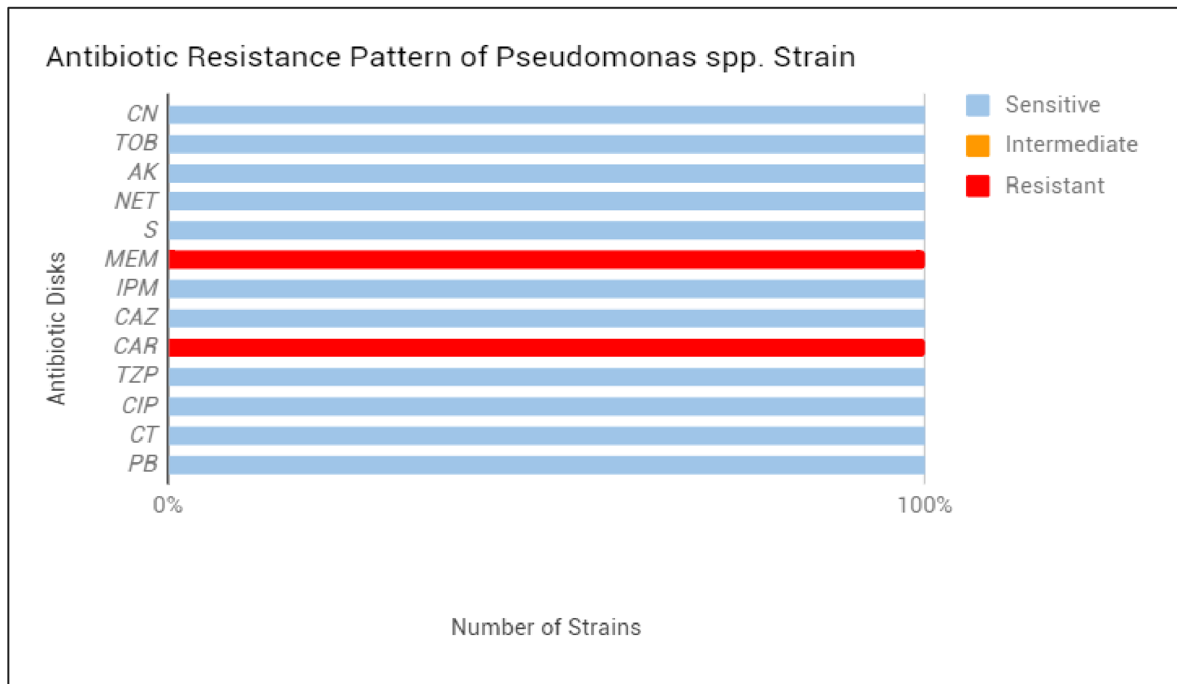

Fig. 3.14. AST resistance profile for *Pseudomonas spp.* strain (n=1) tested in study.

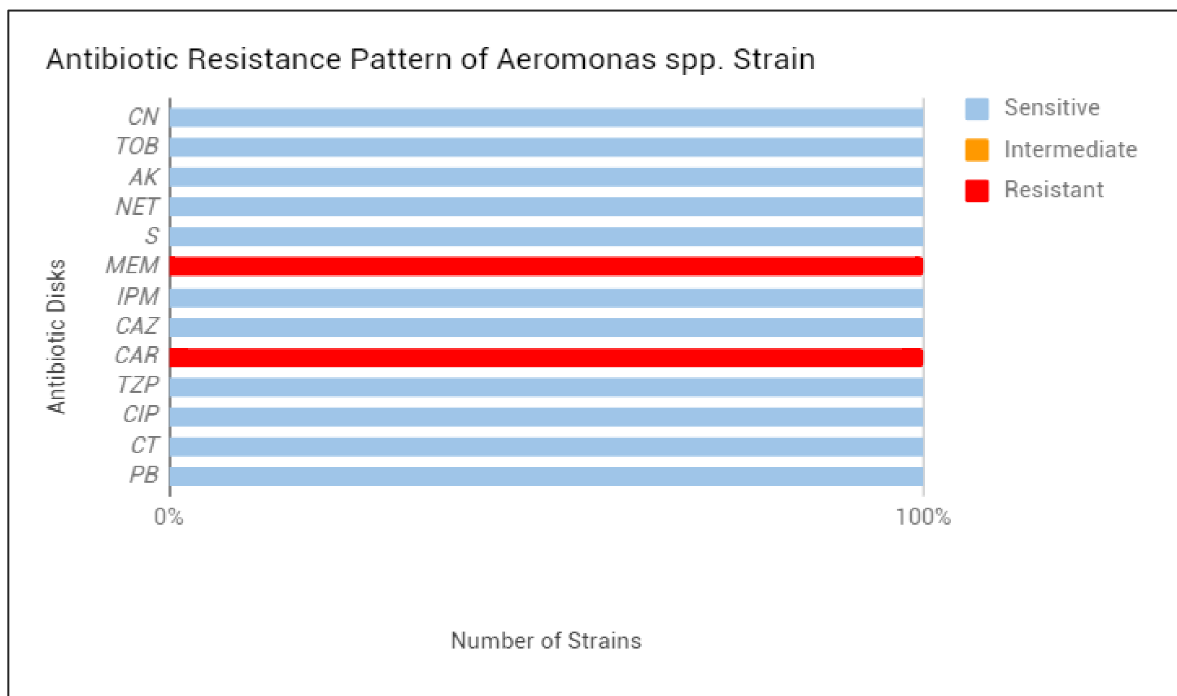

Fig. 3.15. AST resistance profile for *Aeromonas spp.* strain (n=1) tested in study.

3.6.6 Phenotypic Results against Aminoglycoside Antibiotics

Considering only the resistance phenotypes against the five aminoglycosides tested, the frequencies in the order of amikacin, gentamicin, tobramycin, netilmicin and streptomycin will be followed as shown in Figure 15. In that series, ETEC strains demonstrated resistance in frequencies of 16.6%, 50%, 58.3 %, 25% and 41.6%, respectively. *K. pneumoniae* and *P. agglomerans* strains responded equivalently with each other; with rates of 100%, 50%, 50%, 50% and 100%, respectively. As for both *Aeromonas* and *Pseudomonas* species, neither resisted any of the aminoglycosides at all.

3.6.7 Multi-Drug Resistance (MDR) Phenotypes

Among the five species examined, both *K. pneumoniae* species were MDR, resisting against at least 3 different drug classes. Only one of the *P. agglomerans* strains (*P. agglomerans (2)*) was MDR, being fully resistant to every antibiotic tested. As for the ETEC strains, all of them were found to be MDR forms. Neither *Aeromonas spp*. nor *Pseudomonas spp*. specimens were eligible to be classified as MDR types, as they could only resistant against two drugs from two drug classes. Therefore, 100% of ETEC strains; 100% of *K. pneumoniae* strains; 50% of *P. agglomerans* strains, 0% of *Pseudomonas spp*. strains and 0% of *Aeromonas spp*. strains demonstrated classic MDR phenotypes, with a total of 83.3% of all strains collectively labelled as such. These data are also represented in Figure 3.16.

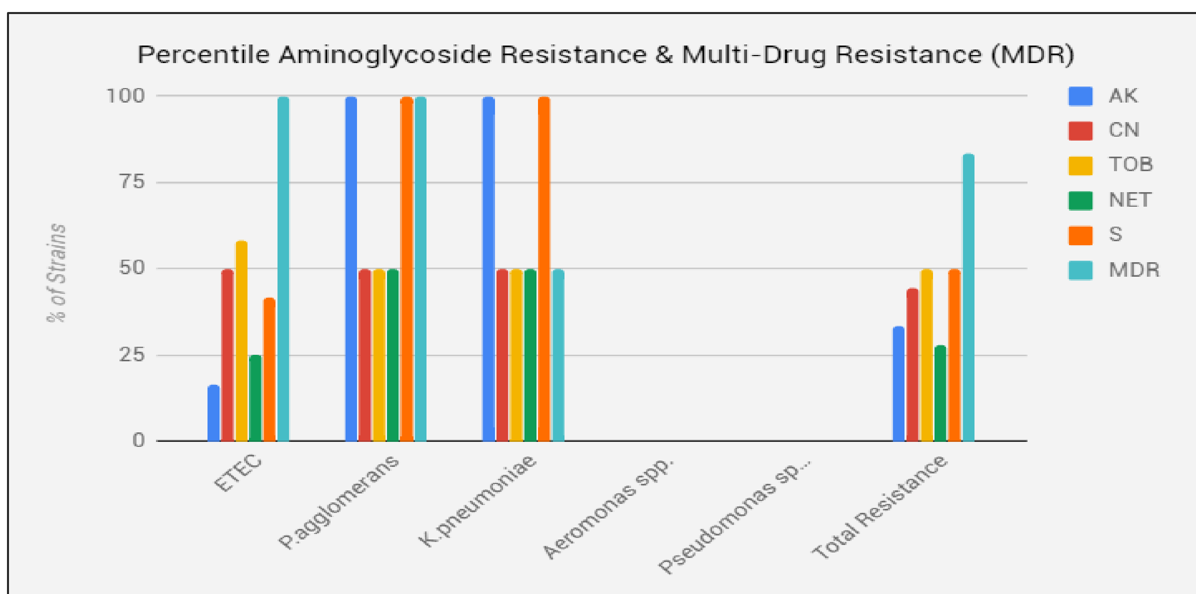

Fig.3.16. Aminoglycoside resistance frequencies and MDR strains.
Represented as percentiles, according to individual species, and as total specimen aminoglycoside resistance towards each drug. This figure also highlights the total percentage of multi-drug resistance for each strain, as well as a sum total of all samples.

Table. 3.1. Employed enzyme encoding resistance genes and their associated aminoglycoside substrate profiles (Shaw et al., 1993).

Resistance Genes	Expected Aminoglycoside Resistance Substrate
Aminoglycoside-*N*-acetyltransferases	
Aac(3')-II	Gentamicin, Tobramycin and Netilmicin
Aac(6')-Ib	Tobramycin, Netilmicin and Amikacin
Aac(6')-II	Gentamicin, Tobramycin and Netilmicin
Aminoglycoside-*O*-phosphotransferase	
Aph(3')-VI	Gentamicin and Amikacin
Aminoglycoside-*O*-adenylyltransferase	
Ant(3")-Ia	Streptomycin
Ribosomal rRNA Methyltransferases	
ArmA	Gentamicin, Tobramycin, Amikacin and Netilmicin
RmtB	Gentamicin, Tobramycin, Amikacin and Netilmicin

3.7 RESISTANCE GENE PCR

Enzymatic modification genes express for proteins that respond to specific aminoglycoside antibiotic substrates which may be inferred from the positive results of each gene from Table 3.1. Genotype analysis of each of the strains was conducted using standard PCR methodology. Of the total of seven genes examined, three AMEs were discovered by this method, while neither of the two ribosomal rRNA methyltransferases genes, *armA* and *rmtB* were observed. Two AMEs, *aac(6')-II* and *aph(3')-VI* were also noticeably undetected. The *ant(3)-I* gene was found only in ETEC 33 specimen (Figure 3.17). A*ac(3)-II* on the other hand was found in a total of three

strains, ETEC 06, ETEC 33 and *P. agglomerans* (2) (Figure 3.18) while the third positive gene, *aac(6)-Ib*, was most commonly detected as present in a total of five strains including ETEC 06, ETEC 12, ETEC 33, ETEC 41 as well as *P.agglomerans* strain (2) (Figure 3.19).

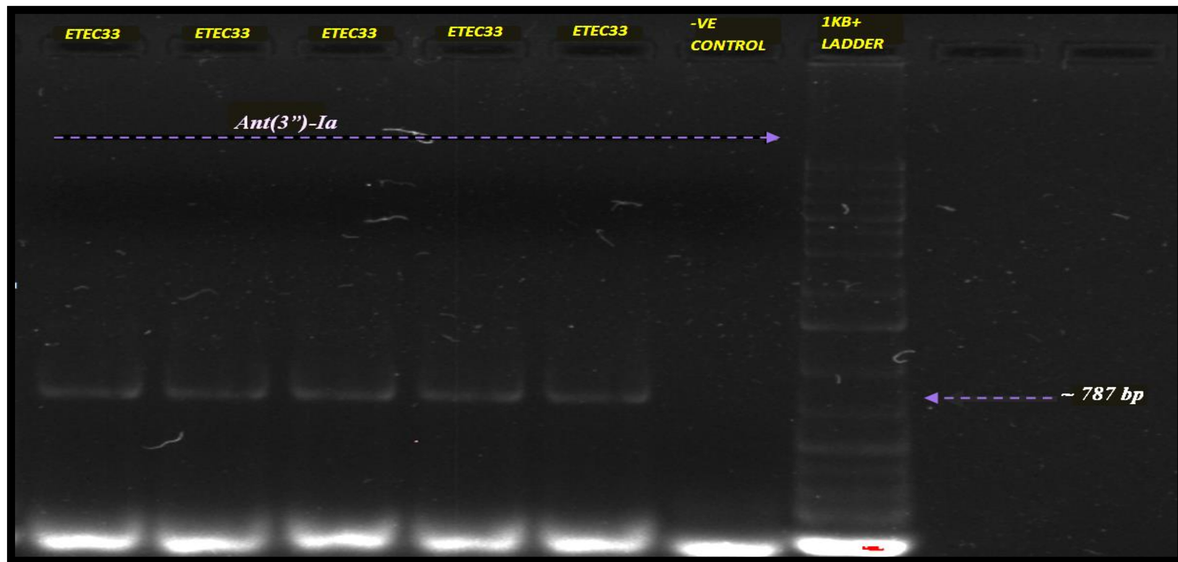

Fig. 3.17. PCR amplification of *ant(3")-Ia* gene.
Only the ETEC 33 strain samples shows clear, single bands in the agarose gel with a size of approximately 787 base pairs (bp), indicating the aminoglycoside resistance gene is present in the strain. No bands were found in the template negative reaction, but primer-dimers were formed, indicating the PCR was successful.

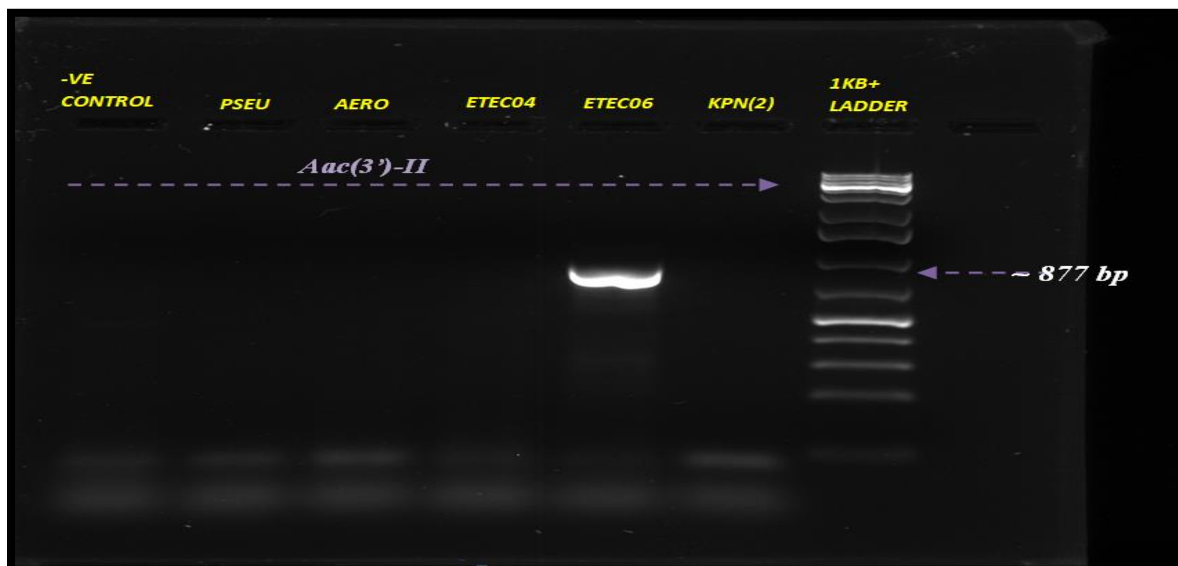

Fig. 3.18. PCR amplification of *aac(3')-II* gene.
Only the ETEC 06 strain sample shows a clear, single band in the agarose gel with a size of approximately 877 base pairs (bp), indicating the aminoglycoside resistance gene is present in the strain. No bands were found in the template negative reaction or in other tested strains during the run, but primer-dimers were formed, indicating the PCR was successful.

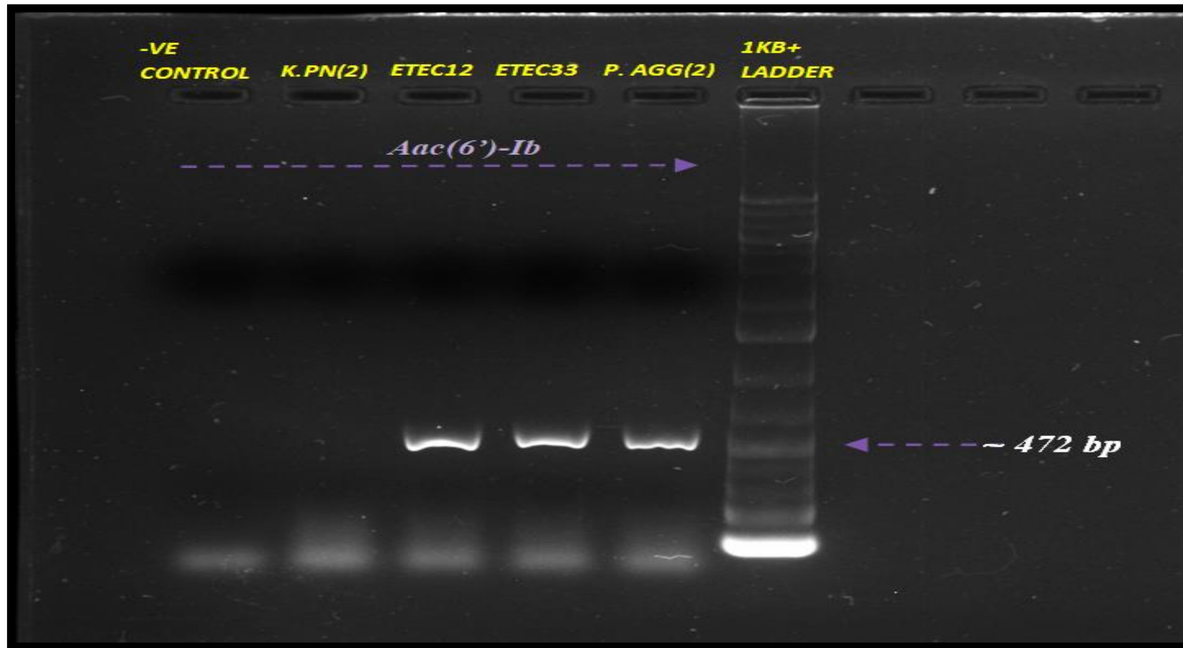

Fig. 3.19. PCR amplification of *aac(6')-Ib* gene.
Only the ETEC 12, ETEC 33 and *P. agglomerans (2)* isolate samples show clear, single bands in the range of approximately 472 base pairs (bp) in the agarose gel, indicating the aminoglycoside resistance gene is present in these strains. No bands were found in the template negative reaction or in the other tested strain during the run, but primer-dimers were formed, indicating the PCR was successful.

3.7.1 Sequencing Data

Sequence reads were initially assessed using Chromas Lite 2.4 software to determine a clean sequence and trimming off the illegible end regions which occur due to the mechanics of the Sanger method. The clarity of the chromatograms generated are illustrated in Figures 3.20, 3.21 and 3.22 below in three of the positive genes sequenced, and confirmed using NCBI BLAST, as exemplified in three MDR strains, *P. agglomerans (2)* for *aac(3')-II*; ETEC 06 for *aac(6')-Ib*; and ETEC 33 representing *ant(3")-Ia*, which will be highlighted for further analysis. The reads generated were clean, clear of dye blobs or significant ambiguous nucleotides, and were all of amplitudes that comply with the accepted norm.

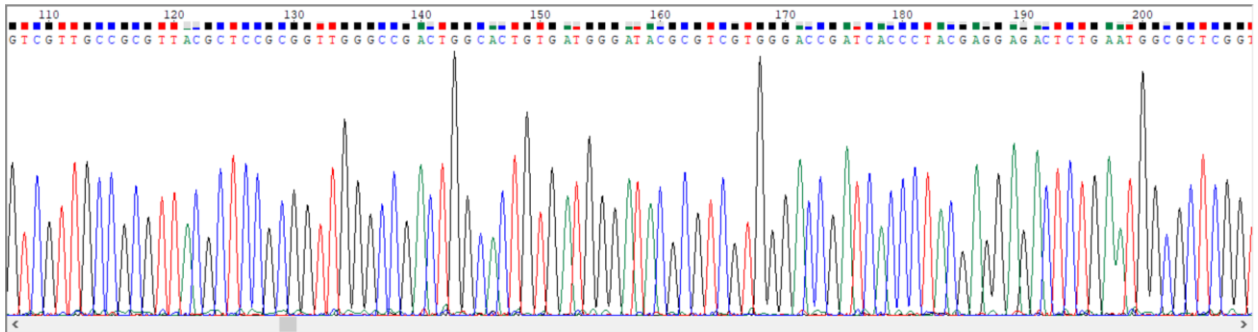

Fig. 3.20. Chromatogram of the sequenced gene *aac(3')-II* detected in *P. agglomerans*.
Visualised using Chromas Lite software- nucleotides are indicated by distinct colours: Adenine (green), Guanine (black), Thymine (red) and Cytosine (blue).

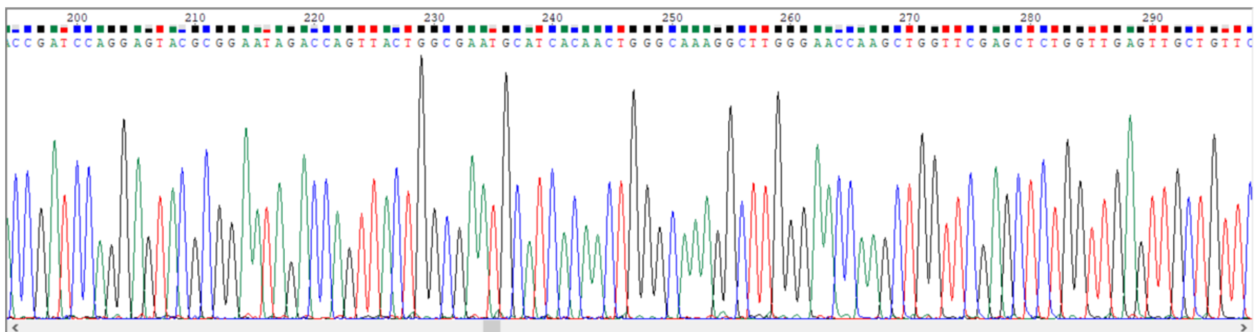

Fig. 3.21. Chromatogram of the sequenced gene *aac(6')-Ib* detected in ETEC 06.
Visualised using Chromas Lite software- the nucleotides are indicated by distinct colours: Adenine (green), Guanine (black), Thymine (red) and Cytosine (blue).

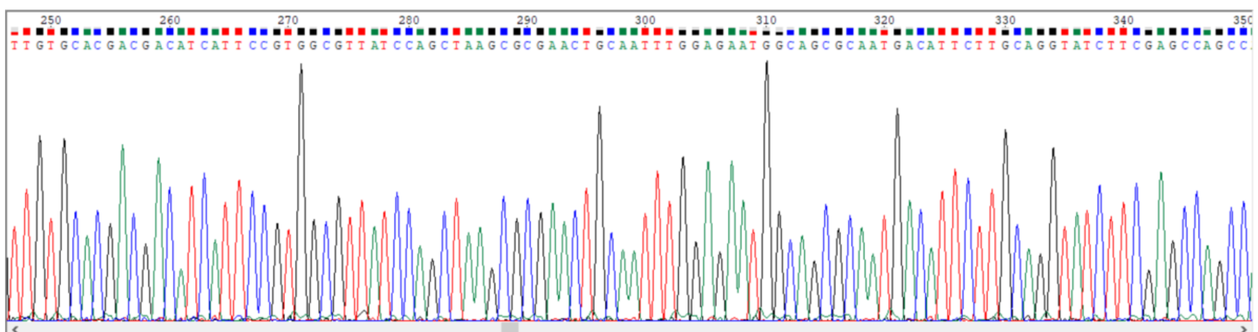

Fig. 3.22. Chromatogram of the sequenced gene *ant(3")-Ia* detected in ETEC 33.
Visualised using Chromas Lite software- the nucleotides are indicated by distinct colours: Adenine (green), Guanine (black), Thymine (red) and Cytosine (blue).

3.8 PHYLOGENETIC ANALYSIS

As Maximum Likelihood (ML) trees are character based rather than distance based and possess no root. As the true ancestor cannot be determined, the outgroup sequence of each strain in all phylogenetic trees described were considered as the hypothetical common ancestral gene. Time was also a factor not considered in the analysis as the General Time Reversible model was not applied, although the accuracy of the changes of the tree were considered to be the most

statistically robust through use of this methodology making this method the most well accepted. Thus, all trees are described according to the number of evolutionary changes involved, as the basis of evolutionary relatedness, as validated by a bootstrap value which ideally would be over 75% for each node.

3.8.1 Multiple Sequence Alignment (MSA)

Sequences compiled were aligned using MUSCLE method, which is a newer, faster and more refined, matrix-based method for aligning multiple sequences of proteins or DNA. Using this method, the alignments were conducted for each sequence by codons, resulting in a biologically accurate model of our protein coding regions. A section of the gene *aac(3')-II*'s alignment is shown below in Figure 3.23 as an example where gaps in sequence represent indels, substitutions and other changes.

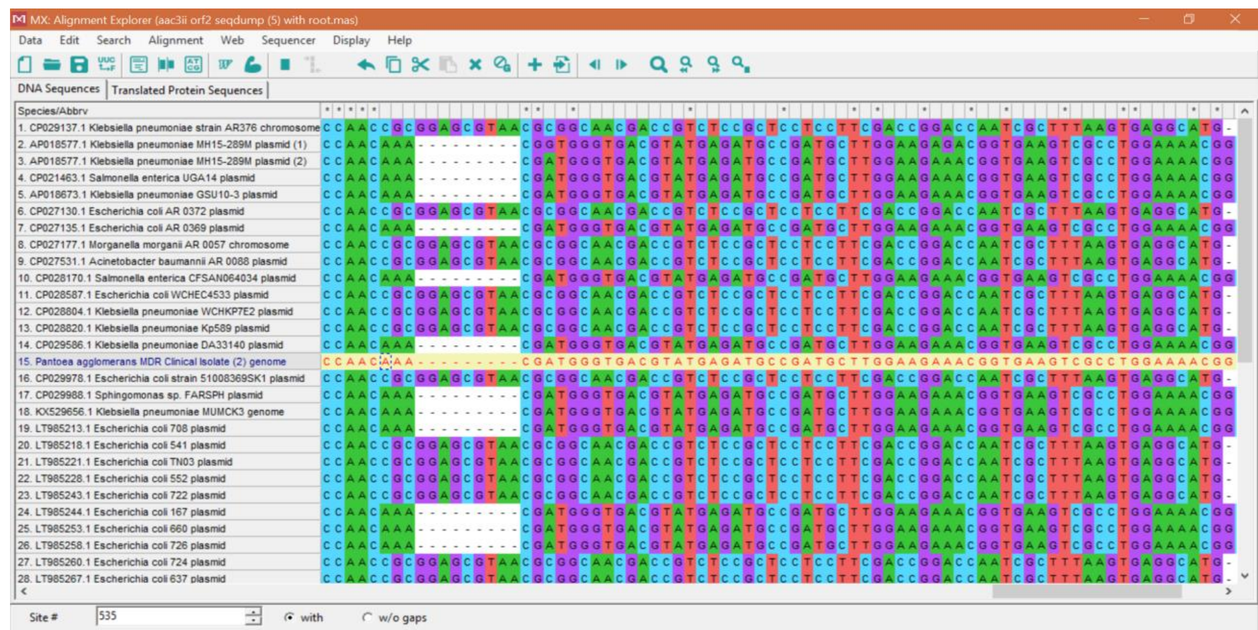

Fig. 3.23. MSA using MUSCLE algorithm of the sequenced *aac(3')-II gene's* ORF2 (in yellow). This was aligned with the most similar sequences in NCBI nBLAST database from top 50 strain hits.

3.8.2 *Aac(3')-II* Gene Phylogenetic Tree Description

The *aac(3')-ii* gene's (consisting of sequences with a 100% identity score and E values of 0.0) phylogram shown in Figure 3.24. can be described according to its topology which segments into two distinct clades, as shown with one outgroup. The bottom clade bifurcates at each point into new divergent forms, while the top clade is more complex, bifurcating into a sub-clade once more after the appearance of the gene in *K. pneumoniae MH15-289M* strain, where it stands alone within *K. pneumoniae AR376* strain as the tree continues diverging below. This entire tree

(based on the Tamura, 1992 evolution model which takes account for transition and transversion biases) is well supported statistically, as validated by the high bootstrap values that did not dip below 87% for any node. For the *aac(3')-ii* gene, the hypothetical ancestor sequence was rooted by using the the gene in strain *E. coli 660* and thus, may be considered the most evolutionarily distinct according to its nucleotide composition. This progenitor sequence was noticeably found within a plasmid, and our study sequence as demonstrated by the MDR strain of *P. agglomerans* differed from this by five consecutive changes. The *P. agglomerans* strain was genetically most closely related to species on the second clade, particularly with *K. pneumoniae DA33140* and *S. enterica CFSAN064034* on top, and *Sphingomonas spp*. *FARSPH* and *K. pneumoniae MUMCK3* strains below; where 3 of the four strains were confirmed to occur on plasmids, while the source of the fourth was not established. The most dominant strains in this gene appeared to be *E. coli* followed by *K. pneumoniae.*

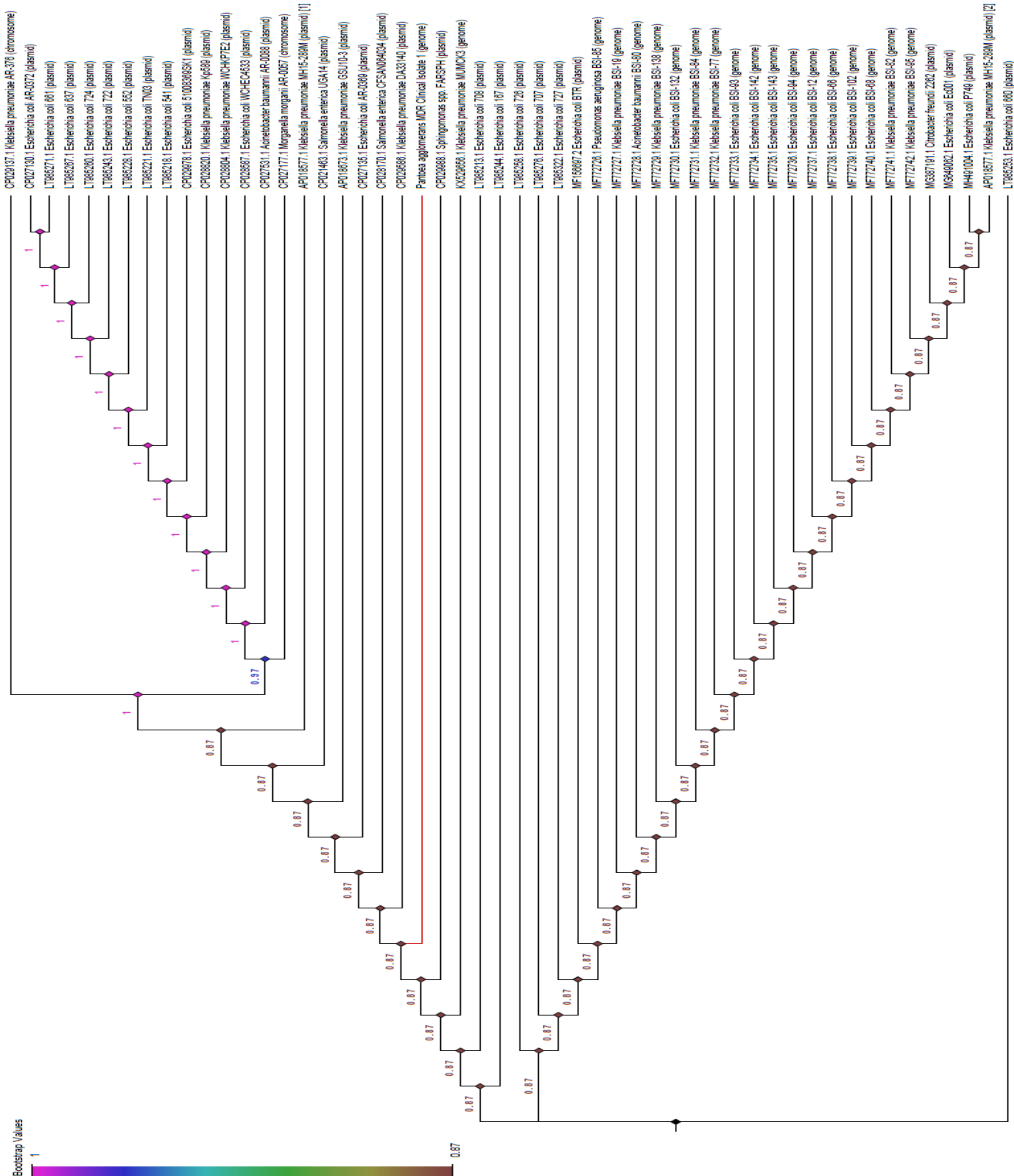

Fig. 3.24. Phylogenetic tree construction (T92 model) for *aac(3')-II* gene using ML method.
The sequenced gene was found in *P. agglomerans (2)* clinical isolate (as represented in the dendrogram by the only red branch). Bootstrap values represent statistical validity of nodes, where 100 tests were run. They are provided in decimal format to represent percentages (0 being lowest value and 1 being highest), as well as represented by colour hues to highlight the evolutionary likelihood of divergences formed at each node. The gene sequences are labelled in order of: Accession ID's, strain name, and source within genome in curved brackets (genome is written when source was unverified) and finally a number designating the copy number from a single strain. The scale on the right represents the number of divergences i.e. 3.0 events.

3.8.3 *Aac(6')-Ib* Gene Phylogenetic Tree Description

In case of the *aac(6')-ib* gene (consisting of sequences with a 99% identity score and E values of 0.0), the hypothetical ancestor was considered to be within the second copy of the *S. enterica MU1* strain which like the previous gene, occurred on a plasmid. According to the phylogram based on the Jukes and Cantor, 1963 evolution model (that considers equal rates for mutations and base substitutions) as shown in Figure 3.25, it took a total of seven evolutionary changes to develop our study sequence as demonstrated by the gene in ETEC *06* clinical isolate. This tree was far more complex than the previous, displaying lower resolution in the initial nodes and the bottom clade with bootstrap values in the 60-70 percentiles; however the statistical validity of the nodes continued to increase to viable levels as the tree branched further onwards. We can observe five clade divergences in total here, with our study strain's gene existing in the final major clade, diverging further on into other predecessors. The gene we found was determined to be most closely evolutionarily linked to *A. hydrophila RJ604* and an *A. caviae* on top, and *E. cloacae AR 0093* and the second sequence found in *A. salmonicida S121* below it. The sources varied from plasmids, class 1 integrons to an unknown genomic source. This tree is composed of a diverse group of bacterial species, with no prominent dominant species.

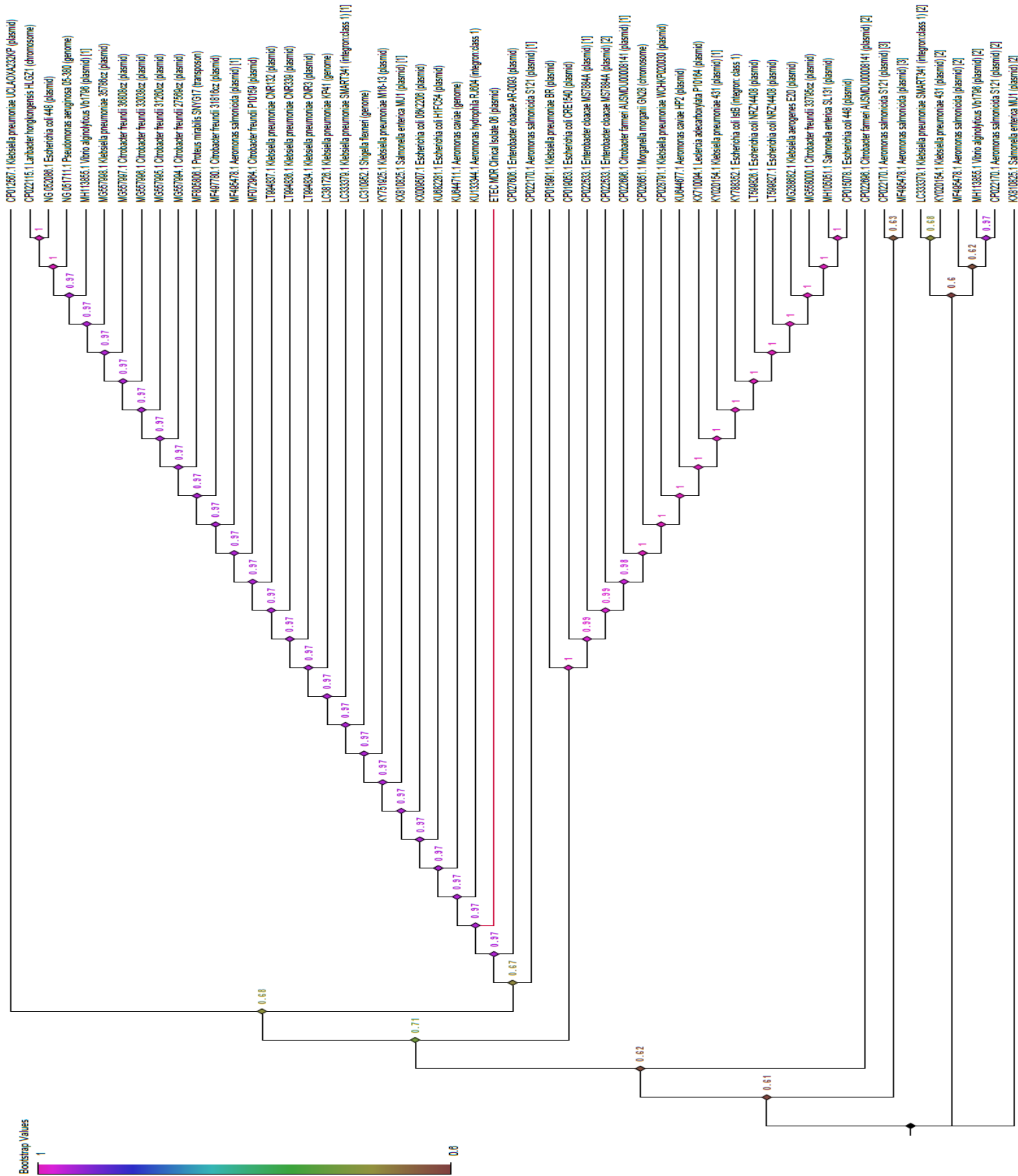

Fig. 3.25. Phylogenetic tree construction (JC63 model) for *aac(6)-Ib gene* using ML method.

The sequenced gene was found in ETEC 06 clinical isolate (as represented in the dendrogram by the only red branch). Bootstrap values represent statistical validity of nodes, where 100 tests were run. They are provided in decimal format to represent percentages (0 being lowest value and 1 being highest), as well as represented by colour hues to highlight the evolutionary likelihood of divergences formed at each node. The gene sequences are labelled in order of: Accession ID's, strain name, and source within genome in curved brackets (genome is written when source was unverified) and finally a number designating the copy number from a single strain. The scale on the right represents the number of divergences i.e. 4.0 events.

3.8.4 *Ant(3")-Ia* Gene Phylogenetic Tree Description

The third gene sequenced in the study, *ant(3")-Ia* (consisting of sequences with a 99% identity score and E values of 0.0) produced the most intricate tree developed from the Kimura, 1992 evolution model (that takes into account transitions, transversions and G+C biases) as shown in Figure 3.26, due to the high number of related sequences populated; although it is largely well supported according to the bootstrap values generated. This tree diverged forming a total of seven distinct clades. From our hypothetical ancestral gene rooted in *A. baumannii XH386* strain found in its chromosome, it took a total of nine evolutionary changes to produce the form found in our study which diverged onwards into several more forms in various isolates down the line. The sequenced gene can be most closely related to the ones found in the first sequence found in strain *E. coli H8* and *K. pneumoniae AR 0140* on top and *K. pneumoniae KPOsh-2k* and *E. coli 552*. Other than *K. pneumoniae KPOsh-2k* which was found on a class 1 integron, the other genes were reported to be found in plasmids. This tree is dominated by *E. coli* strains, *Aeromonas, Salmonella* and *K. pneumoniae* strains.

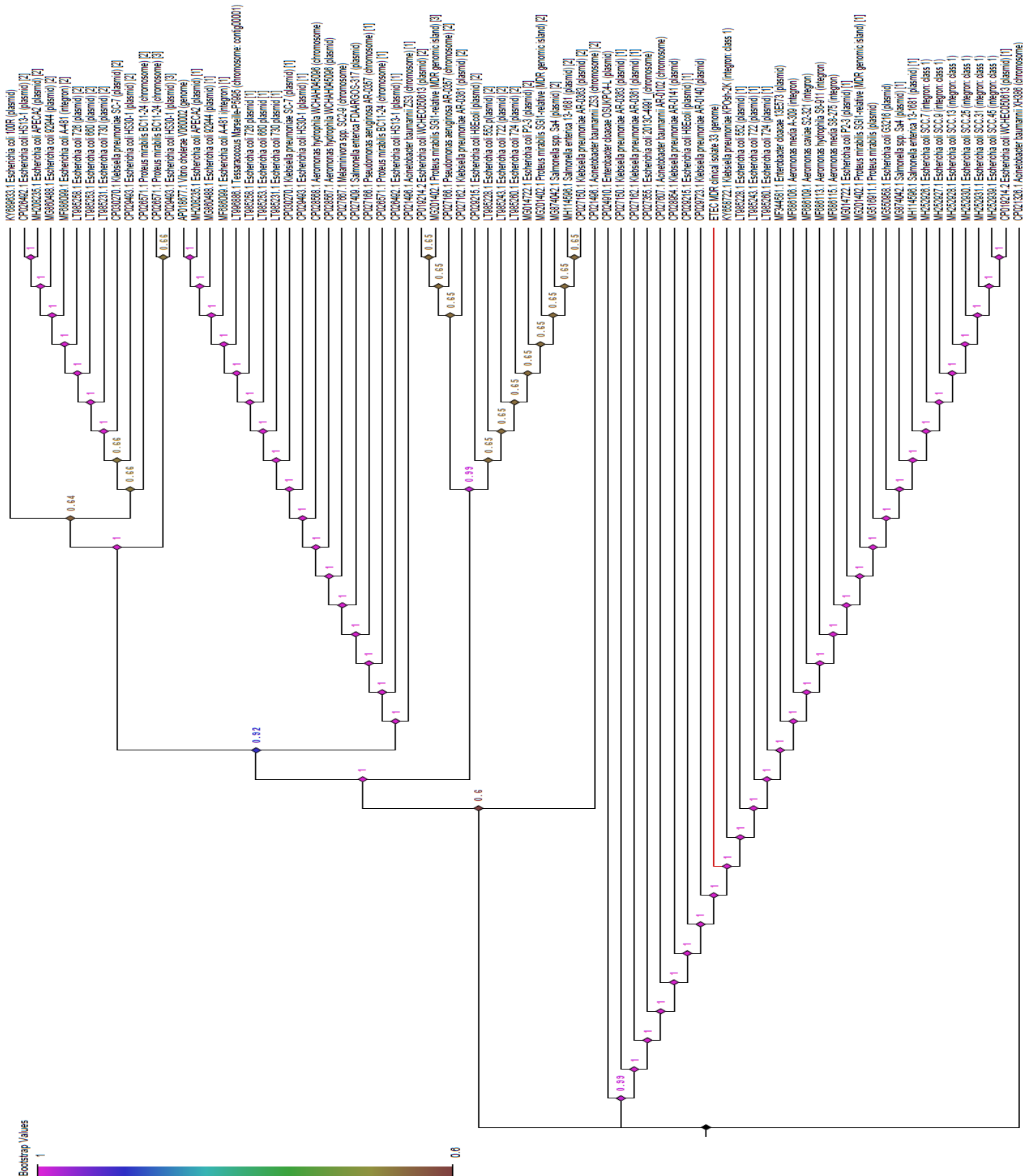

Fig. 3.26. Phylogenetic tree construction (K92 model) for *ant(3')-Ia gene* using ML method.
Phylogram generated from gene found in ETEC 33 clinical isolate (as represented by the only red branch). Bootstrap values represent statistical validity of nodes, where 100 tests were run. They are provided in decimal format to represent percentages (0 being lowest value and 1 being highest), as well as represented by colour hues to highlight the evolutionary likelihood of divergences formed at each node. The gene sequences are labelled in order of: Accession ID's, strain name, and source within genome in curved brackets (genome is written when source was unverified) and finally a number designating the copy number from a single strain. The scale on the right represents the number of divergences i.e. 4.0 events.

Chapter 4

DISCUSSION

4. DISCUSSION

4.1 STATUS OF RESISTANCE IN BANGLADESH AS DETERMINED BY CURRENT STUDY

Undoubtedly, among the 5 species in our eclectic range of clinical isolates, *K. pneumoniae, Pseudomonas spp.* and ETEC species compete for their place among the most prolific pathogens encountered- regularly being discovered around the world in nosocomial settings, especially in ICU wards of hospitals as we have seen. Even *P. agglomerans* and *Aeromonas* species are being isolated more frequently in recent times from hospitalized patients. Moreover, MDR strain frequencies are a present threat in the region making them a prerequisite focus for surveillance studies such as this one.

As it stands, in Bangladesh, ETEC species performs as a year-round pathogen; although its infections peak preferably post-monsoon and during the warmer months spreading easily through contact with contaminated food and water. Respiratory infections caused by *K. pneumoniae* are another major cause of morbidity and mortality in the region with activity peaks observed (in descending order) during the months of September, October, November and January in Bangladesh. *P. agglomerans* infections however were observed to circulate less frequently, although is speculated to peak around August (Bhuiyan et al., 2017). Peak data for the region could not be found for *Pseudomonas* or *Aeromonas* species.

In this particular study, through conducting antibiogram testing for each individual strain against a plethora of drugs popularly prescribed in Bangladesh; it was determined that most were multidrug-resistant varieties, albeit all were resistant to at one member of least two classes of antibiotics. The *Pseudomonas* and *Aeromonas* isolates were the only isolates susceptible to all but two β-lactam antibiotics with the rest being far better equipped. Yet, overall, the most threatening responses were touted by strains of ETEC, *K. pneumonia* and *P. agglomerans*.

In the case of diarrhoeal infections, due to the emergence of MDR strains, the treatment regimen has since shifted to include broad-spectrum antimicrobials consisting of either azithromycin for children; or erythromycin, trimethoprim-sulfamethoxazole, ofloxacin, ciprofloxacin, norfloxacin, doxycycline or rifamycin for adults, which remains effective still, according to Qadri et al., 2005. However we have found that in the case of ETEC-specific diarrhoeal infections, some of these drugs may no longer be useful. In our study, the pathogen had a wide distribution of response to various drug classes, most notably demonstrating high resistance towards macrolides, many cephalosporins, nalidixic acid and ampicillin; the latter to which it was completely ineffective in

100% of our strains. Unfortunately the incredibly high resistance to ampicillin and macrolides is an issue, as it these are common treatment agents for diarrhoea in both children and adults (Qadri et al., 2005). This is in concordance with the fact that ampicillin is also one of the overall most distributed antibiotics by clinicians in the country (Rashid et al., 2017). As for *K. pneumoniae and P. agglomerans,* they were completely resistant to the macrolide azithromycin while only one strain of ETEC was susceptible to it. We have also decided to concur with the findings of Faiz and Bashar, 2017 in demonstrating moderately high rates of resistance by these species towards cephalosporins in all three of these strains reflecting the high rates of usage reported in Bangladesh.

In fact, a startling 83.3% of all isolates studied were determined to be MDR forms, capable of resisting at least 3 classes of antimicrobials but more often impeding a wider array of antimicrobial classes in addition- the most dangerous strain among them being impervious towards every single drug; irrespective of antibiotic class. Of these specimens of interest, one was *K. pneumoniae (2)*, another was *P. agglomerans (2)*, while the rest comprised 100% of ETEC strains isolated. Only one strain of *Pantoea agglomerans (2)* was completely impervious to every single one of the 14 drugs trailed across 10 different antibiotic classes. Even a single such strain is a threatening presence in such a populated society, especially with our knowledge of the potential of bacterial resistance transfer dynamics via HGT.

However, as resistance mounted against aminoglycoside antibiotics was the focus of this project, it is paramount to elaborate specifically on their phenotypes, and associated genes found in this project within examined strains. Firstly, to investigate the phenotypic resistance profiles, five most popular aminoglycoside antibiotics applied in human medicine were included in the AST, in accordance of clinical significance and availability in Bangladesh. These comprised amikacin (AK), gentamicin (CN), tobramycin (TOB), netilmicin (NET) and streptomycin (S). Overall, it was found that 83% (n=15) of total clinical specimens investigated were resistant to at least one aminoglycoside antibiotic tested in this study. Terrifyingly, three MDR strains, *K. pneumoniae (2)*, *P. agglomerans (2)* and ETEC 04 isolate were resistant to all five aminoglycosides interrogated, making for 18% of the total sample population. However, 28% (n=5) of the population were also susceptible to all of them- these accounted for *Pseudomonas spp.*, *Aeromonas spp.;* and ETEC isolates 12, 44 and 46. Moreover, the latter two were remarkably resistant to every other drug other than the aminoglycosides which gleans a ray of hope for their applications in the country, still.

4.2 COMPARATIVE ANALYSIS OF AMINOGLYCOSIDE RESISTANCE PHENOTYPES WITH INTERNATIONAL REPORTS

4.2.1 Global Aminoglycoside Resistance Phenotype Frequencies in *P. Agglomerans*

Trends with resistance phenotype vary across the world during different time periods and according to the differing pathogens studied, with a focus usually directed towards those species with more pressing clinical agendas. For example, albeit being largely an opportunistic pathogen, *P. agglomerans* is especially dangerous for infections in children and most importantly, resistant strains of this species have been described in a report from places like Turkey, where 70% of strains expressed ESBL activity and also included several MDR strains resistant to ciprofloxacin, carbapenems, piperacillin, but were sensitive to the aminoglycoside, amikacin (Büyükcama et al., 2018). Another study in France that tested the aminoglycosides, amikacin, gentamicin and tobramycin found 100% of *P. agglomerans* strains to be completely susceptible to these, although it was not the case for several other antibiotics (Dele´toile et al, 2009). This last study contrasts with our findings, as we have determined 100% of our specimens to be fully resistant to amikacin, and 50% resistant to the other two antibiotics. Our results were more thus more closely linked with the Mexican study by Pococa et al. who cited 100%, 83% and 67% resistance of their studied strains, towards the popular aminoglycosides netilmicin, gentamicin and amikacin, respectively.

4.2.2 Global Aminoglycoside Resistance Phenotype Frequencies in *K. Pneumoniae*

Best known for an outbreak within the healthcare system in Israel in 2006 (Liang et al., 2015), the infamous respiratory pathogen, *K. pneumoniae* as according to one Egyptian study by El-Badawy demonstrated resistance levels of 60% and 26% of isolates resistant to the aminoglycosides gentamicin and amikacin respectively, in 2017; whereas in our study, we found 50% and 100% resistance towards these drugs. Similar to our study, here, *Escherichia coli* (40-78%) and *K. pneumoniae* (50-75%) exhibited much higher resistance rates towards aminoglycosides in Egypt, although contrasts with our study with high levels of resistance also found in *Pseudomonas aeruginosa* (71.1-93.3%), where we found none in Bangladesh. Further research on numerous Gram-negative bacteria, found the highest susceptibilities towards the kanamycin-derived amikacin with only 17.1% of strains resistant. Enhanced resistance towards amikacin were uncovered in places like India and Turkey however, as demonstrated by 55.1% and 49.7% of strains; although this still remained lower than for tobramycin which were in the orders of 83.6% and 82.4%, respectively. Gentamicin resistance in comparison had

demonstrated more variability, with rates of 32.6% for India but as high as 94.5% in Turkey (Gad, Mohamed and Ashour, 2011). One Chinese study looked at ESBL producing *K. pneumoniae* and found 24.7% resistance towards amikacin, with ESBL negative strains showing comparatively lower rates of resistance, highlighting the usual co-association predisposition of antibiotic resistance gene cassettes (Liang et al., 2015). In Sweden these rates are more tempered, as according to Hanberger et al., 2012, resistance to amikacin was determined to be as low as 0.5%, with comparatively higher rates for gentamicin at 7.8%. Interestingly, although world-wide it seems resistance towards amikacin is low today, for amikacin as well as for netilmicin, resistance were extremely high (14-92% and 25-90% respectively) in *Enterobacter*, *Citrobacter* and *Klebsiella* species in parts of South Africa, Europe and Latin America during 1997 (Miller et al., 1997). Although we had only used two strains in our study, it appears the statistics still closely resemble more recent data described for most of the developing world.

4.2.3 Global Aminoglycoside Resistance Phenotype Frequencies in *Pseudomonas* Species.

Another major nosocomial pathogen, the number one reported pathogen among the Pseudomonads is *P. aeruginosa,* occurring in 10-15% of all hospital acquired infections globally is also of interest as we have found one *Pseudomonas spp.* from our clinical sample box. Their infections rely heavily on aminoglycosides, as they are extremely reliable in antipseudomonal therapy (Teixeira et al., 2016). Accordingly, the fact that this species is the dominant agent in lung infections for Cystic Fibrosis patients and commonplace in burn and immunocompromised patients of ICUs makes resistance to aminoglycosides a major challenge. One early study in Spain discovered low incidences of resistance, with 13.5% of 152 *P. aeruginosa* isolates recalcitrant to two or more aminoglycosides, in particular amikacin, tobramycin, gentamicin and netilmicin (Esparragon et al., 1999). Another earlier study in the United States found among 66 specimens collected between the periods of 1976-1977; 79%, 36%; 48% and 82% resistant isolates to gentamicin; tobramycin; amikacin and netilmicin respectively, demonstrating how common they once were in the US and Europe (Weinstein et al., 1980). Closer to home, in Thailand resistance levels were more exaggerated, as shown by 100 isolates examined where 80% were resistant to all aminoglycosides tested. Of these strains, 100% were completely resistant to neomycin, kanamycin and spectinomycin. Rates for other important aminoglycosides were at 96% for tobramycin, 95% for gentamicin, 92% for amikacin and an alarming 99% for streptomycin (Poonsuk, Tribuddharat and Chuanchuen, 2013). Frequencies are also quite high in other developing countries like Egypt according to Gad, Mohamed and Ashour, 2011 who reported high levels of resistances between 71.1-93.3%. Across the world in

Venezuela, resistance patterns against the most common anti-pseudomonal drugs, tobramycin and amikacin revealed moderate levels of 30.7% and 29.9%, respectively among 137 strains, increasing somewhat when compared to an older, more comprehensive study between 1988-1998 where it was 23% and 19% to both these drugs respectively, and gentamicin resistance was at 27% (Teixeira et al., 2016). Although many other studies actually report their continued susceptibility towards aminoglycoside-based antibiotics, thus mimicking the findings of our own study (Igbinosa et al., 2012; Vally et al., 2004; Jones and Wilcox, 1995). Amikacin and tobramycin resistance appears lower, however, with such global variances; it is difficult to make any direct comparisons, and especially considering our study only employed a single specimen which showed no resistance to any aminoglycosides.

4.2.4 Global Aminoglycoside Resistance Phenotype Frequencies in *E. coli* Subtypes

E. coli is also a significant burden, for a multitude of diseases, including diarrhoeal diseases, bloodstream infections, pneumonia, wound and urinary tract infections (UTIs) (Odjana et al., 2018; Akhi et al., 2016; Ho et al., 2010). Where we found 66.7% of *E. coli* strains being MDR, a paper reported recently in Bangladesh by Rabbe et al., testing 50 specimens found that an overwhelming 90% were MDR strains. They also cited studies from the US and Iran where 7.1% and 77% of isolates were MDR, during the same period. Gentamicin was the only aminoglycoside tested however in this particular study, recording just 17% of isolates resistant to it; though they were additionally resistant to almost all other antibiotics of other classes (Rabbe et al., 2016). We found gentamicin incompetence to be almost three times higher in comparison, as 50% of *E. coli* strains were fully resistant. In our study, we found the least amount of resistance towards amikacin and netilmicin at 16.6 and 25%, respectively, with the other agents teetering between 41.6-58.3%. A study in the USA examined 10 isolates, finding resistance rates to be highly variable depending on the aminoglycoside used. For gentamicin, tobramycin, amikacin and netilmicin, they were of 70%, 10%, 20% and 0%, respectively (Weinstein et al., 1980). In Poland, as expressed in one study, the resistance proportions against amikacin, netilmicin, gentamicin and tobramycin revealed respective rates of 11.4%, 43.2%, 59% and 70.5% among 44 isolates with a total of 79.5% of aminoglycoside resistance specimens, exposing the sensitivity of amikacin as the most useful antibiotic (Ojdana et al., 2018). In Japan, they found 15.1% of strains resistant to gentamicin, 13.2% for tobramycin and 1.5% resistant towards amikacin among 212 isolates (Tsukamoto et al., 2013). In Iran, 25.23% of *E. coli* isolates out of 107 were resistant to aminoglycosides, 66.67% of which were also ESBL producers, and were used for AST studies. Here they saw only 1.87% of these resistant to amikacin and 11.2% towards gentamicin (Akhi et al., 2016) while in another Iranian study,

these rates were 6.15%, 3.62%, 23.18%, 21% and 24.6% in order for netilmicin, amikacin, kanamycin, gentamicin and tobramycin (Soleimani et al., 2014). With the exception of Poland, resistance appears lower in developed countries. These global rates are much lower than what we had observed. Therefore, it appears the common theme to be inferred is that that amikacin resistance is generally the lowest, followed by netilmicin in *E. coli*. This fact correlates with our own research findings.

4.2.5 Global Aminoglycoside Resistance Phenotype Frequencies of *Aeromonas* Species

The greatest paucity of data in literature exists in reference to infections caused by *Aeromonas* species, with most of the existing ones investigating non-human infectious sources. However, one study also used tissue from a wound infection to isolate two strains of *A. hydrophila* isolated from a single patient that found resistance to amikacin, gentamicin and tobramycin in 50% of them. This same paper did however mention much older papers- one from 1970s in the USA and another from 1996 from Taiwan. In the first study, one isolate promised resistance to amikacin and gentamicin but not tobramycin, while the latter found three strains resistant to amikacin. Overall however, most pathogenic *Aeromonas* species are aminoglycoside susceptible, as we have also determined (Shak et al., 2011).

4.3 GLOBAL AMINOGLYCOSIDE RESISTANCE GENE FREQUENCIES

4.3.1 Occurrences of Aminoglycoside Resistance Conferring Enzymes among Species Investigated in Current Study

Aminoglycoside resistance gene distributions also vary significantly across the world, much of it reflecting the country's health management and societal nuances. These mechanisms of resistance are not evenly distributed, and vary across species and time (Poonsuk, Tribuddharat and Chuanchuen, 2013; Miller et al., 1997). Examination of resistance genes can aid inferences on resistance substrates, as each gene confers resistance to specific profiles. Gene distributions can be exemplified by comparing frequencies of resistance conferring enzymes in different countries according to various reports.

It has consistently been observed in numerous studies that has studied aminoglycoside resistance gene distributions, that many bacterial species are found to be resistant towards additional drug classes as a universal phenomenon-likely due to multiple resistance gene acquisitions on resistance plasmids, integron cassettes or pathogenicity islands, although in

some cases due to enzymes mediating similar resistance mechanisms. Overlaps of multiple aminoglycoside resistance mechanisms were also found in many studies including several of those cited, in parallel with our findings, in the cases of isolates ETEC 06, ETEC 33 and *P. agglomerans (2)*-a phenomenon reported as far back as in 1993 which can increase the expected substrate resistance range considerably (Teixeira et al., 2016; Kim et al., 2012; Miller et al., 1997; Poonsuk, Tribuddharat and Chuanchuen, 2013; Schmitz et al., 1999).

Moreover, although reports on *P. agglomerans* and *Aeromonas spp.* enzyme mediated resistance towards aminoglycosides were not found from clinical settings; as such the global reports for the remaining strains will be further elaborated on from this point. Although it is fair to note that although we found no AMEs in our *Aeromonas* isolate, we did find two prominent genes, *aac(3')-II* and *aac(6')-Ib* in 50% of our *P. agglomerans* strains.

4.3.1.1 Comparison of Aminoglycoside Resistance Enzyme Frequencies in *K. Pneumoniae*

For *K. pneumoniae* one Egyptian study reported no occurrence of *rmtB* positive isolates, however 14% of the strains investigated contained *armA*. As for the AMEs, they found *aac(6')-Ib* in 88% of isolates, *aac(3')-II* in 58%, *aph(3')-IV* in 50% and *ant(3')-I* in 44% which were much higher in frequency in comparison. Yet they also did not find *aac(6')-II* containing isolates (El-Badawy et al., 2017). Our study found no enzyme-mediated aminoglycoside resistance genes present at all in our *K. pneumoniae* strains; although while the study being compared investigated the genetic basis of these enzymes in over one hundred strains, our study used just two isolates. In China, in a total of 48 isolates including *K. pneumoniae* and *E. coli*, 62% and 38% of strains respectively were found to carry a multitude of RMTases and AMEs. These were *armA*, *rmtB*, *aac(6)-II*, *aph(3')-VI*, *aac(3)-II*, *aac(6')-Ib* and *ant(3")-Ia* in the frequencies of 83%, 29%, 17%, 21%, 77%, 22% and 83%, respectively as a total (Hu et al., 2013). Another Chinese study found *aac(3')-II*, *aac(6')-Ib*, *ant(3")-I*, *ant(2")-I*, *ArmA* and *RmtB* in the rates of 30.2%, 19.8%, 13.6%, 4.3%, 11% and 6.2%, respectively (Liang et al., 2015). The other genes appear more often in Egypt and China. In Korea, *aac(6')-Ib*, *aph(3')-Ia* and *ant(2")-Ia* were found at incidences of 30.4%, 17.4% and 4.3% of ESBL producing isolates (Kim et al., 2012). Overall, the genes *aac(6')-Ib*, *ant(3")-Ia*, *aac(3')-II, aac(6')-II* and *armA* appear to be more commonly found globally among *K. pneumoniae* clinical isolates. Although the genes most frequently identified in other studies were also included in our research, we found none in our small sample size.

4.3.1.2 Comparison of Aminoglycoside Resistance Enzyme Frequencies in *Pseudomonas* Species

A study by Esparragon and colleagues, in as early as 1999 looked into 152 specimens of *P. aeruginosa* isolates, to find 13.5% of resistant strains, of which 55% were positive for *aac(6')-II* and 100% of them containing various isoforms of AAC(3') enzymes. In Thailand, nine AME genes, *ant(3")-Ia* in 84%; *aadB* in 84%; *aadA2* in 67%; *ant(2")-Ia* in 72%; *aph(3")-Ib/aph(6)-Id* in 70%; *aph(3")-IIb* in 57%; *aac(3")-Ia* in 40% and *aac(6")-IIa* in 27% were found in isolates of *P. aeruginosa* specimens highlighting extremely high rates in the region (Poonsuk, Tribuddharat and Chuanchuen, 2013). In Venezuela, the genes *aac(6')-Ib*, *aphA1* and *aadB* were highlighted as the most common with rates of 64%, 30.33% and 30.33%, with *ant(3")-Ia* and *aac(3')-IIa* less commonly observed, as positive in 17.9% and 2.6% of isolates, respectively (Teixeira et al., 2016). We found none of the seven resistance genes employed present in our isolate, and therefore it is not possible to make a solid comparison with these reports. However, for this species, AAC(6') and AAC(3') enzyme isoforms appear to be most prevalent internationally, particularly the AME encoded by the gene *aac(6')-II* and less so for the gene *aac(3')-II* which both were included in our study- the former seminally being first observed as present in *Pseudomonas* strains only according to Shaw et al., 1993.

4.3.1.3 Comparison of Aminoglycoside Resistance Enzyme Frequencies in *E. coli*

In respect to gene distribution studies of *E. coli* in medical settings, one study in Iran found prevalences of *ant(2")-Ia*, *aph(3')-Ia*, *aac(3)-IIa*, *aph(3')-IIa* and *aac(3)-IV* in proportions of 33.3%, 33.3%, 16.67%, 16.67% and 0% respectively among 18 ESBL producing strains (Akhi et al., 2016). However, Soleimani et al. also conducted a study in Iran, finding the genes *aac(3')-II* in a majority 78.87% of isolates, and *ant(2")-Ia* in 47.88% which both exemplifying higher rates of these genes than the previous study by comparing over 200 isolates (Soleimani et al., 2014). Across Asia in Korea, *aac(6')-Ib* was found in 20%; *aph(3')-Ia* in 6.2% and *ant(2")-Ia* in 1.2% among a population of 80 specimens of ESBL producing specimens were found (Kim et al., 2012). In Japan, among 212 strains tested, *aac(3')-II* was found in 10.4% of isolates; *aac(6')-Ib* in only 0.47%; *aac(3')-IV* in 0.47% and *ant(2")-I* in 1.4%, while no *rmtA*, *rmtB* nor *armA* were detected at all (Tsukamoto et al., 2014). Another study in Hong Kong inspected only gentamicin resistant strains in both humans and animals, discovering 81.3% carrying the *aac(3')-II* gene, of which 84.1% were determined in humans. It was thus established that the same genetic mechanisms occur both in humans and animals, primarily due to our close proximity and regularity of contact- highlighting another source of contact for acquisition of resistant strains

among humans (Ho et al., 2010). In that vein, we may also mention that an *armA* gene containing strain was sequenced from pigs in Spain resulting in multiple aminoglycoside resistance (Gonzalez-Zorn et al., 2005). Across Europe, a Polish research found resistance determinants encoded by *aac(6')-Ib*, *aac(3')-Ia*, *aph(3")-Ib* and *ant(2")-Ia*. These occurred in the rates of 59.2% in 26 isolates; 15.9% in 7; 36.2% among 16 and 4.6% among 2 (Ojdana et al., 2018). Overall, it appears *aac(3')-II*, *ant(2")-Ia* and *aac(6')-Ib* are the most commonly distributed resistance determinants in this species. Although our study found neither RMTases, even though they were investigated, nor the invasive *ant(2")-Ia* which was not studied, positive findings of *aac(3')-II*, *aac(6')-Ib* in ETEC strains were comparable, though at lower frequencies of 17% and 28% and 5%, respectively.

4.4 MULTIDRUG RESISTANCE (MDR) & AMINOGLYCOSIDE RESISTANCE PHENOTYPE-GENOTYPE CORRELATIONS FROM CURRENT STUDY

The phenotype of ETEC 12 strain was deemed most peculiar considering we were able to confirm the existence of the prolific *aac(6')-ib* gene from it by sequencing. This strain was also found to contain the ESBL gene, *CTXM-1* according to a previous research conducted in the same laboratory as mentioned before. However, it was also observed to be completely susceptible to all aminoglycoside antibiotics according to our antibiogram, thus impervious to the gene's substrate profile. Quite as the finding of ETEC 12, other phenotype-genotype discrepancies were observed within other ETEC strains (ETEC 06, ETEC 33 and ETEC 41).

In *ETEC 06,* which overall, resisted against 4 out of the 5 aminoglycoside antibiotics tested except for amikacin; it contained both the *aac(3')-II* and *aac(6')-Ib* genes. While the AST profile matched the expected substrate-based resistance profile of the first gene, it did not for the latter case, as amikacin is meant to be included (Ramirez, Nikolaidis and Tolmasky, 2013). As for ETEC 33 which contained all three resistance genes found- *aac(3')-II*, *aac(6')-ib* and *ant(3")-ia*, the resistance profile only paralleled with that of *ant(3")-Ia* substrates.. Once again, the activity of *aac(6')-Ib* was determined to be dysfunctional as in both ETEC 12 and ETEC 41; as only gentamicin but neither tobramycin nor netilmicin was found to be inhibited. Therefore it appears that at least for the *aac(6')-Ib* gene, regardless in which strain it was observed in, was likely not active in spite of being found via PCR investigations. This proves a profound disparity between the observed phenotype and the expectation of the genotype under *in vitro* conditions. This can possibly be attributed to a possible lack of expression of mRNA (Urbaniak et al., 2017) and, or, subsequent translation into a functional protein product in certain strains. Alternatively, this may be accounted by the presence of inactive pseudogene forms of the gene,

with the phenotypes associated with other AMEs not tested for (Davis et al., 2011; Klemm and Dougan, 2016; Lerat and Ochman, 2005). Yet another possible reasoning may be the accumulation of defensive mutations of ribosomal rRNA that inhibit or limit action of certain aminoglycoside antibiotics, or due to the innate resistance mechanisms endemic to prokaryotic cells, that include lack of cell permeability and active efflux-pumps, at least in certain strains.. Moreover, additional resistance to other aminoglycoside antibiotics found may be attributed to such adaptive or intrinsic resistance factors, or due to the presence of existing AMEs or RMTases not tested in this study (Garneau-Tsodikova and Labby, 2016; Teixeira et al., 2016; Morita, Tomida and Kawamura, 2012; Moore et al, 2009; Weinstein et al., 1980).

4.5 PHYLOGENETIC ASSESSMENTS

4.5.1 Phylogenetic Assessment of Prevailing Aminoglycoside Resistant Genes from Our Study

Finally, after having determined phenotypes and genotypes, this project delved into the evolutionary relationships of each gene to understand the variation, sources and typical modes of dissemination of some of our genes' closest counterparts. To do this, phylogenetic analysis was conducted using the MEGAX software. Phylogenetic analysis is one of the most remarkable, yet fairly novel advents in bioinformatics that allow us to use sequencing technology to infer evolutionary relatedness between species and strains. This also allows speculation on how microbial populations, and most importantly MDR bacterial species, are adapting, evolving and disseminating, thus providing crucial epidemiological information. This utility can also extend towards inferences for individual gene dispersals. This eliminates the seemingly abstract nature of strain dispersals and allows us to predict future outcomes, both locally and on a wider geographical scale. Moreover, through phylogenetic network building, it facilitates the determination of emerging strains of clinical importance (Klemm and Dougan, 2016). In our phylogenetics assessment, selecting for only the protein-coding ORF of each specific gene detected through PCR interrogation revealed complex evolutionary relationships as underscored in the phylogenetic trees displayed above. In the case of all three genes found, there were 100% sequence similarities between the closest four sequences, but were discovered in a myriad of species.

4.5.2 Interpretive Evolution of *Aac(6')-Ib* Gene Phylogenetics

With a total in excess of 40 described enzymes, the AAC(6') family are by far the most numerous and widely disseminated groups among the AACs with over 50 variants discovered in Gram-negative bacteria making it the most clinically significant class. Phylogenetically, this particular group has been subdivided into three separate clades, with ambiguous origins (Garneau-Tsodikova and Labby, 2016). Worryingly, the most common gene, *aac(6')-Ib* gene in ETEC, *K. pneumoniae,* to some extent also *Pseudmonas* species and *P. agglomerans* (only in our study) has been also identified in plasmids, co-existing with other clinically concerning genes such as *ndm-1* ESBL. Furthermore, as seen in our study, can occur in plasmids, functional and non-functional class 1 integrons, transposons, but also truncated or disrupted integrons, IS elements, genomic islands and KQ elements (Ramirez, Nikolaidis and Tolmasky, 2013; Ramirez and Tolmasky, 2010). Only in the case of *aac(6')-Ib* were there three distinct nucleotide substitutions from the hypothetical common ancestor, as revealed by n.136 T>A, n.182 C>T and n.370 G>T point mutations from *Salmonella enterica MU1 (KX810825.1)* strain as found within a plasmid in Australia (Abraham et al., 2016). This had likely mutated several times and passed on to its predecessors as new clones, including our sequenced gene clone. These most closely related four genes were found in strains marked by *E. cloacae AR-0093 (CP027606.1)* from the USA (Conlan et al., 2018) and *A. salmonicida (CP022170.1)* (Du and Feng, 2017), *A. caviae (KU644711.1)* (Wen, Zheng and Hu, 2016) and *A. hydrophila RJ604 (KU133344.1)* (Xie et al., 2015) all sourced from China. *E. cloacae* and *A. salmonicida S121* genes were found on plasmids, while the gene in *A. hydrophila* was found in a class 1 integron and the genomic source of *A. caviae's* gene was undetermined. It appears all of the strains containing these genes were reported within the last three years and regularly occurred in transferrable elements. The *aac(6')-Ib* gene was often found as multiple copies in numerous individual strains, as compared to the *aac(3')-II* gene.

4.5.3 Interpretive Evolution of *Aac(3')-II* Gene Phylogenetics

The *aac(3')-II* gene has thus far been discovered in all Gram-negative bacteria, and is commonly integrated into integron gene cassettes in species such as *E. coli, P. aeruginosa, Serratia marcescens* and *Alcaligenes faecalis* (Ramirez and Tolmasky, 2010). It is the second most frequent gene found in our study and especially in *K. pnemoniae and* ETEC reports cited. The *aac(3')-II* gene, occurred on a hypothetical common ancestor *E. coli 660 (LT985253.1)* strain originating in France (Cia and William,2018), encoded within a plasmid; being primarily observed in a multitude of species also occurring on plasmids, although also occurring

chromosomally on fewer occasions. The four closest gene clones were found in strains *Sphingomonas spp. FARSPH (CP029988.1)* from Peru (Bendezu et al., 2018), *K. pneumoniae MUMCK3 (KX529656.1)* from India (Bandyopadhyay, 2016), *K. pneumoniae DA33140 (CP029586.1)* from Sweden (Nicoloff, Hjort and Andersson, 2018) and *S. enterica CFSAN064034 (CP028170.1)* in the USA (Hoffman et al., 2018). These were encoded in plasmids, with the exception of the *K. pneumoniae MUMCK3* who's specific genetic location was unidentified by the authors. It appears that all of these genes emerged, or at least were reported within the past two years.

4.5.4 Interpretive Evolution of *Ant(3")-Ia* Gene Phylogenetics

Also popularly known as *AadA1* in literature, the *ant(3")-Ia* gene is a component of numerous, well studied transposons such as Tn21 and is supposed to be widely disseminated due to co-localization with a toxic metal inhibitor on the same element (Ramirez and Tolmasky, 2010). In our analysis, the most closely related genes for *ant(3")-Ia* were found most frequently occurring either on plasmids or chromosomally as the third most common gene according to many literature reports; although in certain cases was also contained within integrons and MDR genomic islands, with the common ancestral sequence occurring chromosomally within *A. baumannii XH38 (CP021326.1)* strain which originated in China (Hua and Yu, 2017). Although multiple gene copies of all three genes were found in individual strains, for *ant(3")-Ia* in particular we observed the highest number redundant and non-redundant copies of up to 6 sequences occurring within a single strain; although only the unique sequence copies were left for comparative analysis for all individual genes. The four closest gene clones were found in strains distinguished as *K. pneumoniae AR0140 (CP029723.1)* (Conlan et al., 2018) from the USA; *K. pneumoniae KPOsh-2K (KY658722.1)* (Astashkin, Kartsev, and Fursova, 2017) from Russia; *E. coli 552 (LT985228.1)* from France (Cea and Willian, 2018) and *E. coli H8Ecoli (CP029215.1)* (Zheng, 2018) from China. Three of the gene clones were also found on plasmids, as like the ancestral sequence, with the exception in *K. pneumoniae KPOsh-2K* which occurred in a class 1 integron and these strains were all isolated within the past two year period.

4.5.5 Seminal Implications of Phylogenetic Investigations

Phylogenetic analysis alludes to the fact that the MDR strains conferring aminoglycoside resistance found in Bangladesh originated from international sources, particularly France, Australia and China, underscoring the sheer mobility of resistance gene clones, underscoring the deeper problem at hand. These relationships create a vivid picture of the possibility of a single gene to wade out into a very large number of differing species over a very short period of time, using a multitude of horizontal gene transfer methods enabling them to cross international territories easily in the modern era. Many genes were encoded as integrated within the chromosome which also pose risks, as harbours for future transmissions, facilitated by agents such as transposable elements, integrons and conjugative plasmids (Ho et al., 2010; Schmitz et al., 1999; Seward, Lambert and Towner, 1998). The wide-distribution is not surprising, as strains containing resistance enzyme encoded plasmids often been demonstrated in studies to distribute not just to other hospitals but even to other countries (Ho et al., 2010; Arpin et al., 2003). Indeed, the capability for aminoglycoside resistance genes to permeate into a multitude of bacterial species has been confirmed previously in an *A. baumannii* study and another *E. coli* study conducted in Hong Kong (Ho et al., 2010; Seward, Lambert and Towner, 1998) and the greater likelihood of homologous genes being transferred among species is also conveyed by Rodriguez, 2016. Based on the findings of this research, it would be best to contain infections where *P. agglomerans*, *K. pneumoniae* or ETEC species comprise the main etiological antagonist. This may be particularly difficult due to the culture of medical-tourism, which is a popular phenomenon in Bangladesh, and the fact that many Enterobacteriaceae are also commensal members of the normal gut-flora thus serving as potential reservoirs for later spread, especially if opportunistic infections take hold (Vasoo et al., 2015; Islam et al., 2012).

4.6 CLINICAL RECOMMENDATIONS BASED ON LITERATURE REVIEW, ANTIBIOGRAM, MOLECULAR AND PHYLOGENETIC DATA

In knowing resistance profiles, it allows us to advise clinicians on which antibiotic treatment regimens to follow in case of specific infections (Shak et al., 2011). In this study, we have been able to make such inferences based on the phenotyping using AST, but also by molecular profiling of enzymatic aminoglycoside resistance gene elements. Our data clearly shows that discrepancies may arise that make real-life decisions difficult in either case. Yet, as alarming as the findings of this study may appear at first glance, if under any circumstance, aminoglycosides become the best fit choice of therapy; it would be still be recommendable to use netilmicin or amikacin but, as these had the lowest proportion of fully resistant strains with only 25% and

33.3% of strains, respectively shown to be impervious to them. Gram-negative bacilli strains, respond better to these two aminoglycoside antibiotics according to susceptibility rates world-wide, with few exceptions. Netilmicin, streptomycin and amikacin all had only two strains with intermediate level resistance, while the other two aminoglycosides had three which means they may still be effective at higher doses.

The second generation of semisynthetic aminoglycosides released in the 1970s that included the likes of amikacin, netilmicin, arbekacin, dibekacin and isepamicin had initially been observed to possess negligible susceptibility towards AMEs, especially for amikacin and to some extent remains true in the Bangladesh scenario (Gad, Mohamed and Ashour, 2011; Hansberg et al., 2012). Amikacin particularly is widely accepted to possess higher activities due to an aminohydroxybutyryl group which enables it to escape enzymatic modification at several positions, retaining its binding to the ribosomal A site more often (Gad, Mohamed and Ashour, 2011). However, other than streptomycin which surprisingly had the highest percentage of resistant strains at 50% which matched with tobramycin, it would be advisable to try any of the other aminoglycosides first, before resorting to this antibiotic. This is not a surprising finding, as streptomycin was the very first aminoglycoside introduced into clinical settings and therefore has been in use for almost 50 years (Gad, Mohamed and Ashour, 2011). That being said, virtually any of these antibiotics may produce positive clinical outcomes at this point, keeping in mind that a higher dose may be sufficient to fulfil bacterial clearance in the majority of cases if the initial regular dose appears ineffective, due to the relatively high incidences of intermediately resistant strains which can respond to therapy under a higher stress dosage. Thus, although almost all the strains demonstrated the ability to resist some of these antibiotics, these may be cycled, starting from the use of amikacin, followed by netilmicin, tobramycin, gentamicin and finally streptomycin- in that specific order to stave off rapid resistance against all of the drugs in case of non-specific Gram-negative bacilli infections.

This recommendation may vary depending on the pathogen involved, if the species is determined. Only in the case of *P. agglomerans* and *K. pneumoniae* would amikacin or streptomycin be recommended, as all the strains were completely impervious to them, according to this study. It should be noted however that common drug combinations to treat pneumoniae in Bangladesh include the pairing of an aminoglycoside (either amikacin or gentamicin), along with another drug that is usually a 3rd generation cephalosporin. These combinations are usually: cefotaxime plus amikacin; ceftazidime plus amikacin; ceftriaxone plus amikacin; ceftriaxone plus gentamicin; cefotaxime plus gentamicin; ampicillin plus gentamicin (Rashid et al., 2017). As respiratory diseases are the second most common infection type in Bangladesh this explained the relatively high rate of gentamicin and amikacin resistance found,

albeit amikacin resistance was still lower than expected, probably as a result of their semi-synthetic structure as mentioned earlier. On the other hand, for *Pseudomonas spp.* and *Aeromonas spp.*, all the aminoglycosides appear equally applicable.

Only in the case of ETEC infections would streptomycin be more preferable to tobramycin. The Swedish Reference Group for Antibiotics (SRGA) recommends specific treatment guidelines that may be considered the paragon of all guidelines, considering Sweden has some of the lowest rates of aminoglycoside resistance in the world. For *E. coli* infections resistant to cephalosporins; which is a common occurrence in Bangladesh; both according to our study and Rabbe et al., 2016, gentamicin or tobramycin is then preferred over amikacin. Tobramycin is also preferred for *P. aeruginosa* infections, however while acknowledging the higher usage incidences of amikacin in developing countries it, it also notes that amikacin is more useful in treatments involving ESBL or quinolone resistant *E. coli* and is a viable option for *P. aeruginosa* infections as well (Hanberger et al., 2012). Moreover, as it seems the appearance of resistance genes do not always correlate with a given phenotype, it instils faith that a pragmatic regimen may still yield success in most cases, regardless. It would also be recommended to continue their use in concert with β-lactams, penicillins or vancomycin for greater efficacy, due to the cell-wall disruption that should occur enhancing aminoglycoside penetration inside the cell, in case efflux-pumps or lipopolysaccharide alterations are the main arsenal in preventing necessary entry of these drugs, rather than AMEs (Garneau-Tsodikova and Labby, 2016; Shete, Grover and Kumar, 2017; Hanberger et al., 2012; Klingenberg et al., 2004; Gad, Mohamed and Ashour, 2011). Even after more than 30 years applying this classical dual-therapy, it has been observed to still be quite effective, especially in combination with either gentamicin or amikacin. A caveat to this however, is that it is advised to only be applied as empirical therapy in case of very serious, Gram-negative pathogenic infections where the benefits outweigh the burden, as it is associated with adverse effects. If these strains carry plasmids containing β-lactamases, resistance elements that counter aminoglycosides as well as other antibiotics may also exist proximally on the same gene cassette, threatening to offset this dualistic effect, as found in one of our ETEC isolates (Vasoo et al., 2015; Arpin et al., 2003) (Livermore, 2002; Esparragon et al., 1999). Consequently, application of this therapy is only a major concern for strains like ETEC isolate 06, which we found to contain both the *aac(6')-Ib* aminoglycoside resistance gene, as well as the *CTXM1* ESBL gene combined. As such, other drug-drug combinations should also be considered; for example, one Egyptian study has also determined the combination of amikacin and ciprofloxacin to provide significant benefits (Gad, Mohamed and Ashour, 2011) while another American report suggests a combination of aminoglycosides with carbapenems are more viable (Vasoo et al., 2015). For *Aeromonas*-specific infections,

French researchers suggested a combination of aminoglycosides along with either a quinolone or a cephalosporin antibiotic (Shak et al., 2011), although if the AAC(6') variant gene, *aac(6')-Ib-cr* is known to be present, quinolones may also be inhibited along with aminoglycoside antibiotics (Tsukamoto et al., 2014). Furthermore, going by our results, as the two most common RMTases investigated were not apparent in this study, high-level pan-resistance is not expected to occur. This expectation is especially likely; as we have observed very high streptomycin resistance which RMTases do not confer (Nie et al., 2014). Additionally, through our molecular analysis of prevalent genes, it may be inferred that due to the higher incidences of *aac(6')-Ib* and *aac(3')-II* determinants in particular; their substrates consisting of gentamicin, netilmicin, tobramycin and amikacin may all very well falter in terms of efficacy in the future if these are able to spread through HGT, as we suspect it does quite frequently- as established via phylogenetic analysis. However, if the hypothesis postulated by Davis et al., 2011 is true, then resistance may not easily be lost once eventually acquired and greater measures may need to be taken. Yet, the prudent management of such antibiotics is the most logical solution for managing resistance accumulation and spread, considering the dearth of alternative options available, taking into account the normalized status quo, and lack of resources of a developing country such as Bangladesh. Ultimately, next-gen antibiotic development must be expedited as a major priority to combat overall multidrug resistance against aminoglycosides. Thankfully, at least one new aminoglycoside derivative is currently undergoing phase III clinical trials. This sisomicin-derived, broad-spectrum antibiotic named plazomicin, has defined advantages over existing varieties with attenuated nephrotoxicity and ototoxicities, and is less susceptible to AME modifications (Olsen and Carlson, 2014; Rodriguez-Avial et al., 2015; Becker and Cooper, 2012). It is expected to more effectively combat super-pathogens such as MRSA, vancomycin-resistant enterococci and brucellosis when concerted with other antibiotics. In the latter case, gentamicin is usually administered but has serious side-effects due to the long term chemotherapy measures needed (Olsen and Carlson, 2014). According to initial results, it is believed to fare far better than either gentamicin or amikacin towards Gram-negative pathogens and especially the MDR Enterobacteriaceae species as demonstrated against carbapenamase-producing *K. pneumoniae*, especially through synergistic concert with other antimicrobials. One caveat however, is that this drug is still defenceless against the activity of RMTases (Rodriguez-Avial et al., 2015). Other compounds derived from arbekacin, kanamycin, neomycin, paromamine and several gentamicin iterations are also currently in the works and shows promise, however they are not nearly as advanced in terms of research and development as plazomicin (Becker and Cooper, 2012).

4.7 LIMITATIONS OF STUDY AND FURTHER WORK

Based on the results of this study, several further works should be done, and a number of limitations of the study addressed next to create a more comprehensive picture of the current state of aminoglycoside resistance in Bangladesh. It would be necessary to expand investigations to include more hospitals, both within the capital city and the broader territories for active surveillance. As for most of the species studied herein, very few specimens were found; therefore it would be important to generate a better resolution on individual species-biased resistances by increasing the sample size. Moreover, as we have narrowed our study to only seven enzymatic genes, it may be necessary to increase the breadth of primers to include more variations, especially towards other commonly found resistance determinants (especially *AadB*, *Ant-2"-Ia* and *Aph(3')-Ia-b*). The future choice of molecular surveillance may be towards genes for proteins that inactivate substrates which are phenotypically are highly susceptible. The actual expression and translation of functional enzymes may also be determined next, through mRNA, protein and *in vivo* experimentation analysis. The possibility of non-enzymatic inhibitory mechanisms may also require further examination as they may have had greater influence towards the observed phenotypes than the AMEs and RMTases detected, especially towards efflux-pumps and cell wall permeabilities. During phylogenetic analysis, it would also be useful to conduct whole genome sequencing (WGS) followed by pulse-field gel electrophoresis (PFGE) analysis and multi-locus sequence typing (MLST) methods to enable true clonal divergences. This would also be an excellent starting point into examinations of transposable elements and plasmid analysis which would include mating studies for extrapolating inferences on transmission potentials. Pseudogene assessment may also be applied by considering the full length of the gene including early stop codons, truncated gene copies, and examination of promoters and start codons. Therefore, in conclusion it appears that this study is a prudent starting point with more research needed in order to comprehensively accomplish the goals intended.

4.8 CONCLUSION

In Bangladesh, highly dense populations and poor health-care management practices, unpragmatic and indiscriminate use of antimicrobials as well as poor hygiene increases the risk for multiple resistance acquisitions by bacteria. Not all of these are pathogenic species; however, even commensals in our gut and skin can pose as serious opportunistic threats in individuals whose immune systems are attenuated as often found in ICUs of hospitals. Many patients who recover may also carry latent opportunistic pathogens that can readily become

infectious under the correct conditions. Among them, Gram-negative pathogens are most notorious, being complicit in some of the top hallmarks of morbidity and mortality in Bangladesh that includes respiratory and diarrhoeal diseases. These can become impossible to treat when someone's lifeline is ticking against the clock and potent prophylactic strategies must be deployed. As aminoglycosides fit this latter description, and moreover, are an older class of antibiotics believed to have become less relevant in the last few decades, they may hold the potential as the best hope for future treatments. This may especially be true when in conjunction with other antibiotic classes for synergistic effect, and with the correct information novel combinatorial strategies may be developed. The sudden rise in their resistance however, especially in combination with inhibitors of other antimicrobial classes makes life increasingly difficult for both medical practitioners as well as for patients. Exacerbated multidrug resistance frequencies threaten these regimens and novel drugs must ultimately still be pushed through the pipeline if pharmaceutical manufacturers could be motivated. As such this surveillance study finds that resistance rates are relatively high in Bangladesh, with numerous species across various genera resistant at significant levels to this class of antibiotics, along with virtually all other classes. Moreover, resistance gene determinants that predominate on plasmids and other mobile genetic elements are also present as determined by the molecular investigations on numerous aminoglycoside modifying enzymes. One positive is that the pan-resistant class of ribosomal RNA methyltransferases were absent, however discrepancies between phenotype and genotype leaves much room for thought on what other resistance mechanisms are at play, and how many determinants not investigated for may persist. Therefore, surveillance research studies such as this one must rigorously be applied to a broader extent, such that clinical practitioners no longer have to wade through the dark and have realistic information to help decision making during critical situations. Through wide-scale availability of such data, they may more adequately assess which antimicrobial treatments would yield the greatest chance of success with more rapid response times.

Chapter 5

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APPENDIX

Appendix-I

MEDIA COMPOSITIONS:

All media were autoclaved at 121°C for 15 mins under 15 psi of atmospheric pressure. The media compositions are provided below:

1. **MacConkey Agar (BD Difco™, England):**

Ingredients	Amount (g/L)
Peptone	17.0
Protease 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. Mueller Hinton Agar (BD Difco™, England):

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

3. Tryptone Soy Broth (Oxoid, UK):

Ingredients	Amount (g/L)
Pancreatic Digest of Casein	17.0
Papaic Digest of Soybean Meal	3.0
Sodium Chloride	5.0
Dibasic Potassium Phosphate	2.5
Glucose	2

4. STGG Media:

Ingredients	Amount
Skim-milk Powder	2.0 g
Tryptone Soy Broth (Oxoid, England)	3.0 g
Glucose	0.5 g
Glycerol	10 ml
Distilled H_2O	100 ml

5. Motility-Indole-Urease Agar:

Ingredients	Amount	Preparation
Sodium Chloride (Sigma)	5.0g	Prepare up to 900 ml using deionized water for autoclave
Potassium Dihydrogen Phosphate (Fisher Chemical, USA)	4.0g	
Peptone (Himedia, India)	2.0g	
Phenol Red (0.25%) (Sigma, India)	4.0g	
Agar (Himedia, India)	2 ml	
Urea (Ameresco, USA)	20g ml	Prepare up to 100 ml using deionized water for filter sterilization

6. Simmons 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

7. Triple-Sugar-Iron Agar (Difco™, England)

Ingredients	**Amount (g/L)**
Beef Extract	3.0
Yeast Extract	3.0
Pancreatic Digest of Casein	15.0
Protease 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

BUFFERS AND REAGENTS:

Tris-Boric acid EDTA (TBE) Buffer (500 ml): 5.4g Tris-HCl powder, 2.75g Boric acid powder and 2.0 ml of 0.5M EDTA were dissolved in 500ml of Distilled H_2O. The pH was then adjusted to 8.0, autoclaved and stored at r.t.p until use.

Appendix-III

INSTRUMENTS:

Instruments	**Manufacturer**
Autoclave	WiseClave
Refrigerator	Electra, Samsung (4°C)- For reagent storage

	Vestfrost (4°C)- For bacterial growth plates and media storage
Deep-Freezer	Vestfrost (-20°C)- For storage of stock antibiotics, PCR reagents and template
	ESCO (-80°C)-For bacterial glycerol-stock storage
Incubator	Memmert
Shaking Incubator	WiseCube
Oven	WiseVen
Water Bath	WiseBath
Micropipettes	(2-20μl)- Gilson and Costar®
	(20-200μl)- Gilson and Costar®
	(200-1000μl)- Gilson and Costar®
Bio-Safety Cabinet	ESCO Class II Type A2 Labculture® Biological Safety Cabinet
Vortex Mixture Machine	WiseMix
Electronic Balance	OHAUS®
Weighing Paper	Fisherbrand®
NanoDrop™ 2000	ThermoFisher SCIENTIFIC
Centrifuge Machine	ThermoFisher SCIENTIFIC
Light Microscope	OLYMPUS CX41
T100™ Thermal Cycler	BIO-RAD
Gel-Documentation Machine	BIO-RAD
Take-3 Plate	Eon™ Bio-Tek®
Antibiotic disks	Oxoid