AcrAB TolC EFFLUX PUMP GENE IN MULTI DRUG RESISTANT *Escherichia coli* ISOLATED FROM CLINICAL SAMPLES

A Dissertation Submitted to the **Department of Microbiology GoldenGate International College**, Tribhuvan University,Kathmandu, Nepal, in Partial Fulfilment of the Requirements for the Award of Degree of Master of Science in Microbiology (**Medical**)

by

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RECOMMENDATION

This is to certify that **Miss.Alina Koirala** has completed this dissertation work entitled **"*AcrAB TolC* EFFLUX PUMP GENE IN MULTI DRUG RESISTANT *Escherichia coli* ISOLATED FROM CLINICAL SAMPLES"**as a partial fulfilment of M.Sc. Degree in Microbiology (Medical) under our supervision. To our knowledge this work has not been submitted for other degree.

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ABSTRACT

Escherichia coli is one of the most commonly isolated multi drug resistant (MDR) pathogen and has been categorized as the priority pathogen by WHO. Carbapenem is a drug of choice for treatment of *E. coli* but now resistance of these drugs is spreading due to various mechanism such as the presence of intrinsic mechanism of antibiotic resistance efflux pump gene. Efflux pump has been illustrated as one of the key mechanism of antibiotic resistance crucially in the gram-negative pathogens. Forty-two carbapenem resistant *E. coli* were isolated from sample specimens of patients of Kathmandu Model Hospital. Cross sectional descriptive study was conducted and the specimens were processed with respective culture media following the antibiotic susceptibility testing according to CLSI guidelines. Phenotypic detection of ESBL and CRE was done using combination disc diffusion and by using cccp respectively. Chromosomal DNA extraction was done using alkaline hydrolysis method. Amplification of *AcrAB TolC* was carried out by conventional PCR.

Out of 2384 samples the infection rate was found to be (21.8%) that is 520 samples showed significant growth of bacteria.*E coli* being the major isolates constituting of 78% of the total isolates. Bacterial isolates 89.5% were found mostsensitive to PolymixinB and Colistin (99.8%), however was the most and resistant to Amoxycillin (66%). Out of 42 Carbapenem resistant *Escherichia coli* isolates, AcrA gene was prevalent in 23 (54.7%) of the isolates. AcrB gene in 30 (71.4%) and 31 (73.8%) of the isolates were found to be positive for TolC gene. Out of 403 *Escherichia coli* isolates, 42 (10.4%) were found to be Carbapenem resistant. Moreover, out of 147 ESBL isolates, 16 isolates (10.9%) were found to be Carbapenem resistant. This research evaluated the minimum inhibitory concentration (MIC) activity of carbapenem, in the presence or lack of the CCCP (25 g/mL) in order to identify the function of the efflux pump in the carbapenem resistant *E. coli* isolates. The findings demonstrated that most samples had lower MICs in the presence of the pump inhibitor than in the absence of the efflux pump inhibitor.

Key Words: Efflux pump, Carbapenem, antibiotic susceptibility test

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ABBREVATIONS

CRE: Carbapenem Resistant *E coli*

AST: Antibiotic Susceptibility Pattern

MDR: Multi Drug Resistant

CFU: Colony Forming Unit

MHA: Mueller Hinton Agar

CLED: Cysteine Lactose Electrolyte Deficient Agar

SIM: Sucrose Indole Motility

MSU: Mid-Stream Urine

ESBL: Extended Spectrum Beta Lactamase

CRE: Carbapenem-Resistant Enterobacteriaceae

MATE: Multiple Antibiotic and Toxin Extrusion

ABC: ATP binding cassette

RND: Resistance-nodulation-division

SMR: Small MDR Family

MF: Major Facilitator

MIC: Minimum Inhibitory Concentration

MRSA: Methicillin Resistance *Staphylococcus aureus*

LPS: Lipopolysaccharides

CCCP: carbonyl cyanide chlorophenylhydrazone

EPEC: Enteropathogenic *E. coli*

EHEC: Enterohaemorrhagic *E. coli*

ETEC: Enterotoxigenic*E. coli*

EAEC: Enteroaggregative *E. coli*

DAEC: Diffusely adherent *E. coli*

EIEC: Enteroinvasive *E. coli*

AIEC: Adherent invasive *E. coli*

ExPEC :Extraintestinal pathogenic *E. coli*

UPEC: Uropathogenic *E. coli*

NMEC: Neonatal bacterial meningitis *E. coli*

CHAPTER I

INTRODUCTION AND OBJECTIVES

1.1Background

The number of antibiotics to which bacteria have acquired resistance has expanded significantly over the last ten years. As a consequence, some agents are no longer effective at treating infections (Piddock 2006). Additionally, the fact that more and more bacterial species are developing resistance to the several antibiotics including carbapenem is threatening because when it comes to treating patients with serious bacterial infections, especially those brought on by strains that are typically resistant to antimicrobials, carbapenem medicines are widely regarded as the most effective class of antimicrobial drugs. Therefore, it is quite concerning that the prevalence of Enterobacteriaceae species connected to the health care system are becoming more and more carbapenem-resistant (CRE) (Iovleva and Doi 2017).

Antibiotics can be resisted by bacteria using a number of different strategies. These include actively exporting an antibiotic from the bacterial cell and preventing an antibiotic from entering the bacterial cell (efflux of the antibiotic). The fact that such efflux pumps often export a variety of unrelated substances, such as chemicals made by the host organism (such as bile), suggests that these systems may play a part in aiding bacteria to thrive in their biological niche. All species carry the genes and proteins for Efflux-pump. The genes for efflux pumps in bacteria are stored on chromosomes or transmissible genetic units like plasmids. It is widely recognized that efflux pumps can reduce a bacterium's sensitivity to antibiotics; However, such reductions are not necessarily followed by clinically significant antibiotic resistance. Efflux pumps can be tailored to a single substrate or be capable of moving a variety of molecules with varying structural properties, such as antibiotics from several chemical classes. Resistance to several drugs (antibiotics) may be linked to those pumps that transport many substances (MDR) Ball et al (1980) and McMurry et al. (1980) Ball et al (1980) and McMurry et al. (1980) observed the presence of microbial multidrug efflux for the first time in reference to the efflux of tetracycline in

Escherichia coli. The tet (tetracycline) determinants, which were either encoded on plasmids or transposons, were responsible for transfer of resistance between strains (Chopra and Roberts 2001; Roberts 2005).

Additional efflux mechanisms have now been found in both Gram-positive and –negative bacteria, and more recently, in mycobacteria as well. Based predominantly on the homology of the amino acid sequence, bacterial efflux transporters can be divided into five core families. This includes the multiple antibiotic and toxin extrusion (MATE) family, the ATP binding cassette (ABC) family, the resistance-nodulation-division (RND) family, the small MDR (SMR) family, and the major facilitator (MF) superfamily. The first three families use the proton motive force in a proton-drug antiport system to produce the energy needed to extrude a drug out of the cell, meanwhile the MATE family leverages the exchange of either proton or sodium ions. The ABC family, on the other hand, incorporates ATP hydrolysis and drug extrusion. (Piddock 2006) Single transporters from the MF, SMR, or ABC families that is positioned on the cytoplasmic membrane mediates drug efflux from Gram-positive bacteria. But since Gram-negative bacteria have an outside membrane, their efflux pumps are more complicated. They comprise of a tripartite protein channel that requires the proteins membrane fusion protein (MFP), an outer membrane efflux protein (OEP), and the cytoplasmic membrane-located transporter. It is common for an organism to contain the genetic code for many efflux pumps, each of which can either be produced constitutively or involuntarily in reaction to the presence of a substrate. The major RND family member in *P. aeruginosa*, the MexAB-OprM multidrug efflux pump, is constitutively expressed in this organism. P. aeruginosa does, however, also possess the MexXY-OprM pump, which can be activated when any of its substrates, including aminoglycosides, are present. As a result, the intrinsic resistance of P. aeruginosa is influenced by multidrug efflux pumps. (Li et al 2003) 26 The tripartite AcrAB-TolC, MexAB-OprM, and FloR efflux pumps of *E. coli, P. aeruginosa,* and Salmonella enterica serovar Typhimurium, respectively, have received the most attention in investigations of Gram-negative bacteria as they evhibit the ability to export a wide variety of chemically varied substances through their efflux proteins (Poole 2005; Arcangioli et al 1999). Efflux pumps can be made up of a single component or

several components. These efflux pumps are made up of a tripartite system that includes an accessory protein (also known as a membrane-fusion protein) (for example, *AcrA*) that is located in the periplasmic space, an outermembrane protein (also known as an outermembrane protein channel) (for example, *TolC*) that is located in the outer membrane, and a transporter (efflux) protein (for example, *AcrB*) that is located in the inner (cytoplasmic) membrane of the bacterium as an accessory protein (Koronakis et al 2004; Aires and Nikaido 2005).

Due to these bacteria's "intrinsic resistance" to specific antibacterial drugs, certain antibacterial agents cannot be utilized to treat infections caused by certain species of Gram-negative bacteria. This resistance was first attributed to the drug's limited penetration across the bacterial membrane. To the contrary, evidence by Li and colleagues from 1994 showed that efflux was the cause of P. aeruginosa's inherent resistance to a number of antibiotics (Li et al 1994). MexAB-OprM, an operon encoding an efflux system, was reported by Poole and colleagues (Poole et al 1993), in wild-type P. aeruginosa the year prior, and it was demonstrated that deletion of genes encoding components of this system (Livermore 2003), resulted in hypersusceptibility to a number of antimicrobial drugs. It has since been established that efflux is the cause of several drug classes' low performance against Gram-negative bacteria. These include deformylase inhibitors and oxazolidinones (Johnson 2005; Buysse 1996). In a similar vein, there are instances where certain drugs in a class—but not all—have minimal to no action as a result of efflux. Older macrolides (Chollet et al 2004), such as erythromycin, for instance, show negligible to no action against *E. coli* and *Haemophilus influenza* (Peric et al 2003; Sanchez et al 1997). Additionally, Proteus mirabilis and *P. aeruginosa* aren't affected by the new glycylcycline known as tigecycline much at all (Dean et al 2003; Visalli et al 2003) 20-21.

Efflux as an antibiotic resistance mechanism; Baseline levels of efflux are associated with intrinsic resistance to several antimicrobial drugs. In contrast, resistance to antimicrobial agents in bacteria that are typically susceptible to a specific agent is caused by a constitutive increase in expression of the effluxpump protein (the transporter protein), as shown by an increased minimum

inhibitory concentration (MIC; that is, the lowest concentration of an antibiotic that inhibits growth of the organism). The promoter region of the efflux-pump (transporter protein) gene, mutations in insertion elements upstream of the efflux-pump (transporter protein) gene, and mutations in global regulatory genes (transcriptional activators) can all result in a permanent increase of efflux pump expression. When there is a concurrent rise in the MICs of three or more antibiotics for a specific bacteria compared to the MICs of these antibiotics for the parent strain, Efflux is thought to be the mechanism causing antibiotic resistance (Poole 2004; Poole 2005; Piddock 2006).

In the human stomach, *E. coli* is commensal bacteria, but it may also be harmful and frequently results in infections of the urinary system. Furthermore, enteropathogenic *E. coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) are responsible for diarrhea. Depending on the infection kind, cotrimoxazole, nitrofurantoin, or a fluoroquinolone are frequently used as treatments. Invasive or fatal infections can be treated with a third-generation cephalosporin (such ceftriaxone. It should be noted that overexpression of *AcrAB–TolC* alone does not confer clinical levels of resistance; however, when a mutation(s) in a topoisomerase gene (the product of which is a target for the bactericidal activity of fluoroquinolones) occurs in the same bacterium as increased efflux). However, the drug substrate profile of *E. coli TolC*also includes β-lactam antibiotics, along with the antibiotics chloramphenicol, fluoroquinolones and many other substrates (Piddock 2006). Betalactam drug may not be the the choice of drug for the treatment *E. coli* infection, but as a last drug of resort the correlation of carbapenem resistant in CRE strains sure is alarming as there is a huge possibility of dessimination of such gene among wide range of other strains and organism.

1.2 Objective

1.2.1 General objectives

To assess the multi drug resistance pattern of *E. coli* from hospital patients and to identify the efflux pump gene *AcrAB-ToIC* among the isolates.

1.2.2 Specific objectives

- To determine the proportion of *E.coli* among total bacterial isolates from clinical samples.
- To assess antibiotic susceptibility patterns, including ESBL and carbapenem resistance, in *E. coli*.
- To identify the efflux pump gene *AcrAB-ToIC* from the *E. coli* isolates.

CHAPTER II
LITERATURE REVIEW

2.1 *Escherichia coli*

E. coli is the most prevalent commensal of gastrointestinal tract of human and other warm blooded animals. It is the gram negative member of family enterobacteriaceae. Within a few hours after birth *E. coli* typically colonizes the gastrointestinal tract of human infants. These commensal strains of *E. coli* are rarely pathogenic except for some unusual conditions, such as, in immunocompromised host. The bacterium successfully resides in the mucus layer of mammalian colon. Despite multiple theory on its physiology and genetics, one hypothesis that interestingly suggest for its ability to colonize the specific metabolic niche is its natural ability to utilize gluconate more efficiently than any other bacterial flora (Kaper et al 2004). There are several specific kinds of virulence attributes acquired and adapted by multiple strains of *E. coli* which confers them the ability to not just adapt to new niches but also strengthen their pathogenicity responsible for causing disease in host (Nataro 1998).

Previous to the finding of specific virulence factor several pathotypes of *E. coli* were principally classified into the clonal groups characterized by shared 'O' (Lipopolysaccharides LPS) and 'H' (flagellar) antigen (Kaper et al 2004). The antigen O in the bacterium determine its serogroup whereas, the combination of antigen O along with the antigen H determine its serotype (Neidhardt 1996). Some of its prominent characteristics such as; availability of whole genomic sequence, ease of handling, its ability to grow under aerobic and anaerobic conditions, makes it the vital host in biotechnology and also the most used organism in recombinant DNA technology (Yoon et al 2009).

2.2 Major pathotypes of *E. coli*

On the basis of the virulence factors of the bacteria and the clinical symptoms present in host, *E coli* has been classified into seven major pathotypes;

2.2.1 Enteropathogenic *E. coli* (EPEC)

These strains of *E. coli* primarily cause diarrhea in children under the condition of poor hygiene

2.2.2 Enterohaemorrhagic*E. coli*(EHEC)

These strains of *E. coli* produces shiga like toxin causing bloody dysentery and increased risk of haemolytic uremic syndrome (HUS) (Bilinski et al 2012).

2.2.3 Enterotoxigenic*E. coli*(ETEC)

These are the strains that causes traveler's diarrhea in people of all age. These serotypes produce heat liable enterotoxin (LT) and heat stable enterotoxin (ST) and are the leading causing of diarrhea in developing world (Quadri et al 2005; Al-Abri et al 2005).

2.2.4 Enteroaggregative*E. coli*(EAEC)

These pathotypes cause acute and chronic diarrhea in both deveploed and developing world. These are mostly associated with diarrhea in children and HIV infected host and aslo considered second most cause traveler's diarrhea (Weintraub 2007; Nataro & Kaper 1998).

2.2.5 Diffusely adherent*E. coli*(DAEC)

These pathotypes are associated with diarrhea most commonly in children and also considered as strain responsible for causing extra intestinal infections (Servin 2005; Kaper et al 2004).

2.2.6 Enteroinvasive*E. coli*(EIEC)

These oathotypes are closely associated with *Shigella* spp. since it causes syndrome which is identical to shigellosis, with profuse diarrhea and high fever (Kaper et al). They are highly invasive pathotypes of *E. coli* (DarfeuilleMichaud 2002).

2.2.7 Adherent invasive *E. coli* (AIEC)

These are the newly emerged strain which has been associated with the Crohn's disease lesions (Negroni et al 2012).

2.2.8 Extraintestinal pathogenic *E. coli* (ExPEC)

2.2.8.1 Uropathogenic *E. coli* (UPEC)

These strains are distinct from commensal from phenotypic marker as well as from virulence factor. It is the leading cause of urinary tract infection UTIs, comprising around 80% of total human host (Kaper et al 2004; Johnson et al 2000).

2.2.8.2 Neonatal bacterial meningitis *E. coli* (NMEC)

This strain of extra intestinal pathogenic *E. coli* is one of the primary cause of bacterial meningitis in neonates in developing countries, followed by neurological sequelae in many of the survivors (Allocati et al 2013).

2.2.8.3 Avian pathogenic *E. coli* (APEC)

These pathotypes reside as in intestinal micro flora of healthy birds and are accountable for causing extra intestinal infection in avian species (Johnson 2007; Rodriguez-Siek 2005).

2.3 Antimicrobial resistance in *Escherichia coli*

Typically, antibiotic resistance occurs within the few years of introduction of new antibiotics. As most of the antibiotics are derived directly and indirectly from microbial products such resistance is not surprising (Iredell et al 2016). Antibiotics act as centerpiece in the treatment of bacterial infections which makes the worldwide increase in antibiotic resistant bacteria a major concern. Thus haphazard use of antibiotics, along with use of antibiotics in food animals, poor hygienic conditions and overcrowded living conditions are considered to be a major risk factor for the occurrence of antibiotic resistance pattern in bacteria (Erb et al).

The concern with antibiotic resistance pattern is that it is not restricted to pathogenic bacteria but also includes commensals that might significantly increase the population of reservoir resistant strains. *Escherichia coli* is the commensal flora residing in human guts, however is considered pathogenic if found in blood or urinary tract. In fact, it is one of the dominant causative agent urinary tract infections globally (Kahlmeter 2003) and is also one of the most common bloodstream infections causing pathogen (Biedenbach 2004).

2.4 Multidrug resistance (MDR)

Medical literature uses various definition for multidrug resistance (MDR), characterized by different pattern of resistance. The insensitivity or resistance pattern developed in bacteria for two or more classes of antibiotics after previously being sensitive to them, is defined as multidrug resistance or (MDR) (Tanwar et al 2014; Munita & Arias 2016)

Central of disease control and prevention (CDC) defines multi drug resistance (MDR) as, the isolate which is resistant to at least one antibiotic from any three or more type different classes (CDC 2006).

According to WHO, these resistant strains cause persistent spreading of infection since they are able to combat attack by antimicrobial drug. It also claimed such resistance pattern is different for different bacteria, such as *Klebsiella pneumoniae* has higher resistance rate against cephalosporin and carbapenem, *E. coli* against cephalosporin and fluoroquinolones. Whereas, *Streptococcus pneumoniae* against penicillin, *Shigella* species and non typhoidal *Salmonella* against fluroquinolones and similarly, *Mycobacterium tuberculosis* against rifampicin, isoniazid and fluoroquinolones (WHO 2014; Nikaido 2009).

Prolonged exposure of drugs has helped microbes evolve into alarming number of MDR strains, also referred as superbugs. Due to the high level of resistance developed in various strains of bacteria the appropriate doses of drugs fail to function effectively. Such clinical failure deteriorates the medical efficiency which thereby results in uncontrolled increment in medical cost and furthermore, hinder the control and management of the MDR strains and disease causing higher morbidity and mortality (WHO 2014; Olasehinde et al 2014).

Literal definition of MDR is simply, 'resistant to more than one antibiotic'. However, any standardized definitions for MDR have yet to be agreed upon by the medical community. In vitro antimicrobial susceptibility test is one of the various methods used among authors to characterize organisms as MDR (Magiorakos 2012). In another way bacteria can be characterized as MDR, if it is resistant to one key antimicrobial drug (CDC 2008; Seigal et al 2007).

2.5 Multi drug resistance in *E. coli*

During the antimicrobial course of action, both commensal and pathogenic strains of *E. coli* get exposed to the effects of various antimicrobial drugs. Thus, such encounter requires the strains to not just survive but to flourish in the newly established unfavorable environment, which leads them to acquire multi drug resistance characteristics such as drug efflux, enzymatic inactivation, target protection etc (European Food Safety Authority and European Centre for

Disease Prevention and Control [EFSA and ECDC], 2010). One of the major attributes that distinguish normal flora from pathogenic strain is the presence of different virulence factors (toxic, invasive, adhesive etc) in the latter (Szmolka et al., 2012).

Commensals strains are normally overlooked in many aspects due to their lower clinical significance but since they are considered as a potential reservoir of resistance determinants, are still monitored on the regular basis. The dynamics of evolutionary traits changes overtime depending on the host equations and antimicrobials used. Pathogenic strains being on the radar of scientific and medical society, stand primarily on the focus of therapy (European Food Safety Authority and European Centre for Disease Prevention and Control [EFSA and ECDC], 2010).

2.6 Carbapenem

Carbapenems are the beta lactam group of antibiotics, which differ with other classes such penicillins and cephalosporins in their chemical structures. Studies have shown that the oral viability of carbapenem drugs are significantly lower thus, it must be administered orally Michalska et al 2013;Papp-Wallace et al 2011; Papp-Wallace and Bonomo 2016; Shahid et al 2009)

One classification scheme characterized carbapenem drugs into; **Group 1:**

It includes broad spectrum carbapenems, best suited for community acquired infection shown to have limited activity against Gram negative non fermentative bacillus. E.g. Ertapenem.

Group 2:

It includes broad spectrum antibiotics such as Imipenem, Meropenem, Doripenem, which are also effective against non-fermentative Gram negative bacilli.

Group 3:

It is comprised of carbapenems which are effective against methicillin resistance *Staphylococcus aureus* (MRSA). However, it is still under the developmental phase (El-Gamal et al 2017).

Carbapenems in general, tend to have better stability against B- lactamase enzymes as compared to other beta lactam antimicrobial drugs. Despite being highly effective aginst beta lactamase produced by Enterobacteriacea family there are various strains in the family that are able to resist their activity by several mechanisms that includes activity induced by hudrolyzing enzyme and efflux pump. Carbapenem resistance can also be induced by lack of bacterial porins. Presence of bacterial porins render bacteria less susceptible to carbapenem antibiotics because it influences the permeability of bacterial cell membrane. The spread of ESBLs has largely affected the cephalosporin resistance and the rising rsistance pattern of cephalosporin drug in enterobacteriaceae has resulted in extensive use of carbapenem (Pfeifer et al 2010).

Due to the fact carbapenems are sued as last resort for several baxterial infection, the uncontrolled increase in its resistance pattern has alarmed for a severe issue in the context of many hospital and community acquired infection (Nordmann et al 2012).

2.6.1 Meropenem

Meropenem is the parenteral carbapenem drug which exhibit a high quality antibacterial effect in vitro against a widecrange of Gram-positive and negative bacteria including various anaerobes. The reason meropenem covers wider range because it has the penetrating ability into bacterial cell, remarkable stability to beta lactamases, and better affinity for essential PBPs. Its antimicrobial activity against *Enterobacteriaceae* and *Psuedomonas* spp are even better.

2.6.2 Imipenem

Imipenem is the broad spectrum antibiotic drug of the carbapenem class of beta lactam group. The N-formidoy derivative of thinamycin is the precursor of imipenem produced by Streptomyces cattleya. The Wide range of action includes activity against Gram-positive, Gram-negative, aerobes and anaerobes, as well as the various species producing beta lactamases. Imipenem displays bactericidal effect against most species of enterobacteriaceae however is most potent against Gram negative anaerobes.

The resistance pattern of imipenem is not just limited to plasmid or chromosomal mediated beta lactamases, but also includes type 1a beta

lactamases Richmond sykey. Imipenem works against pneumonia, joint infection, endocarditis, intraabdominal infections etc.

2.6.3 Ertapenem

Ertapenem is the broad spectrum, parenteral, carbapenem drug, which demonstrate wide activity against Gram negative pathogens including extended spectrum beta lactamases (ESBLs) and AmpC producing enterobacteriaceae, and also against Gram positive as well as anaerobic pathogens.It is mainly used in patients with complicated intra-abdominal infection (CIAI), acute pelvic infections, and in community acquired pneumonia (CAP).

2.6.4 Doripenem

Doripenem is the newest member of carbapenem class, which closely resembles meropenem and imipenem. It is significantly active against Streptococci, Enterobacteriaceae (including extended spectrum beta lactamase producing strains) methicillin susceptible *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter spp* and *Bacteroides fragilis*.It is clinically not useful against methicillin resistant *Staphylococcus aureus*, vancomycin resistant enterococci, and majority of Gram negative bacteria that are resistant to imipenem and meropenem (Paterson & DePestel 2009)

2.7 Mechanism of resistance against carbapenem antibiotics

Carbapenem demonstrate remarkable potency and broad spectrum of activity against many gram positive band gram negative bacteria, including extensive variety of beta lactam strains.

Primarily there are four different mechanisms by which bacteria can overcome carbapenem antibiotics as described by Babic et al (2006) and Breilh et al (2013)

2.7.1 Enzyme mediated hydrolysis Beta lactamase: AmpC and ESBL

Gram negative bacteria are more susceprible to Carbapenems than any other beta lactam antibiotics mostly because they are resistant to beta lactamases AmpC and ESBLs.The resistance range differ among the carbapenems. Ertapenem exhibit feeble antimicrobial avtivity against ESBL producing strains of *E. coli* and K. pneumonia as compared to any other carbapenem. The variation of activity is also evident in MIC as the MIC of ertapenem rises by one

or dilution whereas, that of imipenem, meropemem and doripenem remains unaffected (Breilh et al 2013).

2.7.1.1Carbapenemase

In simple words, Carbapenemases display the enzymes mediated resistance, that hydrolyze carbapemens together with other beta lactams antibiotics, hence called Carbapenemase.KPC enzymes are the most common type of class A carbapenemase have the ability to cleave the amide bond of most beta-lactam ring (Meletis et al 2012)

The possession of discrete beta lactamases can accelerate rapid carbapenem hydrolysis by metaloenzymes (class B), VIM, IMP, SPM, SIM and GIM (Zhao et al. 2009)

In Some cases, such enzymes are naturally secreted by certain bacterial species as they are encoded in their chromosomal genes making them resistanct to carbapenems for example S. malyophilia and Aeromonas spp. However, in common scenario these genes are naturally carried on Plasmids; which are previously only discovered in *P. aeruginosa* and *A. baumanni* but now are also detected in enterobacteriacea (Chen et al 2011; Kim et al 2006). Almost all the beta lactams encoded by plasmids or transposons are commonly linked with gene encoding for other resistance determinants are hydrolyzed by beta lactamase enzymes.

In the current day, the primary Carbapenemase producers that have been increasingly identified worldwide are *K. pneumonia* and *E. coli*.

2.7.1.2Oxacillinase (Class D)

The hydrolyzing activity of oxacillinase to carbapenem is quite slow in vitro. A significantly high MIC indicates some other resistance mechanism (Babic et al).

2.7.2Chromosomal

SME (Serratia marcescense enzyme), IMI (Imipenem hydrolyzing beta lactamase), NMC (non- metallo enzyme carbapenemase) and CcrA are few classes of enzymes that are found in chrompsomes (Breilh et al 2013).

2.7.3Low affinity of certanin PBPs

Carbapenem do not diffuse easily through the bacterial cell (Martinez et al 2008).It can enter inside either directly transverse through porin channel in the outer membrane of Gram negative bacterial cell walls. Thus the dcreased

expression of outer membrane protein leads to carbapenem resistance in cell (Drawz and Bonomo 2010).

Carbapenem acylate the PBPs permanently after entering into the periplasmic space which includes transglyocolases, transpeptidases and carboxypeptidase catalyze the development of peptidoglycan in the cell wall of bacteria. Carbapenem can inhibit peptide cross linking as well as peptiadase reaction by acting as mechanism based inhibitor of the peptidase domain of PBPs (Pappwallace et al 2011).

Either PBP2a in methicillin resistant Staphylococci or PBP5 in E. faecium do not have high affinity for carbapenem which explains exactly why these species exhibit natural resistance towards carbapenem (Breilh et al 2013).

2.7.4Modification of penicillin binding protein

The affinity for beta lactam antibiotics can also be lowered due to the changes in active site of PBPs which results in subsequent increase resistance to these agents. Point mutation or insertion in Porin encoding genes can also be the reason for lower beta lactam prermeability into the cell, but since this mechanism is not typically enough for producing resistant phenotype it can only act along with beta lactamase expression (Fontana et al 1983; Breilh et al 2013). In both Gram negative and positive organisms, the alteration of PBPs have been described. However, such alteration is assumed to have more impact on Gram positive organism and rarely leads to a significant level of resistance in Gram negative ones (Breilh et al 2013).

2.7.5Decreased expression for outer-membrane proteins

The OprD porin is required for carbapenem penetration into Gram-negative bacteria, and reduction or elimination of OprD synthesis culminates in resistance to imipenem. Despite the fact that meropenem and doripenem's MICs are within the "susceptible" range, other carbapenems' efficacy will also be inhibited. All carbapenems have the ability to use the OprD porin, however Meropenem and Doripenem may also contain alternative points of entry (Breilh et al 2013).

Carbapenems exhibit resistance in *E. coli*, *K pneumonia* due to the loss of OMPs and OprD. This mechanism is also associated with resistance in imipenem and reduced susceptibility to meropenem in *P. aeruginosa*. The loss of CarO and

OMP are also related with resistance in meropenem and imipenem in clinical isolates of MDR *Acinetobacter baumanni* (Drawz and Bonomo 2010).

2.7.6 Efflux pump activities

Gram-negative bacteria are notably tricky to treat because of their underlying antibiotic resistance (Cox and Wright 2013). Their double-membrane structure and underlying tendency to produce efflux pumps allow for the export of antibiotics, which reduces intracellular concentration (Nikaido 2001). The various efflux pumps located in the membranes of gram-negative bacteria transport a wide variety of substances out of the bacterial cell. Antibiotics can be easily transported using these pumps. By expelling antibiotics from the bacterium, the amount of the drug inside the cell is limited, allowing the bacterium to tolerate larger external concentrations of the antimicrobial agent and building resistance (Cox and Wright 2013; Nikaido 2001). Efflux pumps have appeared throughout the three domains of life since the outset of evolution (Saier et al 1998). The physiology of an organism relies on efflux pumps, some of which serve purposes other than imparting resistance to antibiotics. For instance, Gram-negative bacteria's RND efflux pumps are necessary for pathogenicity in their respective host (Bina et al 2008; Piddock 2006)

The main two kinds of efflux pumps are primary transporters and secondary transporters. Members of the first group include ATP-binding cassette (ABC) family transporters, which utilize the energy of ATP binding and hydrolysis for efflux. The energy of the electrochemical potential of the membrane is used by secondary transporters including the major facilitator superfamily (MFS), small multidrug resistance (SMR) family, resistance nodulation division (RND) family, and multidrug and toxic compound extrusion (MATE) family to power efflux (Paulsen 2003; Piddock 2006).

RND family members are the most clinically relevant of these in Gramnegative bacteria. The inner and outer membranes are both served by the tripartite system of these pumps. The *Escherichia coli AcrAB-TolC* system is the most well-studied RND system. It consists of three parts: an outer membrane protein channel (*TolC*), an outer membrane protein channel (*AcrB*), and a periplasmic adaptor protein (*AcrA*) (Du et al 2014). RND systems are highly conserved

across species and comparative genomics reveals that the efflux pumps of *E. coli* (*AcrB*) and several other bacteria such as *Pseudomonas aeruginosa* (MexB), *Pseudomonas aeruginosa, Campylobacter jejuni* (*CmeB*), *Acinetobacter baumannii* (*AdeB*), and *Neisseria gonorrhoeae* (*MtrD*), exhibit high levels of homology (Piddock 2006).

E. coli's tripartite efflux pump *AcrAB-TolC* is comprised of the inner membrane transporter *AcrB*, the outer membrane channel *TolC*, and the periplasmic membrane fusion protein *AcrA*. Although some efflux pumps are substrate-specific, several others recognize a variety of substrates and thus are associated with MDR. Additionally, it has been reported that *E. coli* has intrinsic resistance to several antibiotics as a result of MDR efflux mechanisms. (Chollet et al 2004; Piddock 2006). Benzalkonium, lactams, novobiocin, erythromycin, fusaric acid, fluroquinolones, tetracycline, chloramphenicol, ethidium bromide, acriflavine, crystal violet, SDS, Triton X100, bile salts, triclosan, fatty acids, methotrexate, and linezolid are examples of substances that exhibit efflux pump mediated resistance in *E. coli*(Blair et al 2014).

2.7.7 Efflux pump inhibitors

Due to the obvious role that RND transporters play in the ongoing emergence of MDR clinical bacteria, these efflux pumps have been recognized as "critical" pharmacological targets for the development of antibiotic efflux inhibitor combination therapies (Pagès and Amaral 2009). The combination "lactam lactamase inhibitor" was developed similarly in the past. There are various methods for inhibiting the pump activity of Gram-negative bacteria.

1. Via modification of regulatory processes that control the expression of efflux pumps
2. by impeding the multi-component pump's functional assembly
3. by using a cork to restrict the outer membrane channel (TolC, OprM).
4. by disabling the energy-driven mechanisms of the bacterial transporters, either directly (specifically) via an antiporter site or indirectly (generally) via a collapse of the efflux's energy.
5. by introducing a non-antibiotic molecule into competitive or noncompetitive inhibition at the efflux pump's affinity sites.

6. by altering the chemical structure of earlier antibiotics to decrease their affinity for efflux recognition and binding sites or to inhibit efflux transport

2.7.8Carbonyl cyanide m-chlorophenylhydrazone (CCCP)

The energy necessary for drug transport can be collapsed by a number of different substances. Among them, carbonyl cyanide chlorophenylhydrazone (CCCP) possess a significant impact on the energy level of bacterial membranes and are used in laboratories to completely eliminate drug efflux (Pagès et al 2005).

2.8 Extended Spectrum B-Lactamases (ESBLs)

Clinical microbiologist, doctors and infection control specialists, and antibacterial discovery scientists encounter particular problems from organisms that produce extended spectrum beta-lactamases (ESBLs). Penicillins, broad-spectrum cephalosporins, and monobactams can be all hydrolyzed by ESBLs, which are often produced from TEM and SHV-type enzymes. ESBLs are usually encountered on plasmids that can be transferred between bacterial species and strains. ESBL-producing Enterobacteriaceae have induced several infection outbreaks around the world resulting infection control problems. Multiple resistance generally makes choosing an antibacterial more difficult (Uemura et al 2017). Many ESBL-producing organisms express AmpC -lactamases as well, and they may also co-transmit plasmids that confer aminoglycoside resistance (van Hoek et al 2015). At this time, carbapenems are thought to be the best medication for treating infections brought on by ESBL-producing bacteria. Unfortunately, the development of bacterial species resistant to carbapenems has been linked to their use (Rupp and Fey 2003; Livermore 1995). SHV, TEM, CTX-M, OXA, PER, VEB-1, BES-1 are the various types of ESBLs which are detected in the clinical isolates (Bonnet 2004).

2.9 Global scenario of ESBL

Since the 2000s, there has been an uptick in the colonization and/or infection of these bacteria in the community, which has contributed to their global dissemination (Doi et al 2013).

In the United States, the nosocomial ESBL rates exhibited a substantially rising trend (7.8% in 2010 and 18.3% in 2014). The nosocomial detection rates of

ESBL *E. coli* isolates were 20–40% throughout Southeast and East Asia, and the rates demonstrated a rising trend in many nations (Lob et al 2016; Jean et al 2016).

According to a meta-analysis, the current rates of ESBL detection in long-term care settings are 10–60% in Europe and 50% in China, which itself is consistent with the pattern seen in the data gathered from hospitals (Flokas et al 2017).

In Southeast Asia, both the blaCTX-M-15 and blaCTX-M-14 genes are widespread, albeit CTX-M-15 is more prevalent than CTX-M-14; in contrary, the blaCTX-M-15 genes constitute the majority of CTX-M ESBLs in South Asia, including India (Bevan et al 2017).

The global average rate of ESBL fecal colonization in the community was 14% in a recent systematic review; colonization rates in Europe and North America were 10%; and rates in South, Southeast, and East Asia were 50%. (the highest worldwide) (Karanika et al 2016; Haverkate et al 2017; Madigan et al 2015).

Environmental sources' contribution to the proliferation of ESBLs has also been evaluated. Unsanitary conditions and tainted drinking water have also been highlighted as significant issues in developing countries; large numbers of ESBL-producing *E. coli* in urban wastewater have indeed been observed to be released in a river in a European country (Bréchet et al 2014; Hawkey 2015).

Environmental factors and animals have presumably enhanced the colonization of CTX-M ESBLs, notably CTX-M-15, in human feces in Asia. As a result, Asia is a "key hub" of ESBL genetic evolution and the predominant CTX-M kinds appear to spread internationally (Chong et al 2018).

2.10 Global scenario of *AcrAB TolC* in Carbapenem resistant isolates

The prevalence of resistance to carbapenem specifically, imipenem and meropenem in Enterobacteriaceae showed a steadily rising trend in early 2000. The Enterobacteriaceae genera *E. coli, Klebsiella* species, and Enterobacter species all exhibit a similar pattern. The majority of imipenem-resistant isolates belonged to the *Klebsiella* spp. family, followed by *E. coli* and *Serratia*. The Enterobacteriaceae genus showed the following rank order of imipenem resistance rates from 2000 to 2012: *Serratia* species (1.8%) followed by *Proteus*

species (1.6%), *Klebsiella* species (0.8%), *Citrobacter* species (0.8%), Enterobacter species (0.7%), and *E. coli* species (0.2%) (Xu et al 2015). There have been numerous publications published on the intrinsic and acquired resistance mechanisms of Carbapenem drug in *Escherichia coli*, as well as the involvement of a number of factors which contributes directly or indirectly to these systems (Singh et al 2012).

Recent research, however, demonstrates that a given bacterial species' peculiar phenotype of resistance to antibiotics rests on the coordinated action of several different elements known as the intrinsic resistome. Reduced permeability and higher efflux may be the most important factors, as well as a lack of target modification activity of chromosomally encoded antibiotic inactivating enzymes (Fernández and Hancock 2012). According to a study in India, overexpression of MexAB-OprM efflux pumps was found responsible for meropenem resistance in the absence of acquired resistance mechanism in clinical isolates of *P. aeruginosa* (Choudhury et al 2015). In another study, two novel strains of *Klebsiella pneumoniae* ST1414 and ST1415 were foundresistant to tigecycline in which efflux mediated mechanisms including high expression of AcrAB-TolC and OqxAB efflux pumps appeared to play the key role (Zhong et al 2014).

In a study conducted in US, Carbapenems, such as imipenem and meropenem which are often used to treat multidrug resistance isolates typically strain producing ESBL were found in danger due to the recent appearance of blactamases capable of hydrolyzing carbapenems (Yigit et al 2001). The situation has become more concerning with the arrival of New Delhi metalloBeta lactamases strains in the Indian subcontinent, such as in India and Nepal (Choudhury et al 2015).

Enterobacter aerogenes of clinical origin demonstrated resistance against the lactam and other groups of antibiotics, according to a 2003 study by Charleric Bornet al. These imipenem resistant organisms displayed efflux pump activity against quinolone, tetracycline, and chloramphenicol as well as overexpression of AcrA (Bornet et al 2003).Furthermore, in a recent study conducted in India by *Chetri Shiela et al*,a new finding was observed that showed a strong

correlation was between ertapenem resistance and AcrA over-expression (Chetri et al 2019).Since the time they were first developed, carbapenems— which are used only as a last resort—have been highly successful. The development of a strain of *Klebsiella pneumoniae* that produces carbapenemhydrolyse ß-Lactamase cast doubt on the efficacy of this antibiotic (Blanco et al 2016).

E. coli has been considered the most clinically significant since they are resistant to broad spectrum antibiotics and also possess virulence determinants that can cause extra intestinal infections (Rowe-Magnus 2002). As, Carbapenem is considered the drug of choice by most clinicians for serious infections caused by ESBL-producing Enterobacteriaceae. With the extensive widespread infections with these organisms, it is likely to cause a dramatic escalation in empiric carbapenem use, resulting the emergence of various strain of Carbapenem-resistant Enterobacteriaceae (CRE) (Yigit et al 2001).The efflux pump being an intrinsic resistance mechanism it often remains overlooked in clinical microbiology (Blanco et al 2016).

CHAPTER III

MATERIALS AND METHODS.

3.1 Materials and Equipment

All the materials, equipment, reagents, chemicals and culture media used for the isolation, identification and antimicrobial susceptibility testing of the organism are listed in Appendix-II

3.2 Methodology(s):

This is descriptive cross sectional study conducted in Microbiology laboratory of Kathmandu Model Hospital, Nepal among the patients visiting hospital.

3.2.1 Study design: Study will be laboratory based cross sectional study.

Study population will be in and out patients of Kathmandu Model Hospital

3.2.2 Study subjects

All the patient above the age of 11 months from the birth who are requested for bacterial infection investigation were the study subjects. The demographic and clinical history of the patients were obtained during the sample collection, detailed information of the patients including age, sex and symptoms were collected (Appendix-I)

3.2.3 Sample Size

The researcher intends to include approximately 1500 participants during the study period. However, the following formula will be used for calculation of finite population at the end of the study. Sample size for finite population (n') = n/(1+(n/N)

Where,

N = Population Size n = Sample size

Justification of participant numbers: Sample size (n) = $(Z_{\alpha/2})^2 P(1-P)/d^2$

Where,

P = Prevalence = 71%.[19] d = Margin of error = 5%, based on the prevalence of the previous study. $Z_{\alpha/2}$ = 1.96 at 95% level of significance n = Sample size

Now, $n = (1.96)^2 * 0.71*(1-0.71)/ (0.05)^2 = 316$

The required sample size according to prevalence rate of Kushwaha, 2021 is 316 but all samples collected during the period of research work will be included.

3.2.4 Period of Study

The data for this study was conducted from 1st of April to 1st of September 20th of year 2022.

3.2.5 Inclusion Criteria

Patients above 11 months of age are included in this study.

3.2.6 Exclusion Criteria

Patients below 11 months of age are not included in this study.

Contaminated sample, inadequate information, sample without labelling.

3.3 Method of data collection

Demographic details of the patients were collected from Patients in Kathmandu Model Hospital and also from the software and register where the data of patient's details were registered and is calculated in the form bar graph, pie charts and percentage.

Samples collected in a sterile container were transported directly to the laboratory.

3.3.1 Sample processing

The clinical specimens (sputum, blood, urine, wound/pus swab, tracheal aspirates,endotracheal tips, catheter tips, body fluid) were acquired from the laboratory of the Kathmandu Model Hospital and subsequently inoculated in the respective culture media including Blood agar (BA), Mac-conkey Agar (MA), Cysteine Lactose Electrolyte Deficient (CLED) Agar, Chocolate Agar (CA). All the culture media were then incubated at 37 c. The culture positive samples were further subjected to biochemical testing and Antibiotic susceptibility testing (AST), in order to identify the organism and their antibiotic susceptibility pattern respectively. Afterwards, Phenotypic confirmation for ESBL and Carbapenem resistant strains were carried out followed by Screening and confirmation (PCR) of the *AcrAB ToIC* efflux pump gene among the isolates.

3.3.2 Identification of organism

For the identification of organism standard microbiological procedure was followed. The following examinations were conducted for the identification of organism.

3.3.3 Colony Characteristics

After overnight incubation, for plates significant growth presumptive diagnosis of the organism was made based on the morphology on culture plate (shape, size, appearance, and lactose fermentation). The colony morphology of isolated organisms is mentioned in appendix IV.

3.3.4 Gram staining

The suspected isolated colonies in culture plate were subjected to gram staining procedure. Gram Negative organisms in rods were observed under oil immersion field of light microscope.

3.3.5 Biochemical test

Biochemical tests are the most important methods for the identification of isolated organisms. They shorten the time required to identify microbes, reduce costs, and enhance the accuracy of identification of an unknown microorganism. Isolated colony from the culture plate was selected and inoculated in different types of biochemical test-tubes like TSI, SIM, Citrate and Urease. The result was read after overnight incubation at 37˚C. Different microbes show different types of biochemical reaction for example lactose fermentation, production H2S, utilization of tryptophan etc.

3.3.6 Antibiotic Susceptibility Testing

Antibiotic susceptibility testing is performed by disc diffusion method. In disc diffusion method, the antibiotic is allowed to diffuse through a solid medium so that the concentration is highest near the site of application of the antibiotic disc and decreases with the distance. The disc diffusion method uses filter paper discs charged with appropriate concentration of the drugs. The commonly used method is Kirby-Bauer disc diffusion method.

a)Medium

The medium should support good overnight growth of both the test and the control organisms. Mueller-Hinton agar may be used for testing aerobes and facultative anaerobes. The medium is prepared in petri dish with 4 mm depth.

b)Inoculum

The organisms are isolated in pure culture on a solid medium. Isolated colonies are inoculated in nutrient broth medium and incubated at 37˚C for 46 hours. The density of the organisms in broth is adjusted to approximately 10^7 CFU/ml by comparing its turbidity with that of 0.5 Mc Farland opacity standard tube.

This broth is inoculated on the medium by spreading with sterile swabs. The ideal inoculum after overnight incubation gives even semiconfluent growth.

c)Kirby-Bauer Disc Diffusion Method

The cotton swabs are dipped into inoculum and inoculated in Mueller-Hinton agar by streaking the swab three times over the entire agar surface. The agar surface is allowed to dry for 3-5 minutes. After that, the antibiotic discs are applied on the plate. On a plate of 100 mm, seven antibiotic discs are applied with one disc in the center and six in the periphery. The plates are then incubated at 37˚C for 16-18 hours. The zones of complete growth inhibition around each of the discs are measured. The antibiotics tested were Amikacin (30 mcg), Amoxycillin (10 mcg), Ceftriaxone (30 mcg), Ciprofloxacin (5 mcg), Co-Trimoxazole (25 mcg), Nitrofurantoin (100 mcg), and Ofloxacin (5 mcg). When these drugs were observed to be resistant in some patient, next generation drugs were used which includes Amoxyclav (30 mcg), Cefoperazone/Sulbactum (50/50 mcg), Colistin (10 mcg), Imipenem (10 mcg),
Meropenem (10 mcg), Piperacillin/Tazobactum (100/10 mcg), Polymyxin-B (300 mcg), and Tigecycline (15 mcg).

3.4 Screening of ESBL producers

The Urinary *Escherichia Coli* isolates that were resistant to atleast one of the three indicator 3rd genereation cephalosporins i.e Cefotaxime, Cefpodoxime, and Ceftazidime were screened as ESBL producers and were subjected to phenotypic confirmatory method.

3.4.1 Phenotypic Confirmatory Test for ESBL producers

Phenotypic confirmation of the screened isolates was performed by the Double Disc Synergy Testing DDST method. Bacterial isolates were inoculated in 5ml of nutrient broth and incubated at 37˚C for 2-6 hrs until 0.5McFarland turbidity was attained. Then, lawn culture was prepared by swabbing on MHA using sterile cotton swab. Antibiotic disc containing Amoxycillin Plus Clavulanic acid AMC placed center to center lawn culture on Muller-Hinton Agar (MHA) at a distance of 20mm from the indicator drugs Ceftazidime
30ug and Cefotaxime 30ug. The plates were then incubated at 37˚C overnight. Any enhancement in zone of inhibition of cephalosporins towards the Amoxycillin/ clavulanate was considered as a positive result for an ESBL.

3.4.2 Phenotypic confirmation of Efflux Pump using efflux pump inhibitor:

Meropenem powder (10 g, Himedia, Mumbai, India) was used to detect the efflux pump activity of *Escherichia coli* isolates, both with and without the addition of the efflux pump inhibitor carbonyl cyanide mchlorophenylhyrazone (CCCP), 12.5 µM, also from Himedia, Mumbai, India. Zone of inhibition with the inhibitor and carbapenem alone differ by less than 5 mm, which indicates the presence of efflux pump activity.

3.4.3 Minimum Inhibitory Concentration (MIC)

Minimum Inhibitory Concentration or MIC is the lowest concentration of antibacterial agent expressed in mg/L (µg/ml) which under strictly controlled in-vitro conditions completely prevents visible growth of the test strain of an organism. It is the quantitative method of susceptibility testing, an MIC helps determine which class of antibiotic is most effective.

3.5 DNA Extraction and PCR amplification

3.5.1 Chromosomal DNA Extraction

The chromosomal DNA extraction was performed by Alkaline-Hydrolysis method. Then, the extracted chromosomal DNA were suspended in TE buffer, labeled well and stored at -20˚C. The protocol is maintained in Appendix-V.

3.5.2 Detection of gene by PCR

Amplification of *AcrAB TolC* gene was carried out by conventional PCR using primers Macrogen limited, Korea as;

Table 1: Primer sets for the amplification of *AcrAB TolC* gene

Primer pair	Gene targeted	Sequence 5'-3'	Location	Annealing temperature	reference
Acr AFP	*AcrA*	**CAATTTGAAATCGG ACACTCG**	208-227	Annealing=55 C 45 secs	(18)
Acr ARP		**GGCATGTCTTAACG GCTCCT**	789-807		
Tol CFP	*ToIC*	**TGCTCCCCATTCTT ATCGGC**	49-68	Annealing= 65 C for 45 secs	(18)
Tol CRP		**GCTCTTGCTTGGCG TTGTAC**	1241-1260		
Acr BFP	*AcrB*	**GAAAGGCCAACAG CTTAAC**	674-692	Annealing=55 C 45 secs	(18)
Acr BRP		**GAGCTGGAGTCAG GATCAAC**	1454-1473		

Table 2: Components of PCR

Components	Volume
1x Master Mix	5
Primer-F	0.5
Primer-R	0.5
DNA template	3
Nuclease free water	16
Total	25

PCR assay was performed with primers for *AcrA, acrB, tolC* gene. The thermal cycling conditions were set up as follows; initial denaturation at 94˚C for two minutes, 37 cycles of denaturation at 94˚C for 30 seconds, annealing at 58˚C for 45 seconds and, extension at 72 ˚C for 2 mins and final extension at 72˚C for 10 minutes for gene *AcrA*. Similary, for gene*, acrB* the thermal cycling conditions were set up as follows; initial denaturation at 94˚C for 1 minute, 37 cycles of denaturation at 94˚C for 30 seconds, annealing at 65˚C for 45 seconds and, extension at 72 ˚C for 2 mins and final extension at 72˚C for 10 minutes. Lastly, for gene*, tolC* the thermal cycling conditions were set up as follows; initial denaturation at 94˚C for 30 secs, 37 cycles of denaturation at 94˚C for 30 seconds, annealing at 65˚C for 45 seconds and, extension at 72 ˚C for 2 mins and final extension at 72˚C for 10 minutes. The amplified products were analyzed by gel electrophoresis 2% agarose gel stained with 0.5 mg/l ethidium bromide at 80V for 45 minutes. A 100 bp ladder was used as a DNA marker and visualized through UV transilluminator.

3.6 Quality Control

Quality Control was strictly maintained throughout the process in every step to obtain the appropriate result.

3.6.1 Quality control during isolation and identification

Only pure culture from primary culture media was used for the identification of organism. Work place was ensured to be sterilized, work was performed near the burning flame, inoculating loop was sterilized each timed before and after use.While performing the identification process, control strains were run, *Escherichia coli* ATCC 25922, was used as control.

3.6.2 Quality control during AST

Mueller-Hinton Agar (MHA) from Hi-media was used after checking the lot number and expiry date. ATCC strain was used to check the quality of MHA media. Direct suspension method was used for the inoculums preparation in agar dilution and compared with 0.5 McFarland solution followed by 10x dilution.

3.7 Statistical analysis:

The obtained results will be tabulated, and the percentage of data obtained is calculated based on the infection rate. Data analysis was performed using the SPSS windows version 29.0 software. Tests of significance like Pearson's chisquare test and Fisher Exact test were applied to find out the results. A value of $P<0.05$ was considered statistically significant.

3.8 Implications of study

This study will help to predict the cause of nonspecific symptoms in adults and common infection prevalent in Kathmandu Model Hospital, Nepal.

FLOW CHART METHODOLGY

Sample collection and transport

↓

(Blood, sputum, swab, tissue)(Urine)

↓

MA, BA, CLED

↓

Incubation at 37°C for 24 hours

↓

Observation for the growth of microorganisms and colony characteristics by biochemical tests and Identification of the isolates

↓

Antibiotic susceptibility testing (AST) by Kirby-Bauer disc diffusion method

↓

Interpretation of AST for Multidrug resistant isolates

↓

antibiotic drug by disk diffusion method

↓

Phenotypic Confirmation of ESBL isolates by using combination disc (CTX & CEC, CAZ & CAC) diffusion test

↓

screening of carbapenem resistant isolates

↓

Screening of *AcrAB ToIC* Gene and

↓

Overnight growth of organism in LB broth

↓

DNA extraction by Alkaline Lysis Method Molecular detection of *AcrAB ToIC* Gene by PCR method

↓

Agarose Gel Electrophoresis

CHAPTER IV
RESULT

4.1 Sample positivity

In the period of this study from2022/01/03 to 2022/30/07 total samples registered for the culture was 2384. Out of 2384 samples, the infection rate was found to be(21.8%) that is 520 samples showed significant growth of bacteria and remaining 1884 (78.2%)of patients didn't show bacterial growth which is listed in Table1.

Table 1: Table showing growth and no-growth samples

Variables	Number	Percentage(%)
Growth	520	21.8
No growth	1884	78.2
Total	2384	100.0

4.2 Positive cases according to the age-group

Among the given age-groups in this study, the infection rate was prevalent highest in patients with age group 40-50 years (25.6**%),** followed by the age group above 50 years (24.8**%)**patients, whereas the patients aged 0-10 years showed least infection (10.1**%).** Statistically, we found a significant association between the rate of infection and patients age-groups (P<0.05).The details of the positive cases according to different age group are given in Table2.

Table 2: Growth based on age group

SN	Agegroup (yr)	No of positive cases	Percent(%)	p-value
1	0-10	23	9.3%	
2	10-20	38	18.0%	
3	20-30	106	21.8%	0.00
4	30-40	85	20.15%	
5	40-50	79	27.2%	
6	Above 50	89	25%	

Total	520	100

4.3 Number of positive case according to gender

Out of 520 bacterial isolates, total number of cases for male was observed to be 139 (26.7%), whereas the infection rate was highest for female which was found to be (73.3%). Statistically there was a significant association between growth and gender (P<0.05) which is listed in Table 3.

Table 3: Number of positive cases according to gender

Gender	No. of growth	Percentage (%)	P-value
Male	137	26.7	0.00
Female	382	73.3	

4.4Distribution of Causative agents

Out of 520 bacterial isolates majority of them were found to be Gram negative organism with *E coli* being the major isolates constituting of 78% of the total isolates followed by *Klebseilla* spp 7%, *Pseudomonas* spp 5%, *Acinetobacter*2% and*Enterobacter* spp 1% were among the least frequent. The lists of organism isolated from different specimens are listed in figure 1.

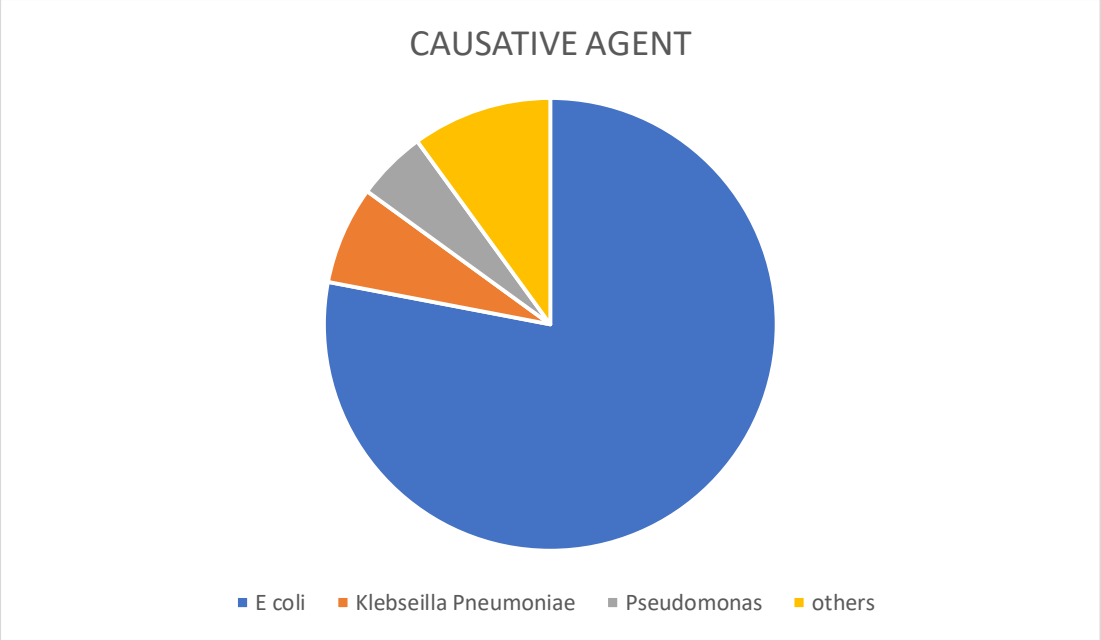


Figure 1: Distrubution of Causative Agents

4.5 Antibiotic Susceptibility Pattern of the isolates

Antimicrobial sensitivity test was performed based on the by Kirby Bauer disc diffusion method. The second line drug was used following the increased resistant pattern with first line antibiotic. Bacterial isolates highest resistance was observed inAmoxycillin 66%, followed by third generation cephalosporin drugs; ceftazidime 48.6%, Cefotaxime/Ceftriaxone 48.1% and cotrimoxazole 34.7%.

Among the first line antibiotics used for the treatment of infection, 86.6% of *E coli* isolates were sensitive to the antibiotic Amikacin, followed by Gentamycin 80.9%, Amoxycillin/clavulanate 66.7%, Cotrimoxazole 65%, Ofloxacin 55.1% and Ceftriaxone 51.9%. Among them, 99.8% isolates showed sensitivity to Polymyxin-B and Colistin, followed by Imipenem and Meropenem 89.6%, whereas 27.8% isolates showed significant resistant to antibiotic Doxycycline .Similarly, 25.8% and 20.8% of the total isolates subjected for the second line antibiotics were found to be resistant to Cefeperazone-Sulbactum and PiperacillinTazobactam.antibiotics respectively.

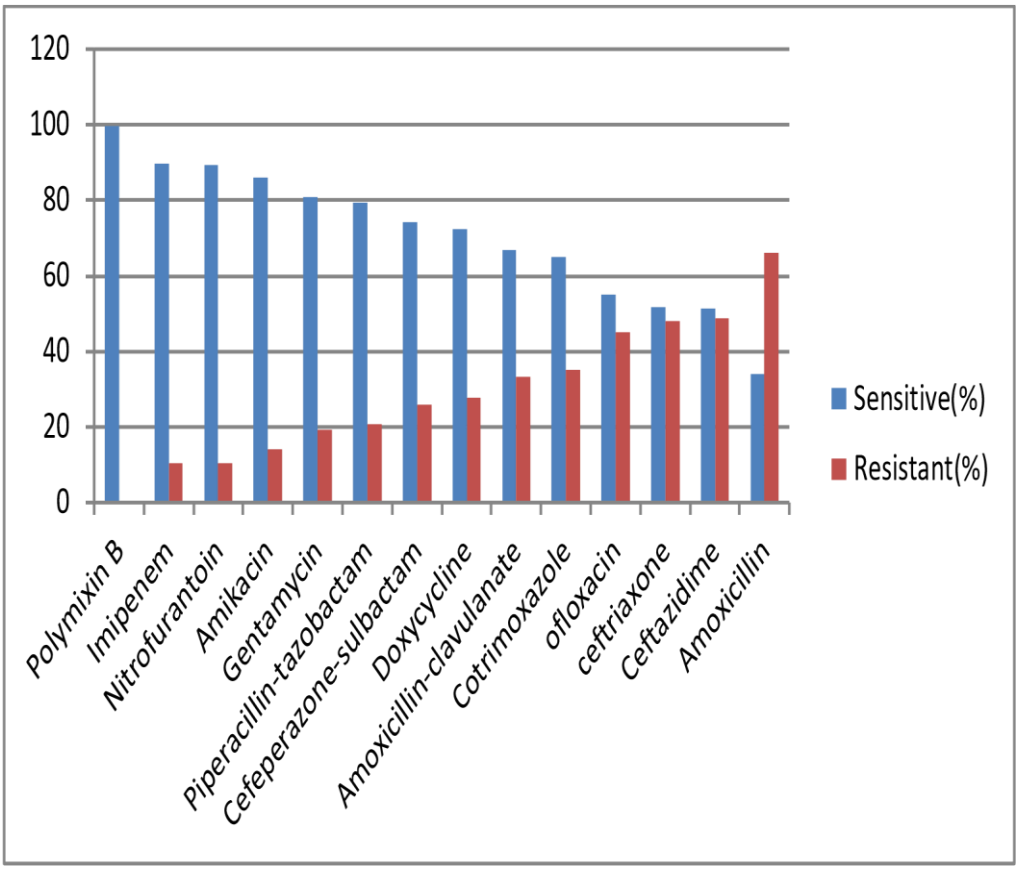


Figure 2: AST pattern of the positive isolates

4.6Association between ESBL *E coli* and MDR *E coli*

Out of 403 *Escherichia coli* isolates, 147 (36.5%)were ESBL producer and 256 (63.5%) were found to be non ESBL producer.Moreover, out of 403 isolates 228 (56.6%) *E coli* were found to be multi drug resistant. Satistically , we observed a significant association between ESBL production and Multi drug resistance which is given in table 4.

Table 4: Association between ESBL *E coli* and MDR *E coli*

Organism	ESBL +ve	ESBL –ve	P-value
MDR *E coli*	147(100%)	81(31.65%)	**<0.05**
Non-MDR *E coli*	0	175(68.35%)	
Total	147(100%)	256(100%)	

4.7Antibiotic Resistance pattern of Carbapenem Resistant isolates

Antibiotic Susceptibility Test of Carbapenem resistant isolates showed that higher percentage of CRE were resistant to all antibiotics used than compared to Carbapenem sensitive *E coli* which is given in the Table 5.

Table 5: Antibiotic Resistance pattern of Carbapenem Resistant isolates

Antibiotics	Carbapenem sensitive *E coli* N(%)	Carbapenem Resistant *E coli* N(%)	P-value
Ceftazidime	156(79.6)	40(20.4)	<0.001
Cefotaxime	155(79.9)	39(20.1)	<0.001
Ofloxacin	134(76.6)	41(23.4)	<0.001
Amoxycillinclavulanate	93(69.9)	40(30.1)	<0.001
Cotrimoxazole	108(77.1)	32(22.9)	<0.001
Doxycycline	76(67.9)	36(32.1)	<0.001

Gentamycin	48(63.2)	28(36.8)	<0.001
Piperacillintazobactam	46(54.8)	38(45.2)	<0.001
Amikacin	31(57.4)	23(42.6)	<0.001
Nitrofurantoin	26(66.7)	13(33.3)	<0.001

4.8 Carbapenem Susceptibility Pattern of *E coli*

Out of 403 *Escherichia coli* isolates, 42 (10.4%) were found to be Carbapenem resistant and 361 (89.6%) were found to be carbapenem sensitive. Moreover In our result, higher percentage of isolates from female patients (9.4%) were found to carbapenem resistant than compared to male (13.83%). Moreover, out of 147 ESBL isolates, 16 isolates (10.9%) were found to be carbapenem resistant. Satistically, we didn't observe any significant association between ESBL production and resistance to carbapenem antibiotics.

Table 6: Carbapenem Susceptibility Pattern with the gender

SN	Gender	Sensitive n (%)	Resistant n (%)	P-value
1	Male	81 (86.17)	13 (13.83)	
2	Female	280 (90.6)	29 (9.4)	0.2

4.9Association between ESBL and Carbapenem Resistance

Out of 403 *E. coli* isolates 42 were found carbapenem resistant, among which 10.9% were ESBL producers which is given in Table 6.

Table 6: Association between ESBL and Carbapenem Resistance

S.N	Organism	Carbapenem Sensitive n(%)	Carbapenem Resistance n(%)	P-value
1	**ESBL**	131(89.2%)	16(10.9%)	0.8
2	**Non-ESBL**	230(89.8%)	26(10.2%)	

4.10 Prevalence of Carbapenem Resistant Genes in *E coli*

Out of 42 Carbapenem resistant *Escherichia coli* isolates, AcrA gene was prevalent in 23 (54.77%) of the isolates. Similarly, 30 (71.42%) *Escherichia coli* isolates was found to be positive for the Carbapenem resistant gene AcrB and 31 (73.80%) of the isolates were found to be positive for TolC gene. Overall, 21 (50%) of the isolates had all three genes present. Moreover, AcrA gene was prevalent in 21 (56.09%) of the MDR isolates.

Similarly, 73.17% and 75.60% of the MDR *E coli* isolates were found to be positive for Carbapenem resistant genes AcrB and TolC respectively. Also, 37.73% of the isolates had both the genes *AcrA* and *AcrB* and 42.59% of the isolates had both the genes *AcrA* and *TolC*. Similarly, 42.62% isolates had both the genes *AcrB* and *TolC*. Satistically, we observed a significant association between Carbapenem resistant genes *AcrA* and *AcrB* (**p<0.05**) and *AcrA* and *TolC* (**p<0.05**) which is given in the Table 7 and 8.

Table 7: Association between *AcrA* and *AcrB* Gene

Gene	*AcrB* Present	*AcrB* Absent	P-value
AcrA Positive	20(87%)	3(13%)	0.01
AcrA Negative	10(52.6%)	9(47.4%)	
Total	30(71.4%)	12(28.6%)	

Table 8: Association between *AcrB* and *TolC* Gene

Gene	*TolC* Present	*TolC* Absent	P-value
AcrB Positive	26(86.7%)	4 (13.3%)	0.003
AcrB Negative	5 (41.7%)	7(58.3%)	
Total	31(73.8%)	11(26.2%)	

4.11 Distribution of Carbapenem Resistant Genes in ESBL *E coli*

In our result, higher percentages of ESBL isolates (69%) were found to be positive for the carbapenem resistant genes than compared to Non ESBL *E coli* (31%). Out of 40 *E coli* isolates, 10(62.5%)of the ESBL isolates were found to be positive for AcrA gene and the frequency of *AcrB* and *TolC* gene in ESBL *E coli* was found to be 93.8%. Satistically, we observed a significant association between ESBL production and Carbapenem resistance for the genes *AcrB* and *TolC* (**p<0.05**).

Table 9: Distribution of Carbapenem genes in ESBL and Non-ESBL *E coli*

Organism	***TolC* present**	***TolC* absent**	***AcrA* present**	***AcrA* absent**	***AcrB* present**	***AcrB* absent**
ESBL producer	16(94.1%)	1(5.9%)	11(64.7%)	6(35.3%)	16(94.1%)	1(5.9%)
Non-ESBL	15(60%)	10(40%)	12(48%)	13(52%)	14(56%)	11(44%)

4.12Phenotypic Confirmation of the bacterial isolates through MIC

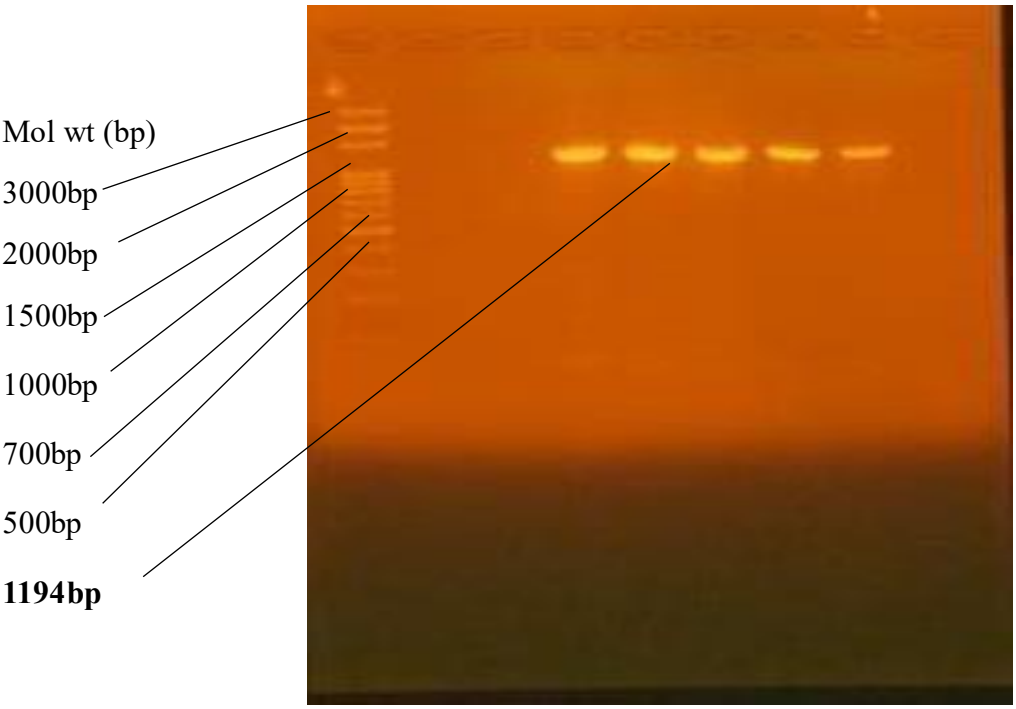
Out of 42 Carbapenem resistant *E. coli,* 33 isolates showed positive results in phenotypic confirmation of efflux pump.

Table 10:Phenotypic Confirmation of the bacterial isolates through MIC

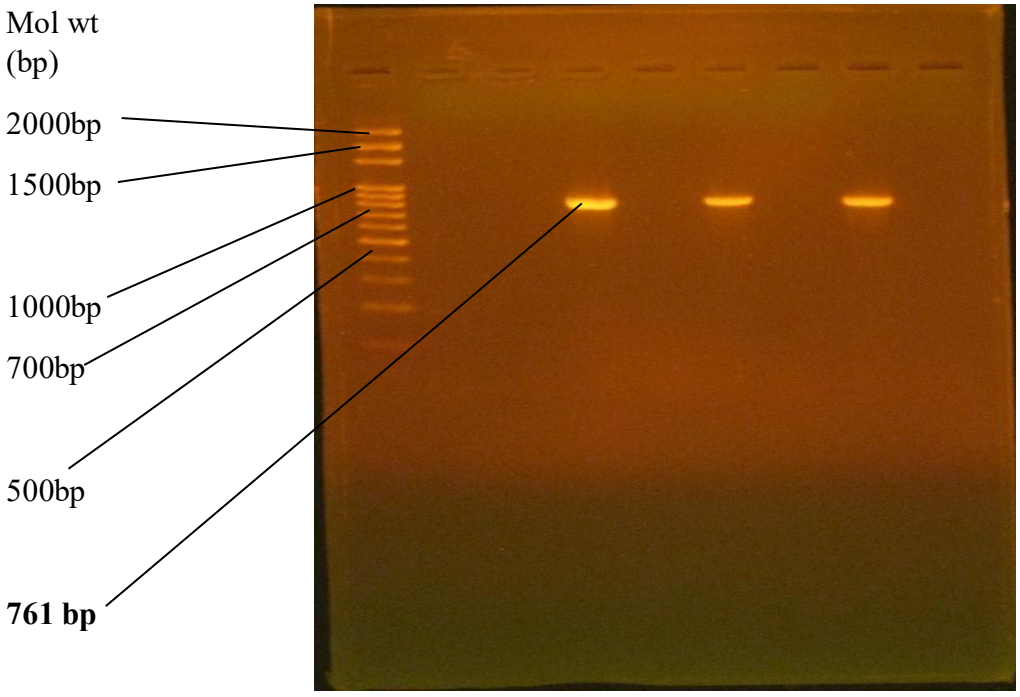
Isolates	Without CCCP	With CCCP
1	16	4
2	8	4
3	256	128
4	256	128
5	64	16

6	8	4
7	128	16
8	8	4
9	512	512
10	512	64
11	256	32
12	32	8
13	256	64
14	512	64
15	512	64
16	512	128
17	256	64
18	64	8
19	256	64
20	32	16
21	16	8
22	512	32
23	64	32
24	1024	256
25	256	64
26	512	128
27	512	256
28	2	NG

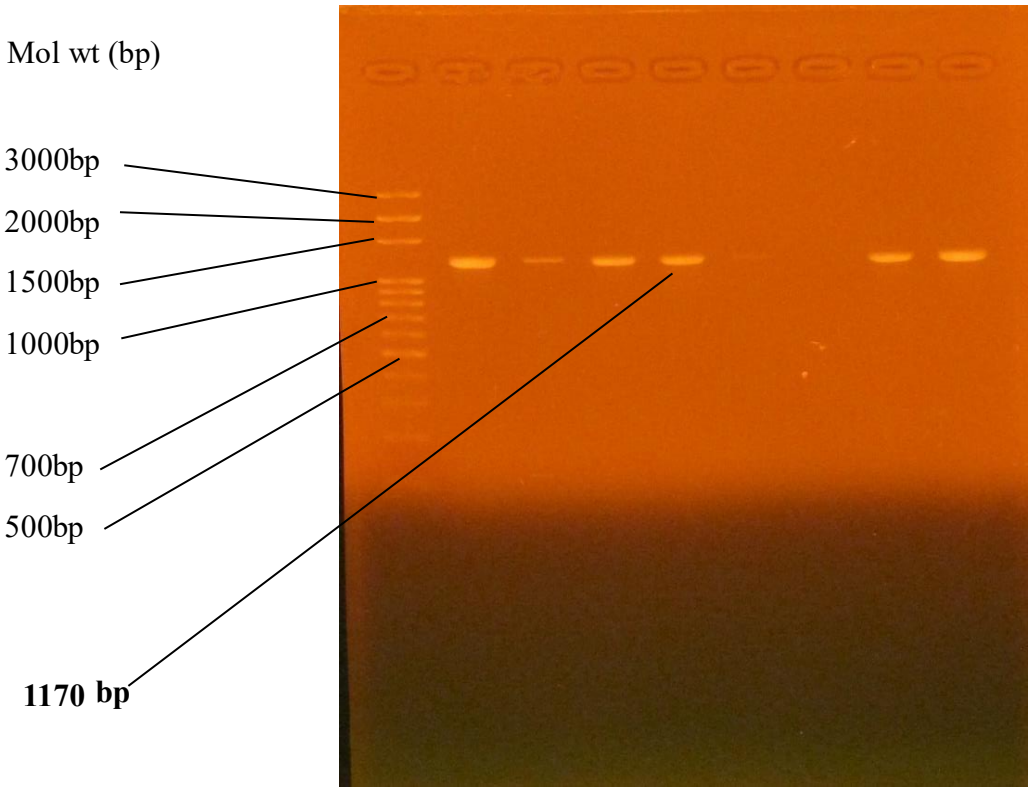
29	8	8
30	64	64
31	512	64
32	256	32
33	16	4
34	128	32
35	8	4
36	128	64
37	256	32
38	1024	64
39	256	32
40	512	64
41	256	64
42	256	256



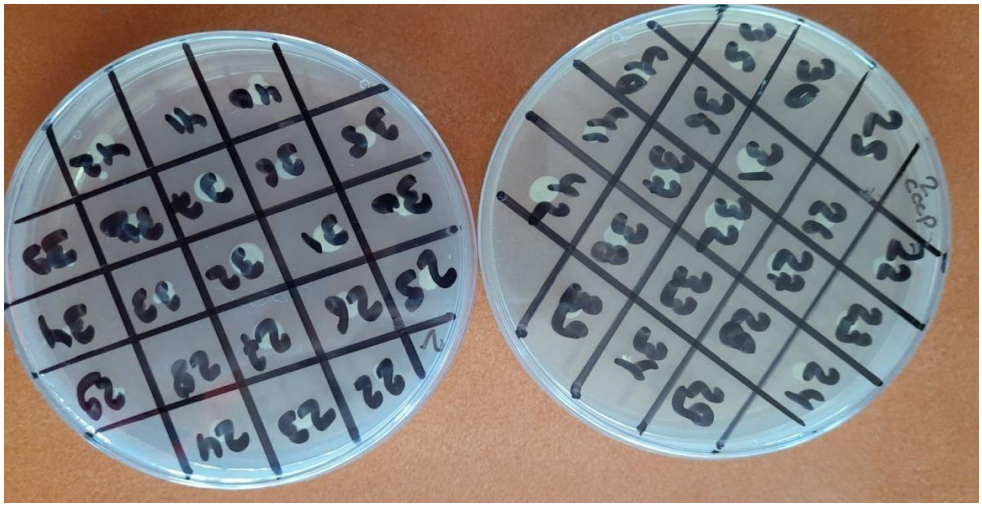
Photograph 1: *AcrA* gene



Photograph 2: *AcrB* gene



Photograph 3: *TolC* gene



Photograph 4: Minimum Inhibitory Concentration with and without cccp

CHAPTER V
DISCUSSION

In our study the significant growth was detected in 520 (21.8%) of the 2384 samples, similarly investigations by Hamid et al. (2011) (24.9%), Pathak and Pokharel (2015) (17.3%), and Poudyal et al. (2011) (16.9%) revealed modest culture positivity from various specimens. The use of antibiotics previous to sample collection, the effectiveness of infection control systems designed to avoid disease, and the management of bacterial infection within hospitals may be the common causes of the low growth rate found. (Hamid et al 2011). Since only aerobic fermentation is employed, anaerobic microbes could also be the reason (Bhattarai 2014). *E. coli* is the most commonly isolated pathogen comprising (78%), followed by *Klebsiella* spp 7%, *Pseudomonas* spp 5%, this result is similar to the study conducted by Daud et al 2005-2012, which shows (60.53-73.98) % the incidence of the *E. coli*, followed by *K. pneumonia* (5.32-8.33) % in UTI patients.

This study depicts the higher occurrence of growth among female 73.3% while compared with male which is only 26.7%. The finding of this study was similar with the recent study conducted by Yadav and Prakash (2017) in which out 321 samples 51.98% significant growth was seen in female and 48.01% in male. In addition to this, similar result was seen in another study (Choudhary et al 2016) where 25.06% male and 74.4% female had the significant growth. Additionally, previous studies conducted by Bomjan 2005 and Baral 2008 produced comparable findings. The shorter urethra and close closeness to the anal orifice that facilitate easy access for coliform bacteria, also the complicated metabolism during pregnancy, as well as the use of contraceptives and other menstrual products, could all be the reasons for the higher growth rate in females. Another explanation for the greater development rate in females could be the relative high proportion of female patients among all patients. Among the (520) 78% isolates of *E. coli* highest growth pattern was seen in the age category above 50 years followed by that of age category 20-30 (22.3) % least growth of isolates (10%), were found in the category (1-10) years. This may be due to the age

extremity factor, as this group is has passive life style and weaker immune system, they also fail to maintain proper hygiene. Being the economically active population, the age group (20-30) is involved in various activities that may lead to accident and or infection leading to the hospital admissions there by increasing their susceptibility towards nosocomial infection. However, the predominant age group may vary according to the type of hospital and its facilities (Bhattrai 2014).

In this study 100% isolates demonstrated highestresistance inAmoxycillin 66%, followed by third generation cephalosporin drugs; ceftazidime 48.6%, Cefotaxime/Ceftriaxone 48.1% and cotrimoxazole 34.7%. Among all second line leading isolates, 99.8% isolates showed sensitivity to Polymyxin-B and Colistin, followed by Meropenem 89.6%, whereas 27.8% isolates showed significant resistant to antibiotic Doxycycline of the total isolates subjected for the second line antibiotics were found to be resistant to Cefeperazone-Sulbactum and PiperacillinTazobactam.antibiotics respectively.

However, another study from tertiary care hospital Kathmandu, showed 73 (52.9%) among 138, were multidrug resistant in which lowest rates of resistance was observed in imipenem followed by piperacillin/tazobactam, amikacin and cefoperazone/sulbactam (Nepal et al 2017). Similarly alarming resistance pattern was seen in resistance patterns was seen for amoxycillin, cotrimoxazole, flouroquinolones and third-generation cephalosporins, among 29 MDR *E. coli* in another study done in urine smples at Kathmandu model hospital (Baral et al 2012).

Out of 403 *E. coli* (10.42%) of the samples tested positive for carbapenem resistance, while 361 (89.6%) samples were carbapenem sensitive. According to our findings, highest resistance was seen in male patients (13.82%) than from female patients (9.2%). In a similar study conducted by Karn et al (2016) the frequency of Enterobacteriacea bacteria was found to be in the range of 4.49% to 20%. Similarly, Indian research Datta et al (2012) found that 7.87% of Enterobacteriaceae family were carabapenem resistant. On the other hand, frequency of carbapenem resistyant *E. coli* isolated from ICU's and departments

of a tertiary care hospital in delhi varied from 13% to 15% according to the research carried out by Wattal et al (2010).

Out of 403, *E. coli* isolates 147 (36.5%) were ESBL producer the prevalence of ESBL producing *E. coli* was found as low upto 18.2% in a study conducted by Raut et al (2015) and high upto 80% for *E. coli* was found as low upto 80% for *E. coli* (Poudyal et al 2011). Global prevalence of ESBL producing presently varies from least than 1% to 74% (Thokar et al 2010). Global prevalence of ESBL among clinical isolates from country to coutry and from institutions to institutions. These difference can be partially due to local antibiotic prescribing habits (Pokharel et al 2006). Two different combination discs that is ceftazidime and cefotaxime with their respective clavulanate were used for the confirmation of ESBL.

The AcrAgene was present in 23(54.77%) of the 42 Carbapenem-resistant *Escherichia coli* samples. The Carbapenem resistance gene AcrB was found to be present in 30 (71.42%) *Escherichia coli* strains, and the TolC gene was found to be present in 31 (73.80%) of the isolates. Overall, all three alleles were found in 21 (50%) of the samples. Furthermore, 21(56.09%) of the MDR samples had the AcrA gene. Similar to this, it was discovered that the Carbapenem resistance genes AcrB and TolC were present in 73.17% and 75.60%, respectively, of the MDR *E. coli* isolates. Additionally, 42.59% of the isolates had both AcrA and TolC, 37.73% of the isolates had both AcrA and AcrB and 42.62% of the isolates had both *AcrB* and *TolC*. we statistically found a significant association between the genes for carbapenem resistance *AcrA* and *AcrB* (p 0.05) *AcrA* and *TolC* (p 0.05) and *AcrB* and *TolC* (p 0.01). Correspondingly in the study conducted by (Fayyazi et al 2020), out all three genes *AcrA*, *AcrB*, and *TolC*, the frequency of *AcrA* was 68.5%, *AcrB* was71.8% and *TolC* was 66.2%, among the uropathogenic isolates of *E. coli*.

Similar to this study,a study conducted by (Maleki et al 2016) showed that, out of total of 78% of the MDR isolates the frequency of the *AcrA* gene was 82.90%, the acrB gene was 95.90% and, *AcrAAcrB* was 95.90%. There was no significant association between *AcrA* and *AcrB* frequency. However, another study

conducted in Egypt by Gawad et al (2018) showed similar prevalence of all three genes (74.84%) among the MDR isolates *E. coli*.

Prevalence of efflux pump genes and carbapenem resistance has been directly linked in a study conducted in India where the association of the *AcrAB-TolC* efflux pump was reported more than 70% with multidrug resistant *E. coli* causing UTI (Choudhury et al 2015).

Similarly, in another study, out of 254 isolates of *Klebsiella pneumonia,* 41 (16.14%) isolates were tigecycline resistant strains among which 9 (21.95%) isolates demonstrated different efflux pump genes of enterobacteriaceae including AcrAB tolC (Zhong 2014).Furthermore, in a recent study conducted in India showed 98 of the 298 carbapenem resistant *E. coli* isolates had efflux pump-mediated nonsusceptibility (Chetri et al 2019).In addition, 16.9% of the 147 ESBL samples were discovered to be Carbapenem resistant. Statistically, we found no significant association link between ESBL production and drug resistance to the carbapenem antibiotic. In a study, 53 ESBL producing *K. pneumonia* 2(3.8%) was tigecycline resistant (Roy et al 2013). Similarly, 1 (14.28%) out of 7 carbapenem resistant *E. coli* was found ESBL positive in a study conducted by Pal et al (2019).

However, a study performed by Nasir et al (2019) showed only 7 (5.03%) out of 139 samples of *E. coli* that produced ESBL and were resistant to carbapenem. This research evaluated the carbapenem MIC in the presence or lack of the CCCP (25 g/mL) in order to identify the function of the efflux pump in the carbapenem resistant *E. coli* isolates. The findings demonstrated that most samples had lower MICs in the presence of the pump inhibitor than in the absence of the efflux pump inhibitor. Similar finding was observed in a study conducted by Moazzen et al (2018).

Some studies have looked into the function of these substances, like CCCP. Rajamohan et al. discovered that the inclusion of CCCP at a dose of 25 g/mL reduced the MIC of various biocides by 2 to 12 folds (Rajamohan 2010). According to Ardebili et al., the majority of samples (86.1%) became less

resistant to ciprofloxacine (2 to 64 times) in the presence of efflux pump inhibitors (Ardebili et al 2014).Based on the findings of Lin et al. (2009), most samples became 2 to 8 times more susceptible to ciprofloxacin when CCCP was present.According to these findings, A. baumannii isolated from hospitalized individuals exhibits significant fluoroquinolone resistance due to the involvement of multidrug efflux pumps. Recent findings suggest that the overexpression of this pump, including AdeABC, is a consequence of drug tolerance. (Marchand et al 2004). The decrease in MIC in the presence of efflux pump inhibitor showed that, efflux pump mechanism has a key role in the resistance of carbapenem in *E. coli*.

CHAPTER VI

CONCLUSIONAND RECOMMENDATION

6.1 CONCLUSION

The current study examined the function of carbapenem-mediated efflux pump resistance in clinical isolates of *Escherichia coli* from a tertiary referral hospital in Nepal.Hence,The study demonstrated that the AcrAB TolC pump plays a significant role in the development of resistance against the carbapenem family of antibiotics and is an important antibiotic resistance determinant in bacterial pathogens. The findings also suggested that most samples had lower MICs in the presence of the pump inhibitor than in the absence of the efflux pump inhibitor.

6.2 RECOMMENDATIONS

- Considering the results from this study, Amoxycillin can no longer be recommended as an empirical prescription for bacterial infection, and instead more effective antibiotic like Nitrofurantoin can be preferred choice of drug.
- Treatment should be started only after performing antibiotic sensitivity testing to minimize the misuse of antibiotics and to prevent increased resistant. Ceftazidime-avibactam, meropenem-vaborbactam, imipenem-relebactam, cefiderocol and novel aminoglycosides and tetracyclines are few of the approved alternatives for carbapenem resistant enterobacterales.

LIMITATIONS

This study does not include the carbapenem inactivation test mCIM , and eCIM, for the detection of carbapenemase enzyme production. Hence, carbapenem resitance pattern among the *E. coli* isolates might also include carbapenem enzyme production. However, phenotypic confirmation of efflux pump had narrowed down the shortcomings of the test since it has clearly been reflected in the result that minimum inhibitory test (MIC) in the presence of cccp had contradicting the effect as compared to the MIC result in the absence of cccp. In order to further limit the shortcomings studies can be conducted narrowing down the other carbapenem resistant mechanisms, including carbapenemase enzyme production.

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APPENDIX I
DATA COLLECTION SHEET

Date……

Demographic Profile

Patient ID No: Age:

Sex: Ward:

Address:

Sample:

Microbiological Profile

Day 1: Culture on CLED

Day 2:

Reading of culture plates:

Shape: Size:

Surface: Elevation:

Edge: Opacity:

Color: Consistency:

Remarks:

Gram staining result:

Catalase: Oxidase:

TSI: SIM:

Citrate: Urease:

Organism identified as:

Antibiotic Susceptibility Testing:

Antibiotic used	Zone of inhibition (mm)	Interpretation

APPENDIX-II

List of materials and equipments used during the study

Equipments

Autoclave

Incubator Refrigerator

Hot air oven

Microscope Weighing machine

pH meter, Thermoscientific, Orian star A111

Centrifuge, Hermle, Z167M

Conductivity meter,

UV- transiluminator, Uvitec

Thermocycler, Veriti, Applied Biosystems

Microbiological culture media (Hi-media)

CLED

Nutrient broth

Mueller Hinton Agar

MacConkey Agar

Chemicals and Reagents

Gram's stain reagent (Crystal violet, Gram's iodine, Acetone:alcohol, safranin)

Catalase (3% hydrogen peroxide)

Biochemical test media (TSI, SIM, Citrate, Urease, etc)

Microscope oil

0.5 McFarland Standard

Agarose; Hi-Media; India

Antibiotic discs; Hi-Media; India

Distilled water

100bp DNA ladder,

EDTA; Hi-media; India
Ethanol, Isopropanol, Tris base, HCL, Boric acid; Hi-Media, India

Ethidium Bromide; Hi-Media, India

Mastermix,

Primer; Macrogen

Phenol; Hi-media, India

Glasswares

Measuring cylinder Test tubes
Beaker Reagent bottles Conical flask Glass rods

Antibiotic discs (Hi-media)

Amikacin (30 mcg)

Amoxyclav (30 mcg)

Amoxycillin (10 mcg)

Ceftriaxone (30 mcg)

Cefoperazone/Sulbactum (50/50 mcg

Ciprofloxacin (5 mcg)

Chloramphenicol (30 mcg)

Colistin (10 mcg)

Co-Trimoxazole (25 mcg)

Imipenem (10 mcg)

Meropenem (10 mcg)

Nitrofurantoin (100 mcg) Ofloxacin (5 mcg) Piperacillin/Tazobactum (100/10 mcg) Polymyxin-B (300 mcg) Tigecycline (15 mcg).

Miscellaneous

Gloves	Distilled water
Rectified spirit	Swab sticks
Immersion oil	Markers
Tube stand	Bunsen burner
Blotting paper	Cotton
Tissue paper	Forceps
Inoculating loop	Staining rack

APPENDIX III

BACTERIOLOGICAL MEDIA AND REAGENTS

Composition and preparation of different media

1.C.L.E.D Agar w/Andrade Indicator

Ingredients	Gms/litre
Lactose	10.0
Tryptone	4.0
Peptone	4.0
Beef Extract	3.0
L-Cystine	0.128
Andrade indicator	0.10
Bromothymol blue	0.02
Agar	15.0
Final pH (at 25˚C)	7.5±0.2

Preparation: As directed by manufacturing company, 36.25 grams of the medium was dissolved in 1000 ml of distilled water and heated to boiling to dissolve the medium completely. The media was sterilized by autoclaving at 15 lbs pressure at 121˚C for 15 minutes.

2.Mueller Hinton agar (Hi-media)

Ingredients	Gms/litre
Beef infusion form	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25˚C)	7.3±0.2

Preparation: 38.0 gm of media was dissolved in 1000 ml of distilled water and dissolved by heating. The media was then sterilized by autoclaving at 15 lbs pressure at 121˚C for 15 minutes

3.Nutrient broth (Hi-media)

Ingredients	**Gms/litre**
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH (at 25˚C)	7.4±0.2

Preparation: 13.0 gm of media was dissolved in 1000 ml of distilled water and dissolved by heating. The media was then sterilized by autoclaving at 15 lbs pressure at 121˚C for 15 minutes.

4. MacConkey Agar

Ingredients	**Gms/litre**
Peptone	20
Lactose	10
Sodium taurocholate	5.0
Sodium chloride	5.0
Neutral Red	0.04
Agar	20
Final pH (at 25˚C)	7.4±0.2

Preparation :24.28 gm of media was dissolved in 1000 ml of distilled water and heated to boiling to dissolve the medium completely. The media was then distributed in test-tubes and sterilized by autoclaving at 15 lbs pressure at 121˚C for 15 minutes. The sterilized medium in test-tubes were set in inclined form to form slant.

5. Simmons Citrate Agar Ingredients Gms/litre

Magnesium sulphate	0.2
Ammonium dihydrogen phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Bromothymol blue	0.8
Agar	15.0
Final pH (at 25˚C)	6.8±0.2

Preparation :24.28 gm of media was dissolved in 1000 ml of distilled water and heated to boiling to dissolve the medium completely. The media was then distributed in test-tubes and sterilized by autoclaving at 15 lbs pressure at 121˚C for 15 minutes. The sterilized medium in test-tubes were set in inclined form to form slant.

6. Triple Sugar-Iron Agar Medium

Ingredients	**Gms/litre**
Beef extract	3.0
Peptone	20.
Yeast extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose monohydrate	1.0
Ferrous sulphate	0.2
Sodium chloride	0.5
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0

Preparation : As directed by manufacturer, 64.42 grams of media was dissolved in 1000 ml of distilled water and heated to boiling to dissolve the media completely. Then the media was distributed in test-tubes and sterilizedby

autoclaving at 15 lbs pressure at 121˚C for 15 minutes. The sterilized medium in test-tubes were set in inclined form to form slant and butt.

7. SIM Medium

Ingredients	Gms/litre
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium thiosulphate	0.025
Agar	3.0
Final pH (at 25˚C)	7.3±0.2

Preparation : As directed by manufacturer, 36.23 grams of media was dissolved in 1000 ml of distilled water and heated to boiling to dissolve the media completely. Then the media was distributed in test-tubes and sterilized by autoclaving at 15 lbs pressure at 121˚C for 15 minutes. The sterilized medium in test-tubes were allowed to cool in an upright position.

8. Urea Base Agar

Ingredients	Gms/litre
Peptone	1.0
Dextrose (Glucose)	1.0
Sodium chloride	5.0
Disodium hydrogen phosphate	1.2
Potassium dihydrogen phosphate	0.8
Phenol red	0.012
Agar	15.0
Final pH (at 25˚C)	6.8±0.2

Preparation : 24.01 gm of media was dissolved in 950 ml of distilled water and dissolved by heating. The media was then sterilized by autoclaving at 15 lbs pressure at 121˚C for 15 minutes. The media was cooled to 45-50˚C and 50

ml of 40 % urea solution was added aseptically and mixed properly. Then the media was dispensed into sterile test-tube and allowed to set in the slanting position.

Composition and preparation of different reagents and Biochemical test media

Gram stain reagent Hucker's Crystal violet stain

Crystal violet stock solution was prepared by dissolving 40 gm of crystal violet (90- 95% dye content) in 400ml of ethanol (95%), filtered and stored at room temperature. For the preparation of working solution of crystal violet, 40 ml of stock solution was added to 160 ml of filtered ammonium oxalate solution (1%).

Gram's iodine

Stock solution of Lugol's iodine was prepared by mixing 25 g of iodine crystals and 50g of potassium iodide in 500ml of distilled water in a brown glass bottle. For the preparation of working solution, 60ml of Lugol's iodine stock solution was mixed with 220ml of distilled water and 60 ml 5% sodium bicarbonate solution. **Acetone alcohol (1:1)**

50 ml of ethanol (95%) was mixed with 50 ml acetone in a brown bottle.The bottle labelled with the date of preparation and stored at room temperature.

Safranin

Stock solution of safranin was prepared by dissolving 5 g of safranin in 200ml of 95% ethanol.For the preparation of safranin working solution,20ml of stock solution was mixed with 180ml of distilled water.

Catalase

Hydrogen peroxide	3 ml
Distilled water	97 ml

Preparation :3 ml hydrogen peroxide was added to 97 ml of distilled water and mixed well.

Oxidase reagent

Tetra methyl paraphenylene-diamine-dihydrochloride	1.0 gm
Distilled water	100 ml

Preparation : The reagent was prepared by dissolving 1gm of reagent

In 100ml of distilled water. To that solution,stripes of Whatman No. 1 filter paper was soaked and drained for about 30sec. Then these stripes were completely dried and stored in dark bottle tightly sealed with a screw cap.

Kovac's reagent

4-dimethyl aminobenzyldehyde	2 gm
Isoamyl alcohol	30 ml
Con. HCl	10 ml

Preparation : In 30ml of isoamyl alcohol, 2gm of reagent was dissolved in clean brown bottle. Then to it, 10ml of conc. HCl was added and mixed well.

Preparation of 0.5 McFarland standard

The stock solution of McFarland standard was prepared by mixing 0.6 ml of 1% (w/v) barium chloride solution and 99.4 ml of 1%(v/v) sulphuric acid solution and mixed well. The stock solution can be stored at 20-28˚C in a wellsealed container for about 6 months.

APPENDIX IV

WORKING PROCEDURES

Sample processing

i. The sample was processed by streaking method.

ii. The plates were labelled and one loopful of homogenized sample was streaked into the desired media prepared aseptically.

iii. Sample was streaked into C.L.E.D agar for the detection of bacterial growth.

iv. The plates were then incubated at 37°C for 24 hrs.

Tests performed for the identification of isolates.

Gram staining

i. Smear was made from pure culture by emulsifying a colony in normal saline and heat fixed.

ii. Smear was covered with crystal violet for 30-40 seconds.

iii. The crystal violet was rapidly washed off with distilled water.

iv. The smear was covered with Lugol's Iodine for 30-60 seconds.

v. Iodine was washed off with distilled water.

vi. Then de-colorization (few seconds) was performed usingacetone alcohol.

vii. The smear was covered with safranin for 2 minutes and thenwas washed off with water and backside of slide was wiped.

viii. The slide was kept in draining rack for the smear to air-dry.

ix. The slide was observed first in 40 X objective and then with oil immersion objective.

Catalase Test

i. 1-2 drops of 3% hydrogen peroxide was taken in a glass slide.

ii. Few colonies of bacterial isolate were picked from the agar plates and mixed in the drop of 3% hydrogen peroxide

iii. The appearance of gas bubbles was recorded immediately.

Oxidase

i. A piece of oxidase test paper was placed in a clean Petri dish.

ii. Using a glass rod, a colony of thest organism was smeared in the oxidase paper iii.Observation was done for the development of blue-purple color within few seconds

Tripe sugar-Iron test

i. The isolated organism was inoculated in TSI by first stabbing through the center of the medium to the bottom of the tube and then streaking the surface of the agar slant by using sterile needle.

ii. The tube was incubated at 37˚C for 18-24 hours iii.Then the tube was observed for the production of gas and H2S.

Simmons citrate agar

i.The isolated organism was inoculated in citrate agar by streaking the surface of the agar slant by using sterile needle. ii.The tube was incubated at 37 for 1824 hours iii.The tube was observed for change in colour to blue.

SIM Test

i. By using sterile inoculating needle, the test organism was inoculated on SIM media by stabbing through the center of the agar.

ii. The tube was incubated at 37˚C for 18-24 hours.

iii. Then, 0.5 ml of Kovac's reagent was added to the tube.

iv. The tube was then observed for the appearance of red colored ring near the surface of the medium.

v. Also, the tube was observed for motility and production of H2S.

Urease Production Test

By using sterile inoculating needle, the test organism was inoculated on theentire surface of the medium.

i.The tube was incubated at 37 for 18-24 hours ii.The colour of slant was recorded.

Antimicrobial Susceptibility Testing

Requirements:

Mueller Hinton Agar

Antibiotic discs

0.5 McFarland Opacity Forceps

1.Preparation of McFarland standard

0.6 mL of 1 w/v solution of barium chloride solution was added to 99.4 ml of 1 w/v solution of Conc. Sulphuric acid. The mixture was shaken well. This stock solution was stored in well-sealed container in the dark. A small amount of the solution was taken at a time in a clean test tube to compare the turbidity of inoculums.

2. Preparation of Inoculum

2-3 morphologically similar colonies were picked using a sterile loop from MA or NA. It was then inoculated in MHB or NB and incubated at 37 for 6 hours. Turbidity was then matched with that of 0.5 McFarland Standard.

3. Kirby Bauer Disc Diffusion method

i.The isolated organism was inoculated in nutrient broth and incubated at 37˚C for 4-6 hours. ii.After incubation, the suspension was compared to 0.5 McFarland opacity standard and was adjusted to match the turbidity.

iii. The suspension was inoculated on MHA by spreading with sterile cotton swab stick. Then the inoculum was allowed to dry for 5-10 minutes.

iv. The antibiotic discs were applied aseptically by forcep with minimum of 24 mm gap between 2 antibiotic disc.

v. The plate was incubated at 37˚C for 16-18 hours.

4. Minimum Inhibitory Concentration with and without cccp (phenotypic confirmation of efflux pump)

Day 1

Task1; Sterilized plates were labelled making boxes for each isolates

Task 2; Stock solution was made dissolving 0.8192gm Meropenem powder in 10ml DMSO.

Task 3; 2.5 mg cccp was dissolved in 500 µl DMSO to make stock solution of 250 µM concentration.

Day 2

- Series of dilution blank tube along with the conical flask containing mha media were subjected to autoclave. At the same time nutrient broth were inoculated with test organisms and subjected to incubation. ○Two sets of dilution flask with and without cccp were labelled ○First set of conical flasks had 37ml of MHA which were added with 1 ml antibiotic solution and 2 ml of cccp solution which the subjected to serial dilution.
- Second set of conical flasks had 39ml of MHA which were added with only 1 ml of antibiotic solution which then subjected to serial dilution. ○ Each flask from both sets were poured to their respectively labelled pour plate and left to get set.
- As soon as the turbidity of the previously inoculated test tubes matched the turbidity of 0.5 mcfarland turbidity they were individually subjected to the respective ranges of prepared agar plates.

- Thus, both with and without cccp sets of plates were then subjected to incubation for 37°C overnight and reading of MIC was taken the following day.

APPENDIX V

Procedure of Chromosomal DNA Extraction

1. 1.5m of overnight LB-broth culture of bacteria was collected in a MFT and spinned at 5000 RPM for 10 minutes.
2. Supernatant was removed and spinned once more with same volume as above to collect more cell mass.
3. Supernatant was removed and 100ul of solution I buffer was added and left at room temperature for 30 minutes.
4. 1/10 volume of 10% SDS was added and swirled to mix intermittently for 10 minutes.
5. 1 ul proteinase K was added and incubated at 37˚C for 30 minutes with gentle shaking.
6. Equal volume of Phenol:Choloroform (1:1) was added and mixed gently, left at room temperature for 10 minutes.
7. Centrifuged at 8000 RPM for 10 minutes at 4˚C and supernatant was collected in a new sterile MFT.
8. Equal volume of 3M. sodium acetate was mixed and left for an hour in ice cold.
9. Spinned at 10000 RPM for 15 minutes at 4˚C and pellet was washed with 70% ethanol.
10. Pellets were dissolved in 50ul TE buffer and stored in deep freeze.

APPENDIX VI

Procedure of agarose gel electrophoresis of DNA

1.1.5% of agarose gel was prepared in 25ml TAE buffer.
2.The resulting solution was boiled until a clear solution was obtained and cooled at 60˚C

3.About 30 ul Ethidium bromide was then added carefully into the gel solution to a final concentration of 0.5 ug/mL.

4.The molten gel was then poured in the clean gel case avoiding the air bubbles and the comb was immediately placed into the gel cast to makewells.

5.The gel was then allowed to solidify at room temperature.
6.After solidification, the gel was mounted in an electrophoresis tank suchthat the wells were positioned towards the negative terminal.

7.The electrophoresis tank was filled with TAE buffer until the gel was completely submerged.

8.The comb was then removed carefully from the gel which left well in thegel.

9.The sample was prepared by taking 20ul of DNA sample and mixed with4ul loading gel buffer 6X

10.The sample was loaded in the corresponding wells. Also, 4ul of DNAladder was loaded in the flanking end.

11.The power cord was attached and electrophoresis at 80V was carried outuntil bromophenol blue migrates to the required distance.

12.The power supply was turned off to terminate and gel was carefully removed from the tank.

13.The gel was taken to the photo-documentation apparatus for the visualization of chromosomal DNA using a UV light.