Molecular characterization of carbapenem- nonsusceptible Enterobacterial isolates of animal origin

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OF

MASTER OF VETERINARY SCIENCE

IN

VETERINARY MICROBIOLOGY

BY

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Enrolment No. V- 2116/19

COLLEGE OF VETERINARY SCIENCE AND ANIMAL HUSBANDRY

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(2021)

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This is to certified that the thesis entitled **"Molecular characterization of carbapenem- nonsusceptible Enterobacterial isolates of animal origin."** submitted by **Dr. Usha Jaiswal,** Enrollment No. V-2116/19 in partial fulfillment of the requirements for the award of the **Master of Veterinary Science in Veterinary Microbiology** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (U.P.),** India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

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ABST	:	Antibiotic susceptibility test
AMP	:	Ampicillin
AMC	:	Amoxicillin-clavulanic acid
AMR	:	Antimicrobial resistance
ATCC	:	American type culture collection
BHI	:	Brain Heart Infusion
BPW	:	Buffer peptone water
CAZ	:	Ceftazidime
CAZ-CLA	:	Ceftazidime/clavulanic acid
Cfu	:	Colony forming unit
CIA	:	Carbapenemase Inactivation Assay
CIP	:	Ciprofloxacin
CLSI	:	Clinical Laboratory and standards Institute
CNP	:	Carbapenemase Nordmann-Poirel
CP	:	Carbapenemase producing
CPD	:	Cefpodoxime
CPE	:	Carbapenemase producing Enterobacteriaceae
CRB	:	carbapenem-resistant bacteria
CRE	:	Carbapenem Resistant Enterobacteriaceae
CRO	:	Ceftriazone
CTX	:	Cefotaxime
CTX-CLA	:	Cefotaxime/clavulanic acid
DDDT	:	Double disk diffusion method
DNA	:	Deoxy-ribonucleic acid
dNTPs	:	Deoxy-nucleotide triphosphates
DR	:	Drug resistant
EDTA	:	Ethylenediamine tetra-acetic acid
EMB	:	Eosine Methylene blue agar
ESBL	:	Extended spectrum b-lactamase
EtBr	:	Ethidium bromide

ETP	:	Ertapenem
GNB	:	Gram negative bacteria
GPB	:	Gram positive bacteria
IMP	:	Imipenam
MBL	:	Metallo-beta-lactamases
MDR	:	Multiple drug resistant
MDR	:	Multidrug Resistant
MHA	:	Mueller Hinton agar
MHB	:	Mueller Hinton broth
MHT	:	Modified Hodge test
MIC	:	Minimum inhibitory concentration
MLA	:	MacConkey lactose agar
MRP	:	Meropenem
NDM	:	New Delhi metallo beta lactmase
NFW	:	Nuclease free water
NSS	:	Normal Saline Solution
PCR	:	Polymerase chain reaction
pH	:	Log hydrogen ion concentration
QR	:	Quinolone reistance
RPM	:	Revolution per minute
spp.	:	Species
TAE	:	Tris-Acetate-EDTA
Taq	:	Thermus aquaticus
TBE	:	Tris-Boric acid-EDTA
Tris	:	Tris-hydroxy methyl aminoethane
TSA	:	Trypticase soy agar
TSB	:	Trypticase soy broth
XDR	:	Extremely drug resistant

bp	:	Base pairs
cm	:	Centimeter
^{o}C	:	Degree Celsius
hrs.	:	Hours
%	:	Percent
$	:	Dollar
M	:	Molar
ml	:	Milliliter
mm	:	Millimeter
µg	:	Microgram
µl	:	Microlitre
mg	:	Milligram
min	:	Minute
ppm	:	Parts per million
Sec.	:	Second
V	:	Volts
gm	:	Gram
N	:	Normality

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Contents

ABSTRACT

Carbapenem-resistant confers resistance to most of the β-lactam antibiotics including penicillin, cephalosporin, and carbapenem, hence responsible for the rise of multidrug-resistant strains. Several different mechanisms are involved in the development of carbapenem resistance which includes, porin channel, efflux pump, and production of carbapenemase. Although these mechanisms are already characterized in human strains there is a lack of a comprehensive understanding of the inherent mechanisms in strains of animal origin, where carbapenems are rarely being used. Hence the present study was undertaken to elucidate the molecular mechanism in carbapenem-resistant bacterial strains isolated from animal's sources. The carbapenem resistant isolates were identified and characterized by morphological, cultural, and biochemical tests. Conventional tests like carba NP test and carbapenemase inactivation assay (CIA) were used to detect the production of carbapenemase production in these isolates. MIC_{IMP} was calculated for all the isolates. The efficiency of the efflux pump to effectively efflux carbapenem antibiotic was evaluated by three independent tests viz. CCCP-IMP/ETP disc synergy test, MIC broth microdilution method, and EtBr cartwheel test. Further, the presence of different carbapenem-resistant genes was confirmed by PCR and gene sequencing. The transcriptional response of various OMP gene and efflux pump genes were evaluated by Q-PCR.

Carba NP and CIA tests could successfully detect 12 and 10 carbapenemase producers, respectively with high sensitivity and specificity. Out of 12 isolates that were producing carbapenemase as observed by carba NP test, among them bla IMP was detected in 7, bla VIM was detected in 4, blaOXA-48 in 3, whereas in 2 isolates PCR experiment could not fetch any amplification, although they were Carba-NP test positive. The sequence analysis of bla VIM, bla IMP and bla Oxa-48 revealed the presence of VIM-2, IMP-8 and Oxa-181 variants. Out of 18 non-susceptible isolates, 5 isolates were found to have the role of active efflux pump mediated carbapenem non-susceptibility. In this study, a strong correlation between imipenem resistance and *acrA* and *acrB* overexpression was observed in all the Enterobacteriaceae isolates. Further, it was observed that imipenem stress decreased *ompF* and *ompC* expression in a majority of isolates. Additionally, the transcription of *ompK35* was insistently increased in *Klebsiella* isolates with a corresponding decrease in the transcriptional level of *ompK36* under carbapenem stress conditions. A corresponding rise in *mexB* efflux pump was noted in Pseudomonas while there was a decrease in *oprD* porin. The study established the involvement of multiple mechanisms in the development of carbapenem resistance. Porins (like OmpF and OmpC) and AcrAB efflux pump are a relevant antibiotic resistance determinant in the bacterial pathogen and have an important role in developing resistance against the carbapenem group of antibiotics apart from carbapenemase production.

Introduction

CHAPTER-1

INTRODUCTION

The world over medical fraternity is deeply worried by the rapidity by which the global emergence of antibiotic resistance bacteria took over in the last decade. Carbapenems are often used as “last-line” agents for treating serious infections caused by multidrug-resistant GNB including ESBL-producing Enterobacteriaceae (Hays et al., 2012). However, in the last decade the prevalence of carbapenem-resistant GNB strains have spread significantly across the globe (Loganand & Weinstein, 2017). The increasing frequency of Gram-negative bacteria producing extended-spectrum β-lactamase enzymes have led to higher carbapenem usage which has resulted in the wider occurrence and spread of carbapenemase-producing *Enterobacteriaceae* (Muller et al., 2018; Kelly et al., 2017). The continued upward trend in the incidence of carbapenemase-producing Gram-negative bacilli (GNB) has troubled the health care professionals in many countries around the world (Cuzon et al., 2015). Most clinically relevant carbapenem resistance appears to have arisen and propagated as a result of clinical carbapenem use in human medicine. Consequently, our knowledge concerning the epidemiology and ecology of carbapenem-resistant bacteria (CRB) is largely limited to human clinical settings (Poirel et al., 2014).

Carbapenems are not approved for use in livestock production anywhere in the world (World Organization for Animal Health (OIE, 2015). Hence, little is known about the prevalence of CRB, and more specifically CRE, in livestock populations and their associated environments. However, several reports from investigators across the world highlighted growing instances of CRE recoveries from animal sources (Fischer et al., 2012 & 2013; Poirel et al., 2012; Wang et al., 2017; Zhang et al., 2013). In India,CRE have been reported in food-producing animals and their environment (Pruthvishree et al., 2017, Nirupama et al., 2018; Ghatak et al., 2013). Carbapenem-resistance has been described in various genera of enterobacteriaceae, mainly Enterobacter, Citrobacter, Proteus, *Klebsiella pneumoniae* and *Escherichia coli* as well as in non-fermentative GNB like *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (Mellouk et al., 2017; Djahmi et al., 2014; Poirel et al., 2012).

The cause for the development of carbapenem resistance in GNB is multi-factorial, but primarily includes several mechanisms such as overexpression of efflux system, permeability reduction outer membrane (OM) porins, production of chromosomal *ampC* β-lactamase (Fernández, and Hancock, 2012; Bhardwaj et al., 2015). More often, CRE with the multidrug resistance phenotype results from the progressive accumulation of different mechanisms of resistance in the same microorganism (García et al., 2011). The acquisition of carbapenem-hydrolyzing enzymes is considered as one of the most important mechanisms of carbapenem resistance. These enzymes are known to hydrolyze all classes of *β*-lactams except monobactams (Palzkill, 2013). Carbapenem resistance in GNB can be mediated by carbapenem-hydrolyzing β-lactamases, including class A β-lactamases (KPC, IMI, and GES), class B metallo-β- lactamases (NDM, IMP, and VIM), and class D β-lactamases (OXA-48, OXA-23, OXA-24, and OXA-58) (Cuzon et al., 2015, Hays et al., 2012, Djahmi et al., 2014, Agabou et al., 2014). Carbapenemase-encoding genes are frequently carried by mobile genetic elements and can spread rapidly such as plasmids, and, as a result, carbapenemase encoding genes are known to jump across members of the enterobacteriaceae family (Kochar et al., 2009). The transfer of carbapenemase-encoding genes between strains and different species represents the most difficult challenge in the strategic management of AMR (Carattoli, 2013). CRE are usually co-or cross-resistant to other clinically relevant antibiotics.

Despite the increasing rate of carbapenem resistance in India, there is still a lack of a comprehensive understanding of the inherent mechanisms especially in strains of animal origin, where carbapenems are rarely being used. This study aims to investigate the molecular mechanisms of resistance to carbapenems among carbapenem-resistant Gram-negative bacilli isolated from animal sources and to identify the underlying genetic mechanisms associated with reduced phenotypic susceptibility to carbapenems.

Objectives:

1) To determine the resistance profile of carbapenem-resistant strains to antibiotics of other classes.
2) To carry out detailed genetic analysis of carbapenem-resistant genes.
3) To identify the main resistance mechanisms of clinical isolates to antibiotics of carbapenem class.

Review of Literature

CHAPTER-2

REVIEW OF LITERATURE

2.1. Beta-lactam antibiotics

Beta-lactams are by far the most used antibiotics worldwide and include the penicillins, cephalosporins, monobactams and carbapenems. They all share a common beta-lactam ring and act similarly by binding to and inactivating the penicillin-binding proteins (PBPs), which are responsible for the formation of the bacterial cell wall. One of the most important therapeutic choices for treating infections in both humans and animals is β-lactam antibiotics. The increase in bacterial resistance to these antibiotics over the few decades is due to selective pressure caused by use and misuse of these antibiotics. The most relevant mechanism of resistance is the production of β-lactamases, which hydrolyze the β-lactam ring of these antibiotics (Medeiros, 1997). Point mutations produce variants which produce the so-called extended spectrum β-lactamases (ESBLs) responsible for extensive drug resistance.

2.2. Resistant to Beta-lactam

The global spread of antimicrobial-resistant bacteria has been progressive and unremitting since the introduction of antimicrobial agents into clinical medicine more than 70 years ago. Not only the dissemination of antibiotic-resistant, Gram-positive cocci is challenging, but also the growing incidence of antibiotic-resistant, Gram-negative bacilli represents an increasingly pressing issue (Vasoo et al., 2015). Specifically, the development of resistance to β-lactams among Gram-negative bacilli is an urgent contemporary threat to medical disease treatments. β-lactams are the most widely utilized antibiotics owing to their comparatively high effectiveness, low cost, ease of delivery, and minimal side effects (Wilke et al., 2005). Unfortunately, bacteria have evolved sophisticated resistance mechanisms to combat the lethal effects of β-lactam antibiotics, and each new compound has been addressed with a diverse and robust array of resistance determinants.

2.3. Carbapenem

Carbapenems (imipenem, meropenem, ertapenem, and doripenem) belong to the β-lactam group of antibiotics and contain in their chemical structure a fused β-

lactam ring and a five-member ring system. They mainly differ from penicillin by being unsaturated and containing carbon instead of sulphur atoms in the β-lactam ring (Shahid et al., 2009). Carbapenems, among the beta-lactams, are the most effective against Gram-positive and Gram-negative bacteria presenting a broad spectrum of antibacterial activity. Their unique molecular structure is due to the presence of a carbapenem together with the beta-lactam ring. This combination confers exceptional stability against most beta-lactamases (enzymes that inactivate beta-lactams) including ampicillin and carbenicillin (AmpC) and the extended spectrum beta-lactamases (ESBLs). The broad spectrum activity for carbapenems cans be attributed to their intrinsic ability to resist the action of the beta lactamase enzymes. This resistance is conferred by the trans-α-1 hydroxyethyl substituent at the position 6 of the carbapenems. Broad spectrum of coverage, includes Gram positive organisms, Gram negative organisms (including *Pseudomonas* and ESBL), and even anaerobes.

2.4. Carbapenem resistant in Gram Negative Enterobactericae

The emergence and spread of resistance to Carbapenem has become a complicated public health alarm (Jeon et al., 2015). In clinical terms, carbapenem-resistant, Gram-negative bacilli, have limited (and sometimes nonexistent) treatment options. Epidemiology of Carbapenem resistant Gram-negative bacilli are spreading worldwide (Nordmann and Poirel, 2014). Genes encoding resistance to carbapenems are often carried on mobile genetic elements that are easily exchanged among Gram-negative bacteria, either within a host or in the environment.

2.4.1. Epidemiology of livestock associated carbapenemase-producing organisms

CRE have been predominantly isolated from humans and environmental samples so far. Recent studies have highlighted the global emergence of CREs in both livestock and companion animals. The first CPE described from livestock were reported by Fischer et al., from poultry and swine in German farms where VIM-1-producing *E. coli* and *Salmonella enterica* were isolated (Fischer et al., 2012, 2013). In particular, recent studies demonstrating the isolation of carbapenemase-producing *Escherichia coli* (NDM-1 and OXA-48) and *Klebsiella pneumoniae* (OXA-48) from clinical infections in dogs (Stolle et al., 2013). Wang et al. (2017) in China, 33.2% CRE isolates were recovered from 739 samples collected from the poultry food

chain. They also identified bla NDM in 161 *E. coli* (21.8%), 55 *K. pneumoniae* (7.4%) and 29 (3.9%) *E.cloacae*. Braun et al. (2016) recovered six *E. coli* isolates from a total of 233 faecal samples collected from cattle in Egypt. The CRE isolates revealed resistance phenotypes against imipenem (42%), ertapenem (35%), doripenem (30%), meropenem (28%), cefotaxime, (59.6%) aztreonam (54.3%) and cefuroxime (47.7%). Another report from Egypt identified blaOXA-48 and blaOXA-181 in different *E. coli* isolates recovered from healthy dairy cattle (Braun et al., 2016). Recently, blaOXA-181 determinant was characterized in an *E .coli* strain from pigs in Italy (Pulss et al., 2017). OXA-48-producing *E. coli* were detected in healthy pets (dogs and cats) and a diseased cat (Yousfi et al., 2016). Recently, OXA-48-producing *E. coli* were recovered in a large set of healthy cats and dogs in France (Melo et al., 2017). The first report of OXA-48 from companion animals found in *K. pneumoniae* and/or *E. coli* isolates, recovered from six diseased dogs admitted to a veterinary clinic in Germany in 2012 (Stolle et al., 2013). Shortly after this episode, in the same region, OXA-48 enzyme was reported in *K. pneumoniae* and/or *E. cloacae* from dogs, cats, and a horse in 2009 and 2011 (Schemiedel et al., 2014).

2.4.2. Epidemiology of livestock associated carbapenemase-producing organisms in India

In India, carbapenem drugs are not used in food animal production and treatment. Hence, the data regarding the prevalence of carbapenem-resistant bacteria in livestock sources in India is largely obscure. However in recent few years, few reports emerged regarding isolation of carbapenem-resistant bacteria from various animal sources. Ghatak et al. (2013) reported isolation of *E. coli* carrying bla NDM gene from milk sample of a dairy cow suffering from mastitis. reported isolation of 23 carbapenem resistant isolates out of which 8 were carrying the New Delhi metallo beta-lactamase (blaNDM) gene from 673 non-diarrheic) and diarrheic fecal sample from piglets (Pruthvishree et al. (2017). Nirupama et al. (2018) screened 741 diarrhoeic and non-diarrhoeic faecal samples from organised pig farms. A total of 27 isolates were phenotypically confirmed as carbapenem-resistant and 3 isolates were found positive for the blaOXA-48 carbapenemase gene.

2.4.3. Mechanism of Carbapenem resistant in Gram Negative Enterobactericae

There are three major mechanisms by which Enterobacteriaceae become resistant to carbapenems: enzyme production, efflux pumps and porin mutations (Haidar et al., 2017).

2.4.3.1. Porins mediated resistance

The bacterial outer membrane (OM) works as a selectively permeability barrier for hydrophobic and hydrophilic compounds (Silhavy et al., 2010; Wiener et al., 2011). OM plays an important role in the susceptibility of the microorganism to antibiotics (Delcour, 2009). Differential membrane permeability of different species of bacteria is characterized by porins, which are specific proteins forming hydrophilic channels (Hancock, 1998). In Enterobacteriaceae, several different families of porins associated with the uptake of antibiotics viz OprD, OmpF or OmpC. OprD is considered as the most important porin associated with significantly decreased susceptibility to carbapenems, especially to imipenem. Isolates belonging to OprD-deficient strains have demonstrated a dramatically increased minimum inhibitory concentration (MIC) of carbapenems (Sakyo et al., 2006). Resistance mediated by porin loss is related to the size of the carbapenem molecule. Usually, high level resistance develops for ertapenem which is a large molecule. While low level resistance is usually observed with the smaller imipenem and meropenem. Carbapenem resistance was initially detected among *Enterobacter spp*., overexpressing the ampC gene and displaying modifications in their porin channels. *Serratia species, Morganella morganii* and some other bacteria were also reported to have similar mechanisms. Alterations in OmpK35/36 in case of *K. pneumoniae* and alterations in the OmpF and OmpC in case of *E. coli* are responsible for this decreased permeability to carbapenems (Nordmann et al., 2012).

Larkin and co-worker describe carbapenem resistance in *E. coli* isolated from urine and identified that *ompC* porin loss plays an important role in resistance (Larkin et al., 2020). Several researchers have highlighted that reduced expression of *OmpF and OmpC* in *E. coli* was frequent in resistant strains (Yoshida et al., 2006).

2.4.3.2. Efflux pump-mediated resistance

Drug efflux pumps are protein complexes which reside in the membrane and remove antimicrobials and toxins, thereby lowering their concentration inside the cell to sub-toxic levels (Poole, 2005). The over expression of these efflux pumps is a nonspecific mechanism of antibiotic resistance (Džidić et al., 2008). Most efflux proteins are divided into five families: resistance-nodulation-cell division (RND), major facilitator (MF), staphylococcal/ small multidrug resistance (SMR), ATP-binding cassette (ABC), and multidrug and toxic compound extrusion (MATE) families (Du et al., 2015). Of the many families of efflux pumps, the members of the Resistance Nodulation Division (RND) super family contribute to antimicrobial resistance and are secondary active transporters exclusively seen in gram-negative bacteria. The overexpression of *acrA* gene which is a component of an efflux pump, has been described as a mechanism of carbapenem resistance in isolates of *Enterobacter aerogenes* (Bornet et al., 2003). The over expression of efflux pumps and loss of OprD porin are the most common mechanism of carbapenem resistance in *Pseudomonas aeruginosa*, notably to imipenem. Other β-lactams may be affected by this mechanism. These mechanisms have been recognised in other organisms such as *Enterobacter aerogenes* and *Klebsiella* species against imipenem agent (Walsh et al., 2000). Generally, Gram-negative bacteria are more resistant to a large number of antimicrobials and other chemotherapeutic agents than Gram-positive bacteria due to cell wall differences, external decreased membrane permeability, efflux pumps and the presence of various broad-spectrum-β-lactamases (e.g., ESBL and/or AmpC cephalosporinase). The resistance may be attributed to the presence of broad-specificity drug-efflux pumps (Wilke et al., 2005). The structural proteins involved in β-lactam resistance are sub-divided; including β-lactamases, PBPs and efflux pump systems.

2.4.3.2.1. AcrAB-TolC efflux pump

The AcrAB-TolC system is a tripartite complex that is widely distributed in Gram-negative bacteria. The three components of the system are AcrB, the inner membrane transporter protein, AcrA, the periplasmic adaptor protein, and TolC, the outer membrane channel (Lobedanz et al., 2007; Touzé et al., 2004). AcrA protein is believed to be involved in vivo in the assembly and maintenance of a stable complex that transmits conformational changes in AcrB and TolC thus leading to opening of

the outer membrane channel (Mikolosko et al., 2006). Several studies have demonstrated that inactivation of the efflux pump AcrAB-TolC by the lack of one of its structural components directly affects the virulence of the bacteria, indicating that this system is required for the bacteria to be pathogenic (Martinez et al., 2009). Although the components of the AcrAB-TolC pump work together as a tripartite system, the individual components may also play certain roles in other efflux systems (Piddock, 2006a). The production of AcrAB-TolC is associated with resistance to some β-lactam antibiotics, including penicillins and cephalosporins (Piddock, 2006b). Pages and co-workers have suggested synergistic effect between AcrAB-TolC and β-lactamase enzymes in conferring resistance to β-lactam antibiotics (Pages et al., 2009).

2.4.3.3. Resistance due to *β*-lactamase production

Carbapenemases are specific β-lactamases with the ability to hydrolyze carbapenems, cephalosporins, and penicillins, except monobactams. Classification based on amino acid homology has resulted in four major classes. Molecular classes A, C, and D include the β-lactamases with serine at their active site, whereas molecular class B β-lactamases are all metalloenzymes with an active-site zinc. Carbapenemases, β-lactamases with catalytic efficiencies for carbapenem hydrolysis, resulting in elevated carbapenem MICs, include enzymes from classes A, B, and D.

2.4.3.3.1. Class A serine carbapenemases

Class A serine carbapenemases have been detected in *Enterobacter cloacae*, *Serratia marcescens*, and *Klebsiella* spp (Medeiros, 1997; Nordmann et al., 1993; Yang et al., 1990). Bacteria expressing these enzymes are characterized by reduced susceptibility to imipenem, but MICs can range from mildly elevated (e.g., imipenem MICs of ≤4 μg/ml) to fully resistant. These β-lactamases, therefore, may go unrecognized following routine susceptibility testing. Three major families of class A serine carbapenemases include the NMC/IMI, SME, and KPC enzymes.

2.4.3.3.2. Class B metallo-β-lactamases

Class B metallo-β-lactamases (MBLs) have a broad substrate spectrum and can catalyze the hydrolysis of virtually all β-lactam antibiotics with the exception of monobactams. They are not inhibited by mechanism-based inhibitors such

asclavulanate, sulbactam, or tazobactam that are effective against serine-based, class A β–lactamases (Drwaz & Bonomo, 2010; Perez et al, 2009)

This class of β-lactamases is characterized by the ability to hydrolyze carbapenems and by its resistance to the commercially available β-lactamase inhibitors but susceptibility to inhibition by metal ion chelators. In addition to the carbapenems, most of these enzymes hydrolyze cephalosporins and penicillins but lack the ability to hydrolyze aztreonam. Major distinctive properties included the requirement of Zn2+ for the efficient hydrolysis of β-lactams and a lack of inhibition by clavulanic acid and tazobactam. The most common metallo-β-lactamase families include the VIM, IMP, GIM, and SIM enzymes, which are located within a variety of integron structures, where they have been incorporated as gene cassettes.

2.4.3.3.3. Class D serine carbapenemases

Class D β-lactamases, also known as OXA-type enzymes or oxacillinases, are widely disseminated in Gram-negative bacteria. They are broadly classified into narrow- and extended-spectrum enzymes based upon the conferred resistance profile against β-lactam antibiotics (Walther and Hoiby, 2006). Class D carbapenemases, also known as carbapenem-hydrolyzing class D β-lactamases (CHDLs), represent a further expansion of the substrate profile of class D enzymes producing resistance to the carbapenems (Poirel et al., 2010). Based on their amino acid sequence identity, CHDLs have been subdivided into several subgroups. Enzymes belonging to the OXA-23, OXA-24/40, OXA-48, OXA-51, OXA-58, and OXA-143 subgroups are of major clinical importance due to their wide dissemination in bacterial pathogens.

2.5. Methods for detection of carbapenemase production by Enterobacteriacae

2.5.1. MIC determination

Elevated carbapenem MICs are generally predictive of Carbapenemase production in the *Enterobacteriaceae*. Carbapenem resistance is defined according to the Clinical and Laboratory Standards Institute (CLSI) criteria, with ertapenem resistance defined as an MIC of ≥2 μg/ml, and meropenem, imipenem, and doripenem resistance defined as an MIC of ≥4 μg/ml (Wayne, 2016).

2.5.2. Phenotypic assays

These tests of varying positive predictive values have been approved for screening in the recent years. According to the CLSI as well as the EUCAST the detection of carbapenemase production is not mandatory for issuing reports on clinical isolates of CRE. However, these tests may still play an important role in epidemiological studies as well as infection control programmes. Molecular detection of genes encoding carbapenemases is considered as the gold standard, however most laboratories still rely on these phenotypic tests for determining carbapenemase production.

2.5.2.1. Modified Hodge test (MHT)

The MHT is used to determine the ability of aarbapenem resistant isolate to produce carbapenemase enzyme. If the test organism produces carbapenemase it inactivates the action of the drug and allows the carbapenem susceptible *E. coli* strain ATCC 25922 to grow towards a carbapenem impregnated disk, resulting in a characteristic cloverleaf-like indentation. The advantages of MHT are that it is simple and at the same time cost effective. Multiple isolates can be tested at the same time. However there are various shortcomings of this tests viz. Time consuming, requires incubation period of at-least 18- 24 hours before results can be interpreted. Class of carbapenemase cannot be determined using this test. False positive results may be obtained in case of AmpC β-lactamase producers having porin deficiency in their cell walls and some ESBL producers (CTX-M-type). In a study by Doyle et al., 2012 MHT was 98% sensitive for detecting KPC producers and 93% sensitive for OXA-48-like enzyme producers. However the sensitivity was only 12% for detecting IMPs, VIMs and NDMs (Doyle et al., 2012).

2.5.2.2. Carba NP test

The Carba NP test is a biochemical test used for rapid detection of carbapnemase production by isolates. It was designed by Nordmann and Poirel in 2012. Bacterial colonies of suspected carbapenemase producers are mixed into a bacterial lysis buffer, and to this is added a solution containing imipenem and an indicator (phenol red) and incubated for upto 2 hours. The carbapenemase enzyme if present hydrolyses the carbapenem agent and this result in a change in the pH of the solution. This change in the pH is then detected by phenol red which turns from red to

yellow. It is an easy and inexpensive test and usually easily interpretable. It can therefore serve as a rapid screening test for CP Enterobacteriacae. The test is very good for detection of NDM and KPC producers (Dortet et al., 2014). The tests have issues in detection of OXA-48-like enzymes attributed to its lower rates of hydrolysis or weak carbapenemase activity (Osterblad et al., 2014, Tijet et al., 2013).

2.5.2.3. Carbapenem Inactivation method (CIM)

The CIM, described in 2015, is a newer method for determining the presence or absence of carbapenemase production by an isolate. In this test, a meropenem disc (AST disc of 10µg drug concentration) is kept immersed in a culture broth of the test isolate and incubated as such for 2 hours. At the end of 2 hours, a lawn culture of a quality control strain of carbapenem susceptible organism is prepared following the standard protocol for disc diffusion testing. The meropenem disc is retrieved from the broth and placed onto this lawn culture. The plate is then incubated overnight at 37°C. On the following day, the diameter of the zone of inhibition of meropenem disc is measured. If the zone diameter falls in the susceptible range it indicates that the drug in the disc was active which implies that the test isolate was not a carbapenemase producer. While if the zone diameter falls in the resistant range, it indicates that the carbapenemase produced by the test isolate inactivated the meropenem in the disc, thus confirming the isolate to be a carbapenemase producer. The advantages of this test are that it is easy to perform, relatively cheaper and the required materials are generally readily available in all laboratories. The major disadvantage being that it requires overnight incubation and is hence not a rapid method. Also like MHT, the class of carbapenemase cannot be determined from CIM (Van der Zwaluw et al., 2015).

2.5.3. Prevalence of carbapenemase genotype in animals

Molecular techniques have become an efficient tool for carbapenemase detection. These are mostly focused on the detection of carbapenemase genes in *Enterobacteriaceae* including their subgroups of carbapenemases (Wang et al. 2012). The most frequently identified carbapenem genes are the ambler class A including bla KPC followed by class B metallo- beta- lactamases (MLBs) such as bla NDM, and the class D OXA-type gene like bla OXA-48. The class B metallo-β- lactamases genes, including bla NDM, bla SME, bla GES, bla VIM, and bla IMP, have also been

disseminated worldwide, blaNDM-1 being the most prevalent worldwide (Nordmann et al., 2012).

After the initial discovery of VIM-1 in Italy during 1997, bacteria with VIM enzymes have been detected worldwide. bla VIM was the first carbapenamase determinant identified in animals. Based on these studies, bla VIM-1 and bla NDM-1 were the most prevalent carbapenemase enzymes among Enterobacteriaceae in food producing animals (Fischer et al., 2012a, 2013, 2017; Poirel et al., 2012; Wang et al., 2012). Nine out of 80 clinical isolates, were found to be VIM-2-producing isolates which were isolated from dogs with pyoderma and otitis in Korea (Hyun et al., 2018). In Asia, bla IMP and bla VIM are prevalent. bla IMP is found mainly in Japan, Korea, China, Taiwan, and Iran (Fang et al., 2008, Peymani et al., 2011, Franco et al., 2010). The prevalence of MBL in India has ranged from 7% to 65% among carbapenem-resistant *P. aeruginosa*. In a study from India, the rate of MBL production was 24.5% among 61 P. aeruginosa isolates, and bla VIM type was the most common (Manoharan, 2010).

OXA-48 is the most efficient class D carbapenemase for imipenem and is one of the most prevalent class D carbapenemases (Jeon et al., 2015). Since its first emergence in Turkey in 2003, the endemic spread of OXA-48 harbouring bacteria has been reported in countries such as Morocco, Libya, Egypt, Tunisia, and India (Nordmann and Poirel, 2014). The prevalence of OXA-48 carbapenemases among carbapenemase-producing K. pneumoniae in Spain and France was particularly high (74 and 78%) respectively (Robert et al., 2014; Palacios-Baena et al., 2016). OXA-181, a derivative of OXA48 with the substitution of a single amino acid, was first identified in India by Potron et al. (2011), and then has been spread to many different countries. Out of the 44 Gram- negative isolates recovered from dogs 28 (76.3%) were positive for at least one tested carbapenemase gene. The highest frequency of carbapenemase recorded was for NDM followed by OXA-181, KPC, OXA-48 and VIM (Sankar et al., 2021)

Materials and Methods

CHAPTER-3

MATERIALS AND METHODS

3.1. Bacterial Strains

Escherichia coli ATCC- 25922, Klebsiella pneumonia ATCC - 700603, *Klebsiella pneumonia* ATCC BAA-1705 and *Klebsiella pneumonia* ATCC BAA-1706, *Pseudomonas aeruginosa* ATCC 10145 procured from Hi-media were used in the study.

3.2. Chemicals and reagents

Nuclease free water, Agarose, Ethidium bromide, Isopropanol was procured from Sigma Aldrich, St Louis USA. 100 bp DNA ladder, 6x loading dye, DREAM Taq Master mix (2x) were procured from Fermentas, USA. Oligonucleotide primers were obtained from Sigma Aldrich and Imperial Life Science. Antibiotic discs were obtained from BD Bioscience. MacConey lactose agar, Trypticase soya broth, Trypticase soya agar, Eosin Methylene blue, Muller Hinton agar and broth were procured from Sigma Aldrich, St Louis USA. Antibiotic powders were procured from Sigma Aldrich /Duchfa /HI Media.

3.3. Glassware and Plasticware

All the glassware's were procured from M/S J-Sil India pvt Ltd. Micro-centrifuge tubes, PCR tubes, Micropipette tips were purchased from M/S Tarsons, India.

3.4. Kits

Qiagen RNeasy Mini Kit (QIAGEN, Germany), Revert-aid® first strand cDNA synthesis kit (Thermo Scientific, USA), QIAquick Gel Extraction Kit (QIAGEN, Germany) were used in the present study.

3.5. List of equipment

Bacteriological incubator (Scintech India), hot air oven (Associated Scientific, India), autoclave (Sonar, India), refrigerated centrifuges (Remi, India), Verity^{Tm} 96-well thermal cycler (Thermofisher, USA), gel documentation system (Uvitec Ltd, UK), submerged horizontal electrophoresis (Atto, Japan), QuantStudio^{Tm} 3 Real-Time

PCR (Thermofisher, USA), Biophotometer plus (Eppendorf, Germany) were used in the study.

3.2. Methods

3.2.1. Revival of bacterial isolates

Brain Heart Infusion (BHI) agar plates and broth were prepared by adding deionized distilled water to dehydrated culture media with or without agar. Media was sterilized by autoclaving at 121ºC for 15 min. The prepared plates and broth were incubated overnight at 37ºC for sterility check. In the present study, bacterial isolates previously isolated and maintained in the department were used. The source details of isolates and source of isolation is included in table 1. Bacterial glycerol stock preserved at -20ºC was taken out, thawed and loop full of culture was inoculated in BHI broth tubes as well as streaked on Brain heart infusion agar plates. The BHI agar plates and broth tubes were incubated overnight at 37ºC for 24 h.

Table 1: Details of carbapenem non-susceptible bacterial isolates used in the study

Sl No	Isolate No	Animal origin	Sample type
1	VS-01	Cow	Uterine washing
2	VS-02	Cow	Uterine washing
3	VA-19	Cat	Faecal sample
4	VS-39	Cow	Uterine washing
5	VS-51	Cow	Uterine washing
6	VA-52	Dog	Faecal sample
7	VA-53	Buffalo	Faecal sample
8	VA-55	Dog	Faecal sample
9	VA-66	Dog	Faecal sample
10	VA-77	Buffalo	Faecal sample
11	VA-99	Buffalo	Faecal sample
12	VA-100	Buffalo	Faecal sample
13	VU-02	Dog	Faecal sample
14	VU-03	Dog	Faecal sample
15	VU-08	Dog	Faecal sample
16	VU-14	Dog	Faecal sample
17	VU-16	Dog	Faecal sample
18	VU-17	Dog	Faecal sample

3.2.2. Morphological, cultural and biochemical identification of isolates

The purity and identity of the cultures were tested by Gram-staining and growth on MLA and EMB agar plates. All the isolates were characterized by a panel of biochemical tests *viz*. catalase, oxidase, ONPG, urease, indole, methyl red, Voges-Prausker, citrate test, H_2S production and sugar fermentation test using adonitol, arabinose, cellibios, dextrose, mellibios, raffinose, rhamnose, lactose, trehalose and xylose.

3.3.3. Antimicrobial sensitivity testing (Kirby-Bauer disc diffusion test)

3.3.3.1. Preparation of culture media

Mueller-Hinton agar (MHA) media was prepared by adding deionized distilled H_2O to dehydrated culture media, sterilized by autoclaving and 65 ml of sterile media was transferred to sterile petri plates (150 mm diameter) to a depth of 4 mm.

3.3.3.2. Preparation of inoculums

For each isolate, three to five morphologically similar colonies were selected from the fresh agar plate and transferred into a sterile capped glass tube containing a saline solution and mixed using a vortex mixer. The suspension's turbidity was adjusted to that of a McFarland Standard 0.5 by adding sterile saline. The bacterial suspension so adjusted having 1 $X10^8$ $cfuml^{-1}$.

3.3.3.3. Protocol

Antibiotic susceptibility test (ABST) was performed as per the CLSI guideline 2017 with the panel of antibiotics, namely ertapenem (10 µg), cefotaxime (30 µg), ampicillin (10 µg), amoxicillin-clavulanic acid (30 µg), ciprofloxacin (5 µg), ceftriaxone (30 µg) and cefpodoxime (30 µg) by disc diffusion method. McFarland adjusted bacterial suspension of all the test isolates and control strains, were used to inoculate MHA plates. The surface was lightly and uniformly inoculated by cotton swab in three directions rotating the plate to ensure even distribution. The plates were incubated for overnight at 37ºC. The zone of inhibition breakpoint was interpreted as per the CLSI 2017 manual.

3.3.4. Phenotypic detection of carbapenemase Production

3.3.4.1. Carba NP test

Modified Carba NP test which is a pH-based test was performed to confirm hydrolysis of the carbapenem β-lactam. CNP-A solution was prepared by adding phenol red (0.05%) and $ZnSO_4.7H_2O$ (0.1 mmol/L) to sterile distilled Water, pH was adjusted to 7.8 ± 0.1 and the solution was stored at 4°C in amber-colored bottles for up to 15 days. The 'B' solution was freshly prepared by adding 12 mg/ml imipenem-cilastatin injectable form (doubling the amount to compensate the cilastatin component; equivalent to 6 mg/ml of imipenem standard grade powder) to 'A' solution and stored at 4°C till it is used. Two calibrated loops full of bacterial culture to be tested from 18 to 24 hrs BHI agar plates were resuspended in 200 µl of solution 'A' and 'B' and vortexed for 5 sec. The tubes were incubated at 37°C and readings were taken at 10 min, 30 min and 120 min. The test was considered positive when the tube "a" was red and tube "b" was orange/yellow. In a negative test, both tubes remained red (*E. coli* ATCC 25922 was used as negative control while *Klebsiella pneumonia* ATCC BAA 1705 was used as positive control) (Rudresh et al., 2017).

3.3.4.2. Carbapenemase Inactivation Assay (CIA)

Carbapenemase Inactivation Assay (CIA) was performed to check carbapenemase producing ability. Briefly, 2-3 loop full of bacterial culture from overnight grown Trypticase soya agar plate was inoculated into 1.5 ml Micro-centrifuge tube containing 400 µl of Mueller Hinton broth (MHB). Ertapenem disc (10 µg) was then placed into each tube and incubated for 4 hrs at 37ºC. *Escherichia coli* ATCC- 25922 inoculum (standardized using 0.5 McFarland standard was plated on to MHA plate. Post incubation, Ertapenem disc was removed from the micro-centrifuge tubes and placed over the MHA plate inoculated with *Escherichia coli* ATCC- 25922.The plates were incubated overnight at 37ºC. *Klebsiella pneumonia* ATCC BAA-1705 (Carbapenemase-KPC) and *Klebsiella pneumonia* ATCC BAA-06 was used as positive and negative control respectively.

3.3.5. Phenotypic evaluation of efflux pump activity

3.3.5.1 EtBr-agar cartwheel method

Trypticase soy agar (TSA) media was prepared by adding deionized distilled H_2O to dehydrated culture media. Media was sterilized by autoclaving at 121°C for 15 min. Molten agar was allowed to cool down to 55°C and EtBr was added to reach a concentration gradient ranging from 0.5 to 2.0 mg/l in different plates. The plates were prepared fresh on the same day of the experiment and kept protected from light by wrapping the plates in aluminum foil (Martins et al., 2013).

3.3.5.1.1. Preparation of inoculums

For each isolate, three to five morphologically similar colonies were selected from the fresh agar plate and transferred into a sterile capped glass tube containing a saline solution and mixed using a vortex mixer. The suspension's turbidity was adjusted to that of a McFarland Standard 0.5 by adding sterile saline. The bacterial suspension so adjusted having 1 $X10^8$ $cfuml^{-1}$.

3.3.5.1.2. Protocol

The TSA plates containing EtBr were divided into 12 sectors by radial lines (cartwheel pattern). The bacterial inoculum was swabbed onto EtBr agar plates starting from the centre of the plate and spreading towards the edges, as indicated by the arrowheads. One reference strains each, as positive and negative controls were included in the experiment which served as a comparative control. The swabbed EtBr plates were then incubated at 37ºC for 16 hrs and examined under a UV light and index of efflux activity of the MDR strains (the capacity to efflux EtBr of each bacterial strain was ranked relative to the reference strain according to the following formula:

$$\text{Index}=MC_{Etbr}\,(MDR)-MC_{Etbr}\,(Ref)/\,MC_{Etbr}\,(Ref)$$

3.3.5.2. CCCP-broth micro dilution method

Mueller Hinton Broth media was prepared by adding deionized distilled H_2O to dehydrated culture media with or without agar. Media was sterilized by autoclaving at 121°C for 15 min.

3.3.5.2.1. Preparation of bacterial inoculum by colony suspension method

For each isolate, three to five morphologically similar colonies were selected from the fresh agar plate and transferred into a sterile capped glass tube containing a saline solution and mixed using a vortex mixer. The suspension's turbidity was adjusted to that of a McFarland Standard 0.5 by adding sterile saline. The bacterial suspension so adjusted having 1 $X10^8$ cfuml-1 was further diluted by a factor of 1:100 by adding 200 µl bacterial suspension to19.8 ml sterile MHB.

3.3.5.2.2 Protocol

Two sterile 96-well microtiter plates were used for each bacterial isolate to be tested. The test was performed in duplicate plates with and without CCCP. Sterile 96-well Microtiter plate was labelled with the respective antibiotic concentration. 50 µl of MHB was taken into all the wells from column 2-11. 100 µl of MHB was added to column 12 which serves as sterility control well. Column 11 is used as growth control well. 50 µl of each antibiotic working solution into the first well of each row in column 1 was taken. Using a multichannel pipette 50 µl of the content from the first column was transferred and mixed to the second column to achieve 2 fold dilutions. Further two-fold dilution was done by repetitive transfer and mixing of solution from 2-10 column. From column 10, 50 µl of content was discarded so that each well from column 1 to 10 have 50 µl each content. 10 µl of CCCP (20 µg/ml) was added to each well of the duplicate plate. 50 µl of final adjusted bacterial suspension was added to each well of both the plates, except column 12 which serves as growth control well. The plates were covered by sterile covers and incubated at 37ºC for 18-24 h.

3.3.5.3. Carba-CCCP disc synergy test

Mueller Hinton agar media was prepared by adding deionized distilled H_2O to dehydrated culture media with or without agar. Media was sterilized by autoclaving at 121°C for 15 min.

3.3.5.3.1. Preparation of inoculums

For each isolate, three to five morphologically similar colonies were selected from the fresh agar plate and transferred into a sterile capped glass tube containing a saline solution and mixed using a vortex mixer. The suspension's turbidity was adjusted to that of a McFarland Standard 0.5 by adding sterile saline. The bacterial suspension so adjusted having 1 $X10^8$ $cfuml^{-1}$.

3.3.5.3.2. Protocol

This test performed as the standard disk diffusion assay. The adjusted bacterial suspension was plated onto MHA plates. The surface was lightly and uniformly inoculated by cotton swab in three directions rotating the plate approximately, to ensure even distribution. Ertapenem (10µg) and Imipenem (10µg) disk with or without CCCP (@ 20µg) were placed in the plates. Zone of inhibition noted. The difference in zone diameter>5 mm signifies efflux pump inhibition. The plates were incubated for overnight at 37ºC

3.3.6. Molecular characterization of isolates

3.3.6.1. DNA isolation by snap-chill method

Loopful of each isolate was mixed with the nuclease free water thoroughly in each micro-centrifuge tube. The suspended isolates in micro-centrifuge tubes were denatured at 100ºC in boiling water for 10 minutes. After heat treatment boiled cell lysate tubes were placed immediately in ice for 10 min and centrifuged at 12,000 rpm for 2 min. 2.0 µl of the supernatant was taken as DNA template.

3.3.6.2. Polymerase chain reaction (PCR)

PCR assay was carried out in 0.2 ml thin walled tubes (Granier). Total of 25 µl of PCR mixture was prepared by using DNA template 2 µl, 2 X Dream Taq master mixes 12.5 µl. The primers were diluted upon arrival to 100 pmol/µl stock and 10 pico-mol/µl working concentration in sterile nuclease free distilled water. Thermo-cycling was carried out in a T100 thermal cycler (Technique TC- 5000 PCR Thermal Cycler, UK). The primer concentration and amplification conditioned for the PCR reactions used throughout the study are detailed in table 2 and 3. Appropriate negative and positive controls were included in each PCR assay. The resulting PCR products were analyzed in 1.5% Agarose gel, using the following electrophoresis: 30 min, 100V, TAE buffer 1X. Ethidium bromide (EtBr) was used for visualization of amplified products under UV light.

3.3.6.3. Agarose gel electrophoresis

For agarose gel electrophoresis 1.5% agarose gel was prepared using TAE buffer. Ethidium bromide (10mg/ml) was added to the molten agarose gel at 55ºC. Agarose gel was poured on the gel casting tray with the gel comb. Electrophoresis was carried out at 70 volt for 45 minute. The gel was visualized under UV light.

3.3.6.4. Gel-extraction of PCR product

The gel pieces containing target DNA was excised using sharp scalpel blade and collected in 2 ml microcentrifuge tube. The gel extraction of PCR product was carried out using QIAquick gel extraction kit (QUIAGEN, USA), as per the protocol provided by the manufacturer. The gel slice in a microcentrifuge tube was weighed. Three volume of Buffer QG was added to the tube per 1 volume of gel. The tube was incubated at 50°C until the gel slice has completely dissolved with intermittent mixing. The tube was vortexed every 2–3 min during the incubation to dissolve the gel completely. After the gel slice has dissolved completely, 1 gel volume of isopropanol was added to the sample and mixed thoroughly. QIAquick spin column was placed in the 2 ml collection tube provided with the kit and the sample was applied to the QIAquick column, and then centrifuge for 1 min at 8000 rpm. Flow-through collected in collection tube was discarded and QIAquick column was placed back into the same collection tube. 0.75 ml of Buffer PE (Wash Buffer) was added into the QIAquick column and centrifuge for 1 min at 8000 rpm. Flow-through collected in collection tube was discarded and QIAquick column was placed back into the fresh 1.5 ml collection tube. 50 µl of Buffer EB (Elution Buffer) was applied to the centre of the QIAquick membrane, and then centrifuge the column for 1 min at 12000 rpm. The gel purified PCR product was stored at -20ºC till further processing.

3.3.6.5. Sanger sequencing of the gel eluted PCR product

50 µl of Amplicons (50-100 ng/ µl) were sent to Bioserve Biotechnologies India Pvt Ltd, Hyderabad for Sanger sequencing using forward and reverse primer. Ambiguous reads were trimmed off and processed raw sequence was subjected to similarity search using NCBI-BLAST.

3.3.7. Quantitative PCR for OMP and Efflux pump gene expression

3.3.7.1. Total RNA extraction

Total RNA extraction was performed using Trizol^{TM} and QIAamp RNA Mini Kit (Qiagen, Germany) with slight modification. Briefly, 1-2 colonies of each bacterial culture from overnight grown Trypticase soya agar plate was inoculated into 5 ml of Trypticase soya (TSB) containing subinhibitory concentration of imipenem (1µg/ml). 1.5 ml of mid log phase culture was taken in microcentrifuge tubes and pelleted by centrifugation at 7000 rpm for 6 min. The

supernatant was removed completely and carefully without disturbing the pellet. The lysis buffer provided with the QIAamp RNA Mini Kit was heated at 90ºC. 200µl of preheated lysis buffer was added to the bacterial pellet and the pellet was resuspended completely using micropipette. 20µl lysozyme and 10µl proteinase-K was added to the resuspended culture, mixed thoroughly and incubated at 90ºC for 10 minutes or till the complete bacterial cell lysis has occurred. 1 ml Trizol solution (Invitrogen) was added in lysed suspension and incubated at room temperature for 5 min. The tube was further incubated for another 2-3 minutes, following addition of 200 µl chloroform followed by centrifugation at 12,000 × g for 15 minutes. The resulting mixture was separated into a lower red phenol-chloroform, an interphase, and a colourless upper aqueous phase. Carefully the upper aqueous phase containing the RNA was transferred to a new tube without touching interface. 560 µl ethanol was added and the tube was mixed thoroughly using vortex. The content was transferred in the 2 ml spin columns provided with the kit and centrifuge at 8000 rpm for 1 min. The follow through was discarded. 500 µl of Buffer AW1 was added to the QIAamp Mini column and centrifuge at 6000 x g (8000 rpm) for 1 min. The follow through was discarded. 500 µl Buffer AW2 was added to the QIAamp Mini column and centrifuge at 10,000 rpm for 1 min. The follow through was discarded. A dry run was performed using empty spin column at 10,000 rpm for 1 min to remove residual wash buffer. The QIAamp Mini column was placed in a fresh clean 1.5 ml microcentrifuge tube and 60 µl of Buffer AVE (elution buffer) equilibrated to room temperature was added. The QIAamp Mini column was centrifuged at 8000 rpm for 1 min. The column was discarded and the purified RNA collected in tubes were marked and stored at -20ºC till further use.

3.3.7.2. Quantification and RNA purity check

The quantity and purity of purified RNA samples were analysed in Bio-Photometer plus™ (Eppendorf, Germany) by measuring absorbance at a wavelength of 260 nm, 280 nm and 230 nm. The A260/A280 and A260/A230 ratio was used to assess RNA purity.

3.3.7.3. cDNA preparation

cDNA synthesis was carried out from the mRNA present in the total RNA using Revertaid® first strand cDNA synthesis kit following manufacturer's

instructions. Briefly, 1000 ng RNA was mixed thoroughly with 1 µl of random-hexamer primer (20 pmol/µl) and nuclease-free water added to make the volume to 12 µl. The solution was incubated at 65°C for 5 min in thermo-cycler and immediately cooled on ice. To this, 2 µl dNTPs (10 mM), 4 µl reaction buffer (5X), 1 µl RevertAid M-MuLV Reverse Transcriptase enzyme (200 U/µL) and 1 µl RiboLock™ RNase inhibitor were added to make the total volume 20 µl. Afterwards, the tubes were incubated for 5 min at 25ºC followed by 60 min at 42ºC in a thermo cycler. The reaction was terminated by heating at 70ºC for 5 min. The prepared cDNA was stored at -20ºC.

3.3.7.4. Standardization of Primer by gradient PCR

Gradient PCR was used for standardization of amplification temperature for various primers. PCR assay was carried out in 0.2 ml thin walled tubes (as described previously at different anneling temperature ranging from 58ºC to 64ºC with gradient of 2 ºC. Most suitable anneling temperature characterized by absence of non-specific amplification, amplification of target gene was further used for Q-PCR analysis.

3.3.7.5. Reverse transcription real-time quantitative PCR (RT-qPCR)

RT-qPCR was performed using 2X SYBR Green master mixes (Thermo Fischer Scientific, USA). Each sample was run in duplicate in 20 µl reaction. The 20 µl reaction mixture consisted of 10 µl SYBR Green master mix, 10 pmol final concentration of forward and reverse primers, and 1 µl of (1:4 dilution) of cDNA. Final volume was made up to 20 µl with nuclease free water (NFW). The real-time PCR was performed on QuantStudio™ 3 Real-Time PCR System (Applied Biosystem, USA). The real-time PCR reaction was, 95 ºC for 2 min followed by 40 cycles of amplification with denaturation at 95 ºC for 15 sec, annealing at 60 ºC for 15 sec and extension at 72 ºC for 1 min each. To assess the specificity of the amplified product, dissociation curve was generated at temperature of 60 °C through 95 °C. The results were expressed as threshold cycle values (CT). The threshold, automatically adjusted by the instrument, was used for the generation of CT values. The formula used to calculate the fold change in gene expression was “fold change = 2-ΔΔCT,” (where ΔΔCT = [(CT target gene - CT ref) treatment - (CT target gene - CT ref) control]. The gene-specific amplification was corrected for the difference in input of RNA by taking housekeeping gene *rspL* gene to account. Evaluation of 2-ΔΔCT

indicates the fold change in gene expression relative to control group (i.e., fold change in control=1). The results were analyzed in comparison with the CT (minimum threshold of amplification) value of the target gene and the reference gene.

3.3.7.6. Statistical Analysis

The log2 fold mRNA expression of target gene is correlated with MIC_{IMP}. Pearson's correlation coefficient was calculated using Graph Pad Prism 5 (USA).

Table 2: Details of primers used for β-lactamase genotypic study in the study

PCR Name	β-lactamase (s) targeted	Primer Name	Sequence (5'-3')	Primer Concentration (Pmol)	Amplicon Size	PCR Conditions	Reference
blaCTX-M	CTX-M Universal Primer	CTX-M uni_F	CGA TGT GCA GTA CCA GTA A	15	580	94^0C for 40 s, 57^0C for 40 s 72^0C for 1	Hopkins et al., 2007
		CTX-M uni_R	TTA GTG ACC AGA ATC AGC GG	15			
Multiplex I TEM and SHV	TEM and SHV variants	MultiTEM_for	CATTTCCGTGTCGCCCTTATTC	15	800	94^0 C for 40 s, 60^0 C for 40 s 72^0 C for 1	Dallenne et al., 2010
		MultiTEM_rev	CGTTCATCCATAGTTGCCTGAC	15			
		MultiSHV_for	AGCCGCTTGAGCAAATTAAAC	15	713		
		MultiSHV_rev	ATCCCGCAGATAAATCACCAC	15			
OXA- 48-like	OXA-48-like	OXA-48_for	GCTTGATCGCCCTCGAT	15	281	94^0 C for 40 s, 55^0 C for 40 s 72^0 C for 1	
		OXA-48_rev	GATTTGCTCCGTGGCCGAAA	15			
Multiplex I IMP, VIM, and KPC	IMP variants	MultiIMP-F	TTGACACTCCATTTACDGa	15	139		
		MultiIMP-	GATYGAGAATTAAGCCACYCTa	15			
	VIM	MultiVIM-F	GATGGTGTTTGGTCGCATA	25	390		
		MultiVIM-R	GATGGTGTTTGGTCGCATA	25			
	KPC-1 to KPC-5	MultiKPC-F	CATTCAAGGGCTTTCTTGCTGC	25	538		
		MultiKPC-R	ACGACGGCATAGTCATTTGC	25			

Table 3: Details of primers used for sequencing of carbapenem genes

β-lactamase (s) targeted	Primer Name	Sequence (5'-3')	Primer Concentration (Pmol)	Amplicon Size	PCR Conditions	Reference
Bla-VIM	VIM-2-SQ-F	ATGTTCAAACTTTTGAGTAAG	10	801	94^0C for 40 s, 52^0C for 40 s 72^0C for 1	Fiett et al., 2006
	VIM-2-SQ-R	CTACTCAACGACTGAGCGAT	10			
Bla-OXA-48	IMP2-SQ-F	GTTTTATGTGTATGCTTCC	10	678	94^0C for 40 s, 55^0C for 40 s 72^0C for 1	
	IMP2-SQ-R	AGCCTGTTCCCATGTAC	10			
Bla-OXA-48	blaOXA-48 -SQ-F	TTGGTGGCATCGATTATCGG	10	743	94^0C for 40 s, 55^0C for 40 s 72^0C for 1	Shibata et al., 2020
	blaOXA-48 -SQ-R	GAGCACTTCTTTTGTGATGGC	10			

Table 4: Details of primers used for Q- PCR for quantitative estimation of outer membrane porin gene

PCR name	Primer Name	Sequence (5'-3')	Primer Concentration (Pmol)	Amplicon Size	Reference
Klebsiella ompK35 gene	*ompK35/ompF-F*	TCCCTGCCCTGCTGGTAG	10	**124 bp**	Doumith et al., 2009
	ompK35-R	CTGGTGTCGCCATTGGTGG	10		
Citrobacter/Enterobacter ompF gene	*ompK35/ompF-F*	TCCCTGCCCTGCTGGTAG	10	**139 bp**	
	ompF-R	TAAGTGTTGTCGCCATCGTTG	10	**180 bp**	
Citrobacter/Enterobacter ompC gene	*ompK36/ompC-F*	GCGACCAGACCTACATGCGT	10		
	ompC-R	TTCGTTCTCACCAGAGTTACCCT	10	**113 bp**	
Klebsiella ompK36 gene	*ompK36/ompC-F*	GCGACCAGACCTACATGCGT	10		
	ompK36-R	AGTCGAAAGAGCCCGCGTC	10		
Escherichia coli ompF gene	*E. coli OmpF F*	AAGTAGTAGGTTGCGCCCAC	10	**118 bp**	Chetri et al., 2019
	E. coli OmpF R	AGTTCGATTTCGGTCTGCGT	10		
Escherichia coli *ompC* gene	*E. coli OmpC F*	ATTCTGGCAGTACGTCGGTC	10	**125 bp**	
	E. coli OmpC R	AAACAACTCCTGGACCCGTG	10		

Table 5: Details of primers used for Q- PCR for quantitative estimation of efflux pump gene

PCR name	Primer Name	Sequence (5'-3')	Primer Concentration (Pmol)	Amplicon Size	Reference
Pseudomonas membrane efflux pump	*mexB--Fwd*	CAACATCCAGGACCCACTCT	10	167	Serra et al., 2019
	mexB-Rev	AGGAAATCTGCACGTTCTG	10		
Pseudomonas outer membrane porin	*oprD--Fwd*	GCCGAAGCCGATATAATCAA	10	157	
	oprD-Rev	CATCTACCGCACAAACGATG	10		
Enterobacteriace efflux pump AcrA	*acrA-Fwd*	CTCTCAGGCAGCTTAGCCCTAA	10	107	Swick et al., 2011
	acrA-Rev	TGCAGAGGTTCAGTTTTGACTGTT	10		
Enterobacteriace efflux pump AcrB	*acrB--Fwd*	AGCTTCCTGATGGTTGTCGG	10	107	
	acrB--Rev	ACGGCTGATGGCATCTTTCA	10		
30-S ribosomal protein gene	rpsL--*Fwd*	GCAAAAACGTGGCGTATGTACTC	10	104	
	rpsL-*Rev*	TTCGAAACCGTTAGTCAGACGAA	10		

Results

CHAPTER-4

RESULTS

4.1. Revival and identification of bacterial isolates

A total of eighteen carbapenem non-susceptible isolates (zone of inhibition <19 mm on disc diffusion test) were selected for further characterization. Isolates were subjected to Gram staining for morphological examinations. Isolates were characterized as Gram-negative rod-shaped organism based on microscopical examination (Figure 1). All the isolates were characterized by a panel of 14 biochemical tests. All the isolates showed typical results during their biochemical characterization. Results were interpreted by visualizing change in color of respective growth media (Figure 3). The CRE samples comprised of *Pseudomonas aeruginosa* (n=1), *Enterobacter* spp. (n=1), *Citrobacter spp.* (n=2), *Klebsiella spp.* (n=2) and *Escherichia coli* (n=12). The details of biochemical test results are included in table 6.

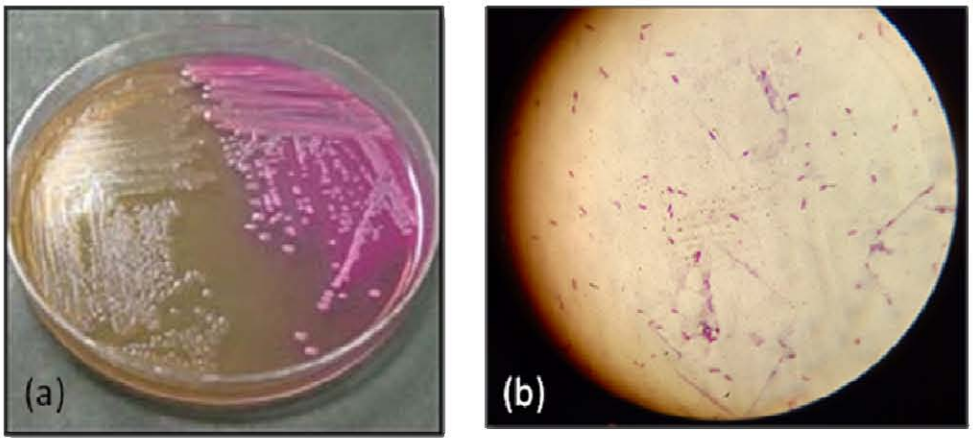


Figure 1a & 1b. Colony morphology and Gram staining result of test isolates

4.2. Antibiotic susceptibility tests

All the eighteen isolates were subjected to antimicrobial sensitivity testing using Kirby-Bauer disc diffusion method (Figure 3). The result was interpreted as resistance, intermediate and sensitive strains for all the isolates examined on the basis of zone interpretation criteria given in CLSI 2017 (Table 7). The susceptibility tests showed that 38.8% (n=7/18) isolates were resistant to the entire panel antibiotic tested

while 27.7% (n=5/18) isolates were resistant to all the antibiotics except ertapenem and characterized as multiple drug resistant. All the isolates were found to be resistant to 2 or more classes of antibiotics. The resistance pattern within the class varied. The highest resistance was observed among penicillin and cephalosporin. None of isolates were found susceptible to either ampicillin or amoxicillin-clavulanic acid. A relatively high rate of resistance was observed together for all cephalosporin antibiotics (cefotaxime (94.4%, cefpodoxime (94.4%) and ceftriaxone (88.8%). The heat map of antibiogram is shown in Figure 4.

4.3. Phenotypic detection of carbapenemase production

All the 18 isolates were subjected to phenotypic detection of carbapenemase production.

4.3.1. m-Carba NP Test

The mCarba NP test is a biochemical test for rapid detection (≥2 h) of carbapenemase production on Gram-negative bacilli. It is based on *in vitro* hydrolysis of imipenem by a bacterial lysate, which is detected by changes in pH values using the indicator phenol red (red to orange/yellow). Out of 18 isolates, 12 isolates showed positive reaction in m-Carba NP test (Figure 5).

4.3.2. Carbapenemase Inhibition assay

The CIA consists of the incubation of a potential carbapenemase producer with ertapenem discs and use of the resulting supernatant to challenge a susceptible indicator strain. Out of 18 isolates, 10 isolates showed positive reaction in carbapenemase inhibition assay (Figure 6). The details of result of carba NP test and CIA test is given in table 8.

4.4. Phenotypic Evaluation of efflux pump activity in multidrug resistant isolates

4.4.1. CCCP-IMP/ETP disc synergy test

The antibiotic sensitivity assay was performed in the presence ertapenem and imipenem alone, or, in combination with CCCP (Figure 7). The difference in the zone of inhibition (ZOI) in the presence and absence of CCCP (IMP – IMP+CCCP) was measured. The ZOI difference of ≥4 mm was considered significant. Among 18 carbapenem non-susceptible isolates under study, 27.77% (5/18) isolates showed ZOI

Table 6: Details of biochemical tests results of test isolates

Isolates	*Catalase*	*Oxidase*	*ONPG*	*Motility*	*TSI*	*Indole* test	*MR* test	*VP* test	*Citrate* test	*Xylose*	*Arabinose*	*Cellobiose*	*Rhamnose*	*Raffinose*
VS_01	+	+	-	+	K/K	+	-	-	-	-	-	-	-	-
VS_02	+	-	+	+	*A/A/Gas*	+	+	-	-	+	+	+	+	-
VA_19	-	-	+	+	*A/A/Gas*	+	-	-	-	+	+	+	+	-
VS_39	-	-	+	+	*A/A/Gas*	+	-	+	+	+	+	+	+	-
VS_51	-	-	+	+	*A/A/Gas*	+	-	-	-	+	+	-	+	-
VA_52	-	-	+	+	*A/A/Gas*	+	+	_	+	+	+	-	+	+
VA_53	-	-	+	+	*K/A*	+	+	-	-	-	-	-	-	-
VA_55	-	-	+	+	*A/A/Gas*	-	-	-	-	+	+	-	+	+
VA_66	-	-	-	-	*A/A/Gas*	+	+	-	-	+	+	-	+	-
VA_77	-	-	+	-	*A/A/Gas*	+	-	-	+	+	+	-	+	+
VA_99	-	-	+	+	*A/A/Gas*	+	+	-	-	+	+	+	+	+
VA_100	-	-	+	+	*A/A/Gas*	+	+	-	-	+	+	+	+	-
VU_02	-	-	+	+	*A/A*	-	-	-	-	+	+	+	-	-
VU_03	+	-	+	-	*A/A/Gas*	+	+	-	+	+	+	+	+	+
VU_08	+	-	+	+	*A/A/Gas*	+	+	-	-	+	+	+	+	+
VU_14	-	-	+	+	*A/A*	+	+	-	-	+	+	+	+	+
VU_16	-	-	+	-	*A/A*	+	+	-	-	+	+	+	+	+
VU_17	+	-	+	+	*A/A*	+	+	-	-	+	+	+	+	+

*+ positive reactions, - negative reaction; K/K: Alkaline over Alkaline; K/A/Gas: Alkaline over acid and gas; A/A/Gas: acid over acid and gas; A/A, acid over acid and no gas

Table 7: AST Zone diameters for control strain *Escherichia coli* (ATCC®25922™) and the test isolates* used in this assay

Antibiotic	Disc Code	Antibiotic Concentration	Control Strain Zone Diameter	Control Strain Diameter observed	Test Zone diameters (mm)		
		(µg)	(mm)	(mm)	Resistant	Intermediate	Susceptible
Amoxicillin / Clavulanic acid	AmC-30	20/10	18-24	22	<= 13	14--17	>= 18
Ampicillin	AM-10	10	16-22	20	<= 13	14--16	>= 17
Cefotaxime	CTX-30	30	29-35	34	<= 14	15--22	>= 23
Ceftazidime	CAZ-30	30	25-32	29	<= 14	15--17	>= 18
Cefpodoxime	CPD-10	10	23-28	25	<= 17	18--20	>= 17
Ceftriaxone	CRO-30	30	29-35	29	<= 13	14--20	>= 21
Cefoxitin	FOX-30	30	23-29	24	<= 14	15--17	>= 18
Ciprofloxacin	CIP-5	5	30-40	30	<= 15	16--20	>= 21
Gentamicin	GM-10	10	19-26	17	<= 12	13--14	>= 15
Ertapenam	ETP-10	10	29-36	32	<= 15	16--18	>= 19

*Adapted from M100, Performance Standards for Antimicrobial Susceptibility Testing; 27th Edition, (Published by Clinical and Laboratory Standards Institute, CLSI 2017, Pennsylvania, USA

Table 8: Details of the phenotypic tests performed on carbapenem resistant isolates.

Isolate No.	VS 01	VS 02	VS 39	VS 51	VA 19	VA 52	VA 53	VA 55	VA 66	VA 77	VA 99	VA 100	VU 02	VU 03	VU 08	VU 14	VU 16	VU 17
m-Carba test	+	+	+	+	+	-	+	+	-	-	-	+	+	+	+	-	-	+
CIA	+	+	+	+	+	-	-	+	-	+	-	-	+	+	-	-	-	+

Table 9: AST zone diameter of *tests* isolates with and without CCCP tested by Kirby-Bauer disc diffusion method.

Isolates No.	IMP	IMP+CCCP	ETP	ETP+CCCP
VS 01	10	12	7	8
VS 02	10	10	6	6
VS 39	10	10	6	6
VS 51	10	11	6	7
VA 19	10	10	6	6
VA 52	11	18	19	21
VA 53	11	21	25	25
VA 55	10	18	16	17
VA 66	10	10	6	6
VA 77	10	12	16	18
VA 99	10	15	24	25
VA 100	10	11	22	22
VU 02	10	19	28	28
VU 03	10	10	6	6
VU 08	11	11	7	8
VU 14	10	11	20	22
VU 16	10	10	14	18
VU 17	10	10	6	6
KPC 1705	10	10	6	6
KPC 1706	28	28	26	26
E coli	29	29	32	33
P.A	27	27	24	24

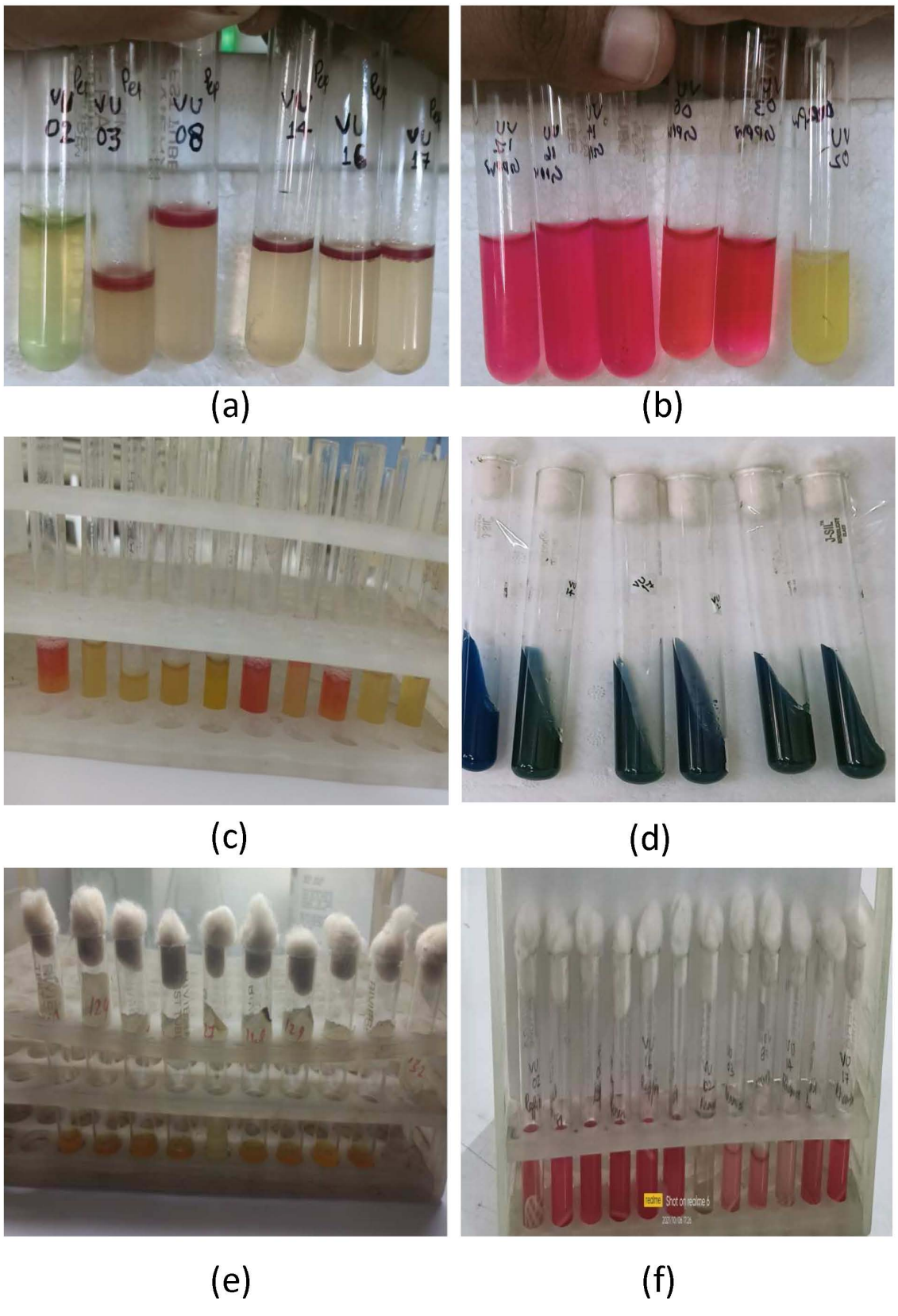


Figure 2: Results shown by isolates (a) Indole Test, (b) Methyl Red Test, (c) Voges Proskaeur Test, (d) Citrate Test, (e) ONPG Test, (f) Sugar Fermentation Test.

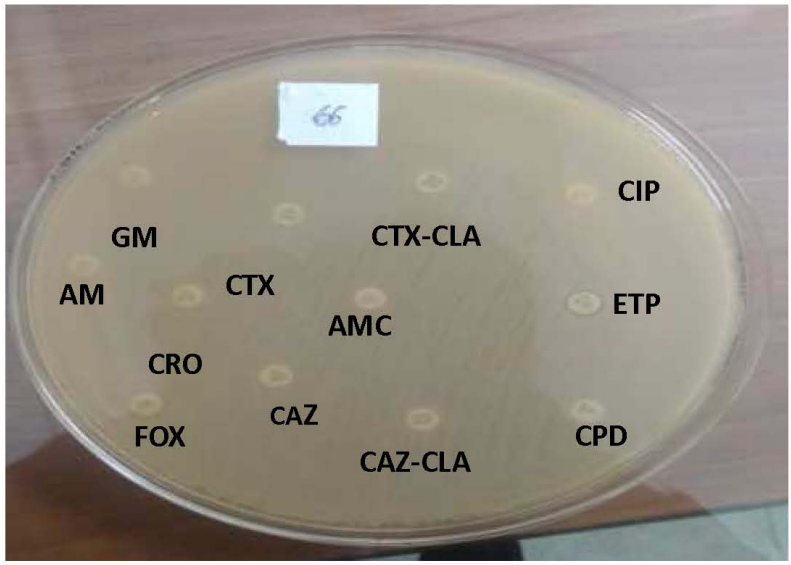


Figure 3. Antibiotic sensitivity test results of representative isolates. The zone of inhibition diameter was mesured and the results were interpretated as Resistant (R), Intermediate (I) and Sensitive (S) based on interpretation criteria.

Isolates codes	Ceftriaxone (CRO 30)	Ampicillin (AM)	Cefotaxime (CTX 30)	Amoxicillin (AMC 30)	Ciprofloxcin (CIP 5)	Cefpodoxime(CPD 10)	Eetapenem (ETP 10)
VS 01	R	R	R	R	R	R	R
VS 02	R	R	R	R	R	R	R
VS 39	R	R	R	R	R	R	R
VS 51	R	R	R	R	R	R	R
VA 19	R	R	R	R	R	R	R
VA 52	R	R	R	R	R	R	S
VA 53	R	R	R	R	R	R	S
VA 55	R	R	R	R	R	R	I
VA 66	R	R	R	R	R	R	R
VA 77	S	R	S	R	S	S	I
VA 99	R	R	R	R	R	R	S
VA 100	R	R	R	R	R	R	S
VU 02	R	R	R	R	R	R	R
VU 03	R	R	R	R	R	R	R
VU 08	R	R	R	R	I	R	R
VU 14	S	R	R	R	S	R	I
VU 16	R	R	R	R	R	R	I
VU 17	R	R	R	R	R	R	I

Figure 4 : Antimicrobial susceptibility profiles are shown as a heatmap within susceptible (green), intermediate (yellow) and resistant (red) categories Abbreviation: AMP, ampicillin; AMC, amoxicillin-clavulanic acid; CIP, ciprorofloxacin; CTX, Cefotaxime; CRO, Ceftriaxone; CPD, cefpodoxime, CAZ, ceftazidime; ETP, ertapenam; FOX, Cefoxitin; GM, gentamicin.

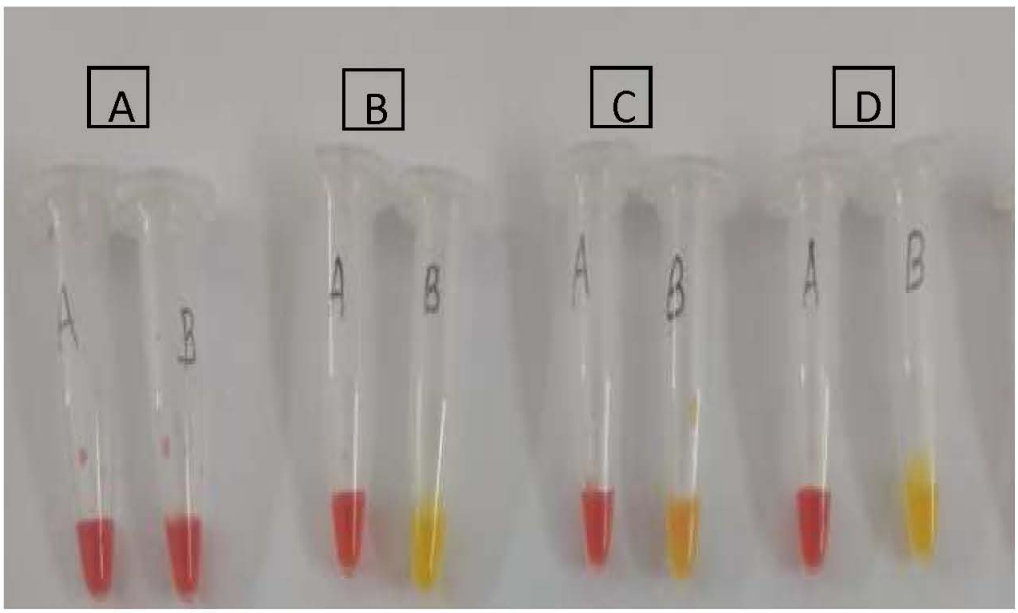


Figure 5. Modified Carba NP test results of representative isolates (A) Negative Control E. coli ATCC 25922 (B) Positive Control *Klebsiella pneumoniae* ATCC BAA 1705 (C) Isolate VMCM-19 (D) Isolate VMCM-55. (a) Tube containing phenol red solution 0.1 mM $ZnSO_4$ (pH 7.5 (b) Tube containing phenol red solution 0.1 mM $ZnSO_4$(pH 7.5) supplemented with 6 mg/mL of Imipenem

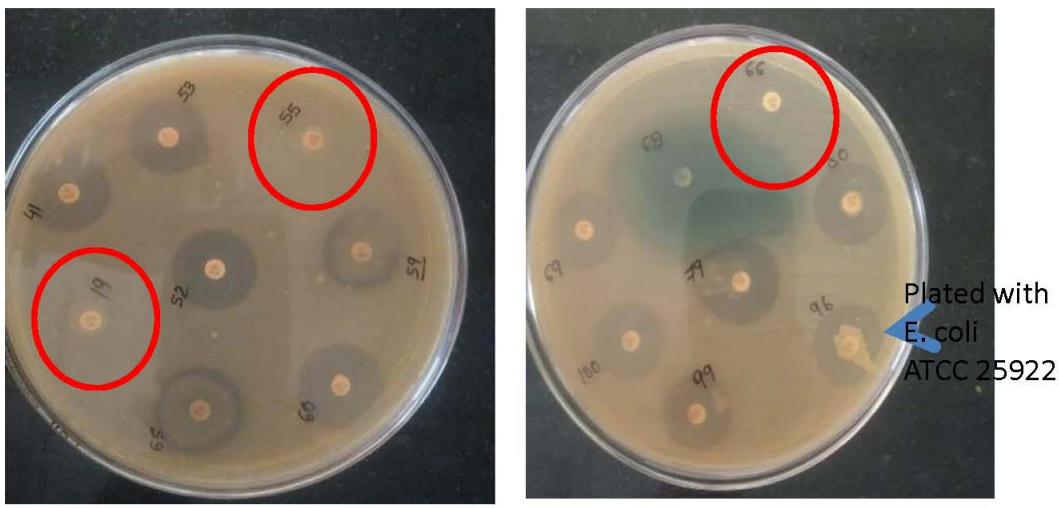


Figure 6. Carbapenem Inactivation method (CIM) of representative isolates . (A) Test isolates showing positive results with absence of an inhibition zone (B) Test isolates negative results appeared >20 mm of inhibition zone diameter. (C) Control Strain *E. coli* ATCC 25922

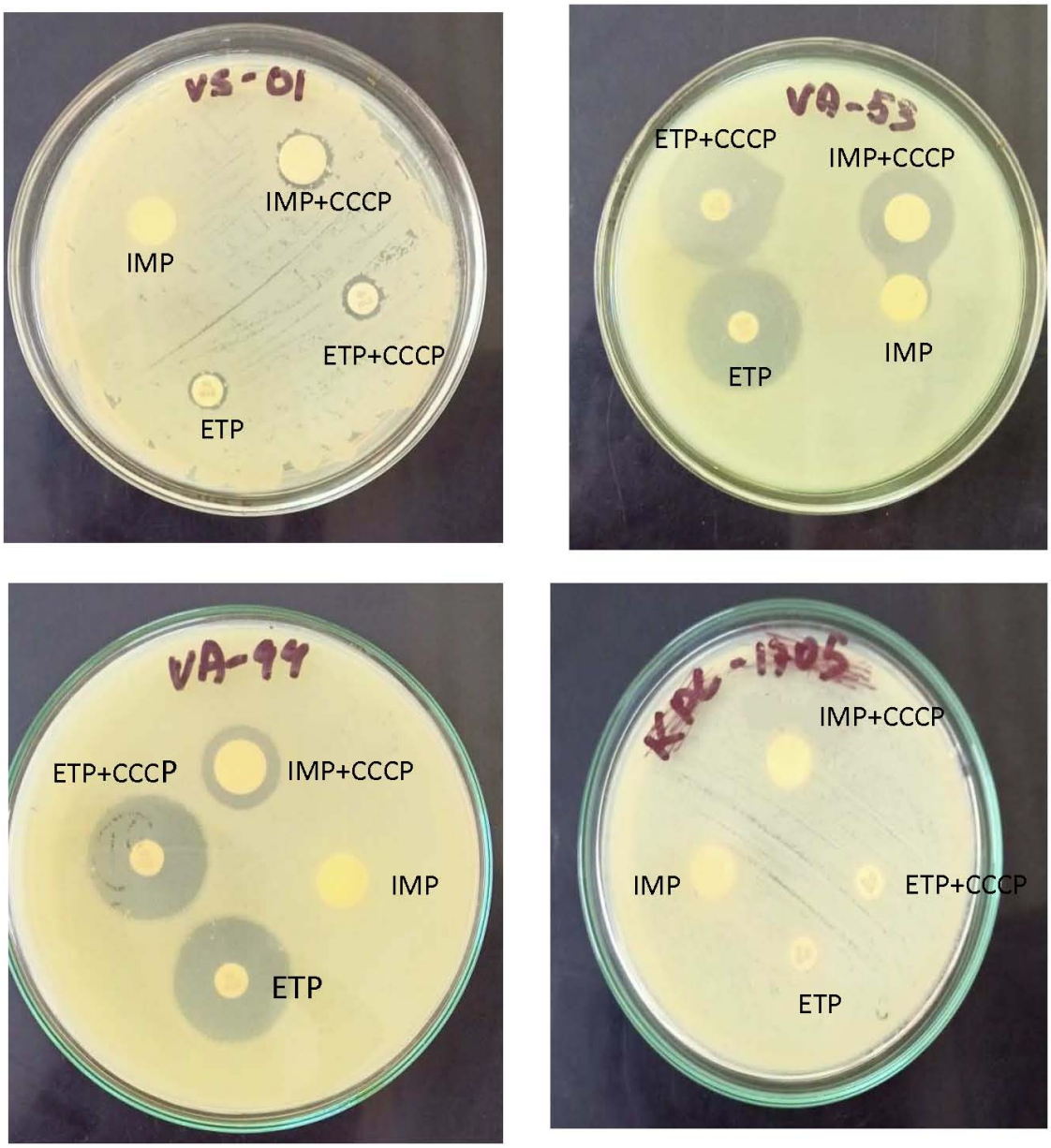


Figure 7. CCCP-carba disc synergy test result of representative isolates Disc containing 30µg IMP and ETP with and without CCCP (20µg/ml).The inhibition of efflux pump activity by CCCP is interpreted by increase in zone of inhibition by ≥4 mm in presence of CCCP. Control Strain *Klebsiella pneumoniae* ATCC BAA1705

≥4 mm indicating that these isolates were efficiently effluxes IMP out of the cells (Table 9, Figure 8). However, none of the carbapenem non-susceptible isolates showed considerable efflux pump inhibition when ertapenem was used in similar experiment.

4.4.2. CCCP-IMP broth microdilution assay

The minimum inhibitory concentration (MIC) for imipenem was calculated for each isolate in the presence and absence of CCCP (Figure 9). The imipenam demonstrated relevant direct antibacterial activity against the test strain, with MIC values ranging from 0.625 to 80 µg/mL (Table 10). MIC result revealed that in 88.8% isolates (n=17/18) MIC were found to be above the break point against imipenam (2µg). When the antibiotic was tested in association with CCCP efflux pump inhibitor, a reduction in the antibiotic MIC was observed in three out of eighteen isolates (16.66 %), with inhibition of the antibiotic resistance mechanism being observed. The MICs of Imipenem (IMP) and the differences in the MIC values for each strain with and without the inhibition of CCCP are shown in table 10 and figure 10.

4.4.2. EtBr cartwheel method

High efflux pump activity is one of the mechanisms of antibiotic resistance. The efflux activity of isolates was determined by the ability of an organism to pump out EtBr out of the cell. The cartwheel test was performed at different concentrations of EtBr (Figure 11). At 1 µg/mL, 1.5 µg/mL and 2.0 µg/mL of EtBr, the efflux activities of isolates were not distinguished. Out of 18 MDR isolates, 3 isolates did show reduction of MC_{EtBr} activity indicating pronounced activity of efflux pump inhibitor CCCP which was used to assess the efflux activity (Table 11).

4.5. Genotypic characterization of carbapenem-resistant gene

Multiplex PCR was carried out for typing of beta-lactamase gene using a set of primers specific for bla OXA-48, bla IMP, bla VIM, and bla KPC. All the genes were characterized by observing specific amplification of 281bp, 139bp, 390bp and 538bp amplified products for OXA-48, bla IMP, bla VIM, and bla KPC respectively (Figure 12 & 13). *Klebsiella pneumonia* ATCC BAA1705 (bla KPC^{+}) and *Escherichia coli* ATCC 25922 were used and positive and negative control

respectively. Out of 18 CRE isolates, bla IMP was detected in 8 isolates while 7 isolates carried bla VIM gene. Three isolates carried bla OXA-48 gene. Incidentally, all three bla Oxa-48 isolates were recovered from companion animals. Three out of 18 isolates carried both bla VIM and bla IMP gene while co-existance of bla IMP and bla Oxa-48 was found in one isolate. Three out of 18 isolates carried one or more extended spectrum beta lactamase gene apart from carbapenemase gene. The details of distribution of carbapenem beta lactamase gene in test isolates are given in table 12.

4.6. Genotypic characterization of ESBL-resistant gene

Multiplex PCR was carried out for typing of beta-lactamase gene using a set of primers specific for bla SHV, bla TEM, and bla CTX. All the genes were characterized by observing specific amplification of 800 bp, 713 bp and 580 bp amplified products for bla SHV, bla TEM, and bla CTX respectively (Figure 14 & 15). *Klebsiella pneumoniae* ATCC 700603 (blaTEM+) and *Escherichia coli* ATCC 25922 were used and positive and negative control respectively. Out of 18 CRE isolates, 4 were harbouring bla CTX-M while 3 were found positive for bla TEM. None of the isolates were positive for bla SHV gene. The details of distribution of extended spectrum beta lactamase gene in test isolates are given in table 12.

4.7. Sequence analysis of carbapenemase encoding genes

bla VIM, bla IMP and bla-Oxa-48 of representative isolates were amplified and PCR amplicons were purified using gel extraction kit (Figure 16 & 17). The Sanger sequencing of PCR products were analysed by NCBI-BLAST tool. The sequence alignment analysis revealed presence of bla VIM-2, bla IMP-8 and bla Oxa-181 (Figure 18, 19 and 20).

4.8. Relative quantification of OMP and efflux pump gene by Q-PCR

4.8.1 Total RNA isolation

Total bacterial RNA was isolated from all the bacterial isolates using combined use of Trizol and QiaAMP RNA isolation kit. The extracted RNA samples were subjected to concentration and purity check. All the RNA samples were found to be optimum quality (ranging from 1.7-2.0). The concentration of various samples

Table 10: MIC values of *tests* isolates with and without CCCP tested by broth micro dilution method.

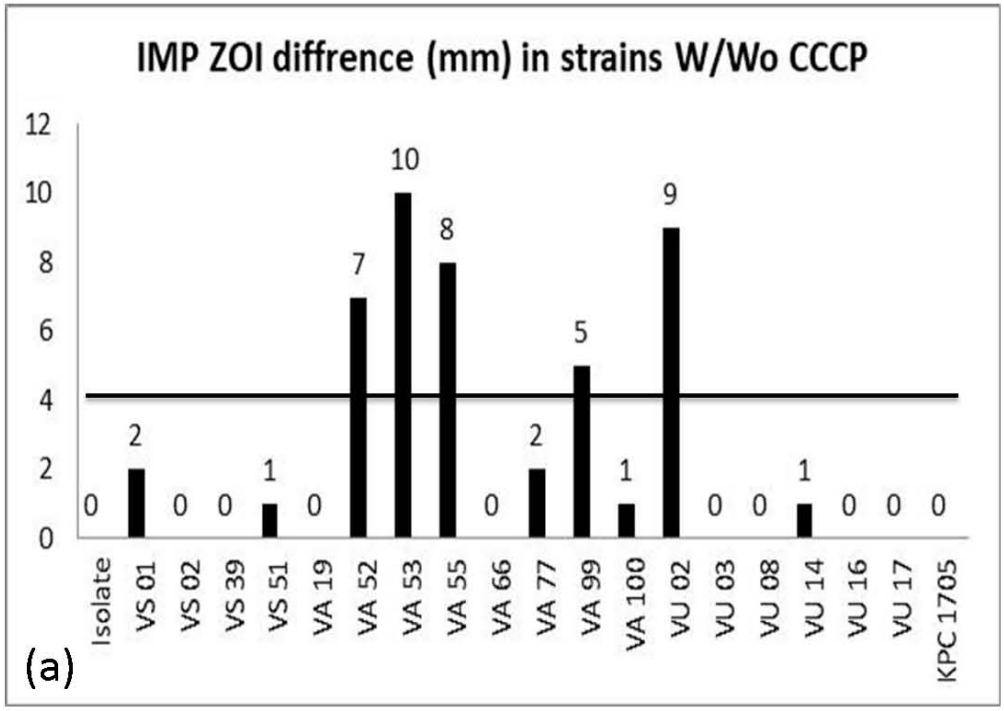
Isolates No	Imipenem(µg/ml)	Imipenam+CCCP(µg/ml)
VS 01	40	40
VS 02	20	20
VS 39	20	20
VS 51	20	20
VA 19	40	40
VA 52	5.0	05
VA 53	2.5	0.625
VA 55	0.625	0.625
VA 66	20	4.0
VA 77	2.5	2.5
VA 99	5.0	0.625
VA 100	5.0	5.0
VU 02	40	40
VU 03	40	40
VU 08	80	80
VU 14	1.25	1.25
VU 16	5.0	5.0
VU 17	20	20
KPC 1705	20	8.0
KPC 1706	1.25	<0.0625
E coli	0.6	<0.0625

Table 11. Relationship between MC_{EtBr} and efflux activity.

Isolate No	MC_{EtBr}	*Index*	MC_{EtBr} *with CCCP*	*Index*
VS 01	0.5	0	1.0	1.0
VS 02	1.0	1.0	1.0	1.0
VS 39	1.0	1.0	1.0	1.0
VS 51	1.0	1.0	1.0	1.0
VA 19	1.0	1.0	1.0	1.0
VA 52	1.0	1.0	1.0	1.0
VA 53	0.5	0	1.0	1.0
VA 55	1.5	1.0	1.0	1.0
VA 66	1.0	3.0	1.0	1.0
VA 77	0.5	0	0.5	0
VA 99	0.5	0	0.5	0
VA 100	0.5	0	0.5	0
VU 02	0.5	0	0.5	0
VU 03	0.5	0	0.5	0
VU 08	0.5	0	0.5	0
VU 14	1.5	2.0	0.5	0
VU 16	1.0	1.0	0.5	0
VU 17	0.5	0	0.5	0
KPC 1705	0.5	0	0.5	0

Table 12: Detail of distribution of β-lactamase gene in test isolates

Isolate No.	Organism	Source	bla CTX-M	bla TEM	bla SHV	bla VIM	bla IMP	bla KPC	bla Oxa48
VS 01	*Psudomonas sp.*	Cow	-	-	-	+	+	-	-
VS 02	*E. coli*	Cow	-	-	-	-	+	-	-
VS 39	*E. coli*	Cow	+		-	+	+	-	-
VS 51	*Enterobacter sp.*	Cow	-	+	-	+	+	-	-
VA 19	*E. coli*	Cat	-	-	-	-	-	-	+
VA 52	*E. coli*	Dog	-	-	-	-	+	-	-
VA 53	*Citrobacter sp.*	Buffalo	-	-	-	-	+	-	-
VA 55	*E. coli*	Dog	-	-	-	-	-	-	+
VA 66	*Klebsiella sp.*	Dog	-	-	-	-	-	-	-
VA 77	*Klebsiella sp.*	Buffalo	-	-	-	+	-	-	-
VA 99	*E. coli*	Buffalo	-	-	-	-	-	-	-
VA 100	*E. coli*	Buffalo	-	-	-	-	+	-	-
VU 02	*E. coli*	Dog	+	+	-	-	+	-	+
VU 03	*Citrobacter sp.*	Dog	-	-	-	+	-	-	-
VU 08	*E. coli*	Dog	+	-	-	-	-	-	-
VU 14	*E.coli*	Dog	-	-	-	-	-	-	-
VU 16	*E.coli*	Dog	-	-	-	-	-	-	-
VU 17	*E.coli*	Dog	+	+	-	-	-	-	-



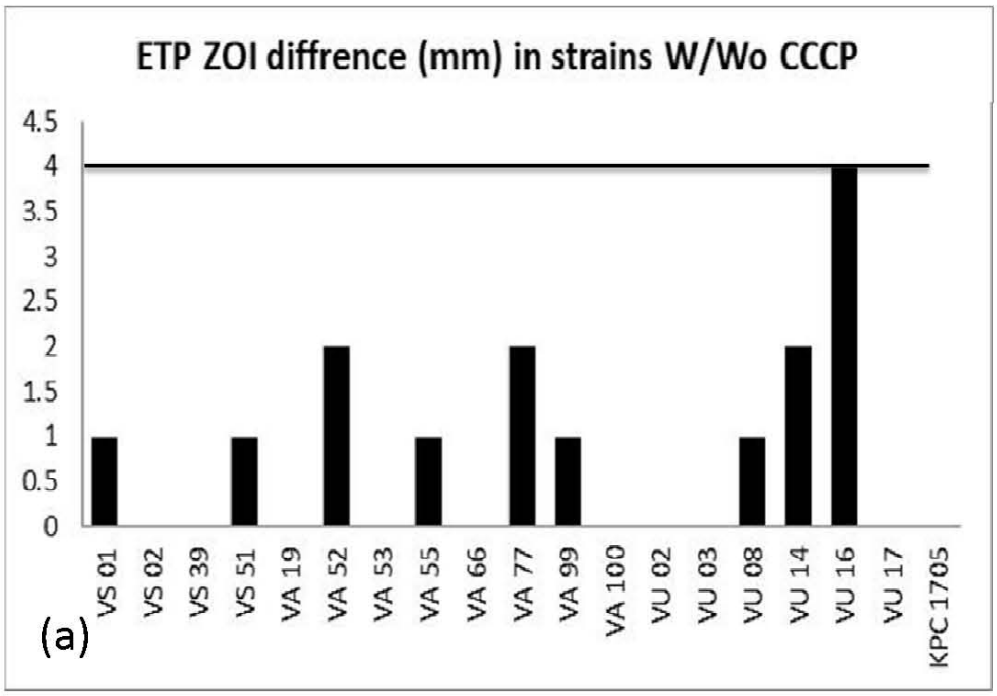
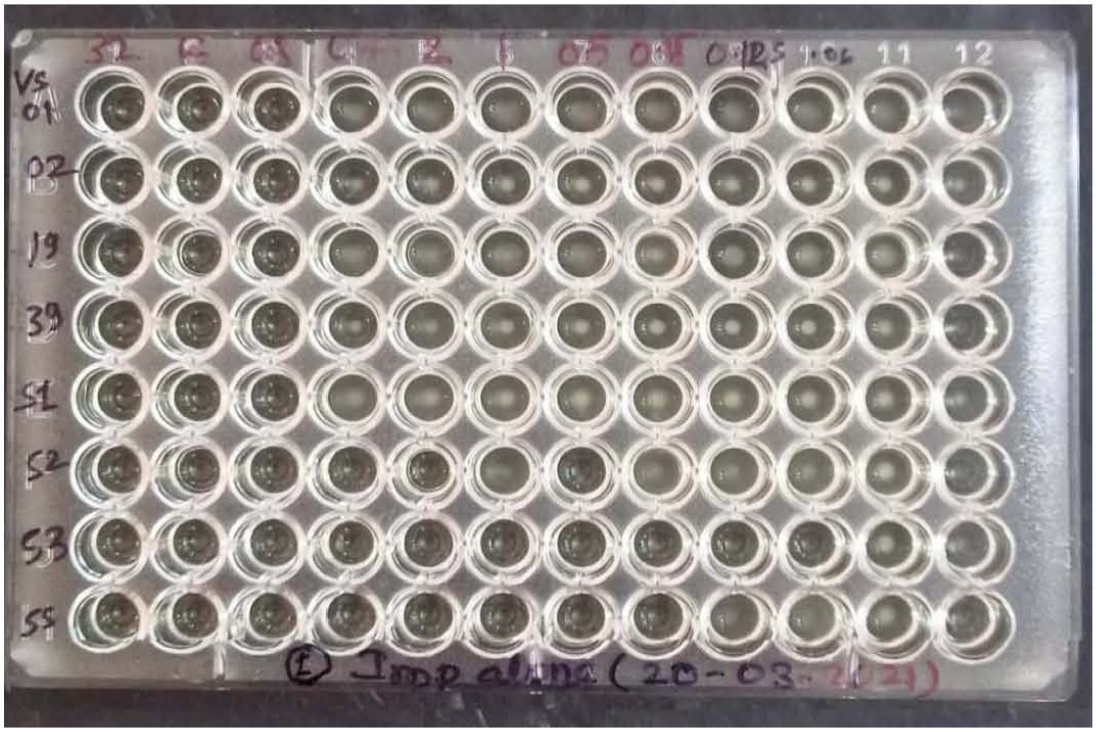
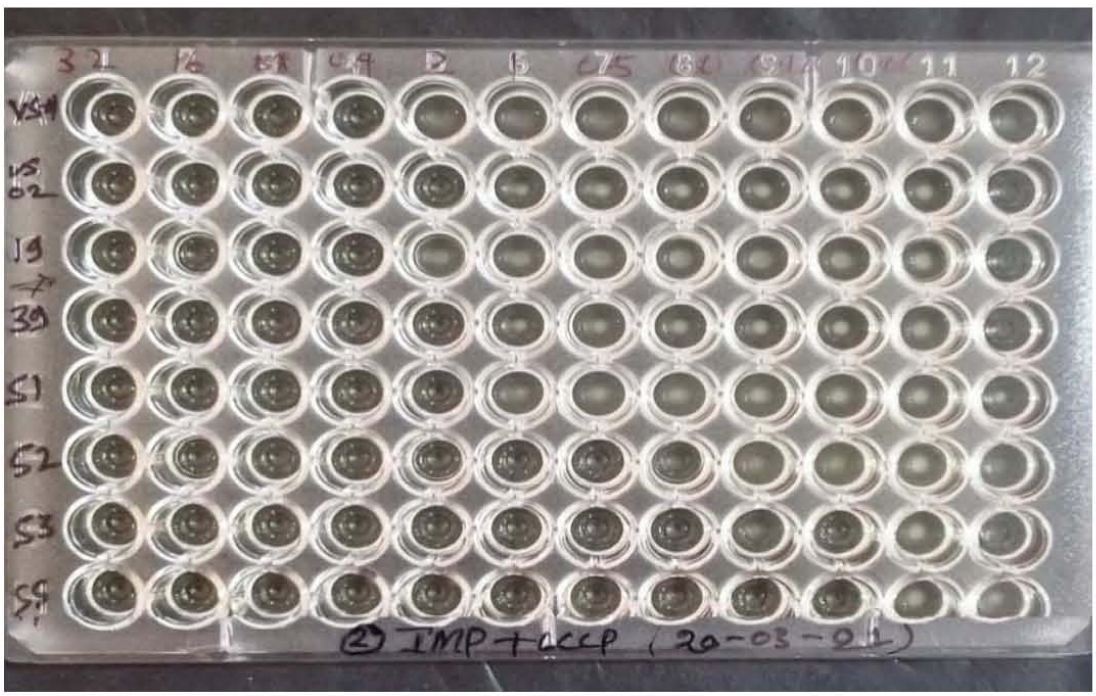


Figure 8. Difference (in mm) in Zone of inhibition (ZOI) . (a) Disc containing IMP (30µg) and **(b)** ETP (10µg) with and without CCCP (20µg/ml).The inhibition of efflux pump activity by CCCP is interpreted by increase in zone of inhibition by ≥4 mm in presence of CCCP. Control Strain *Klebsiella pneumoniae* ATCC BAA1705



(a)



(b)

Figure 9. CCCP-carba broth micro dilution result . MIC of imipenem was calculated by making serial two fold dilution of Imepenem with **(b)** or without CCCP **(a)** (20µg/ml).The efflux pump activity is interpreted by decrease in MIC by 2 fold in presence of CCCP: Control Strain *Klebsiella pneumoniae* ATCC BAA1705

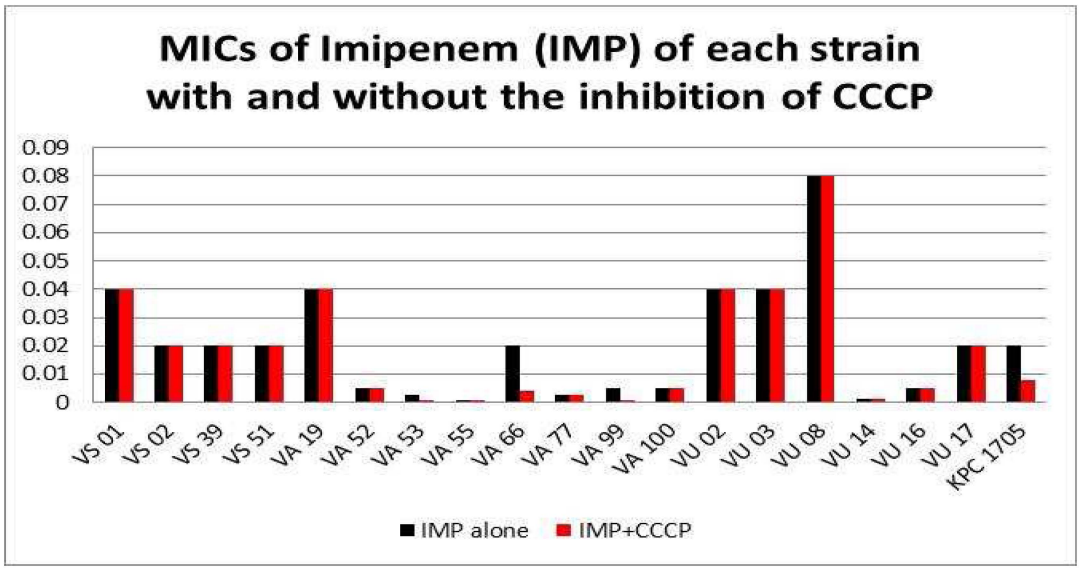


Figure 10. Bar diagram showing MIC (mg/ml) of imipenem with or without CCCP. The efflux pump activity is interpreted by decrease in MIC by 2 fold in presence of CCCP.

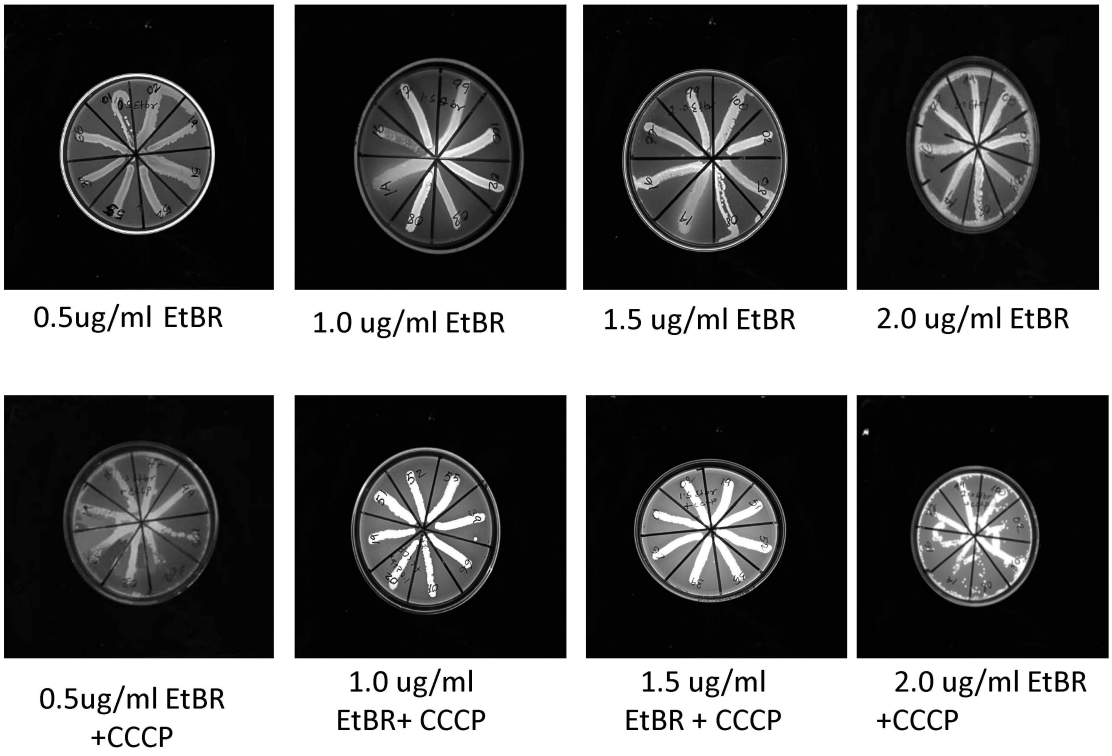


Figure 11. EtBr cartwheel agar method test reults for representative isolates . The activity of efflux pump was evaluated for its ability to extrude EtBr dye along concentration gradient. The MIC of EtBr was calculated as minimum concentration of EtBr effectively removed by efflux pump. Control Strain *Klebsiella pneumoniae* ATCC BAA1705

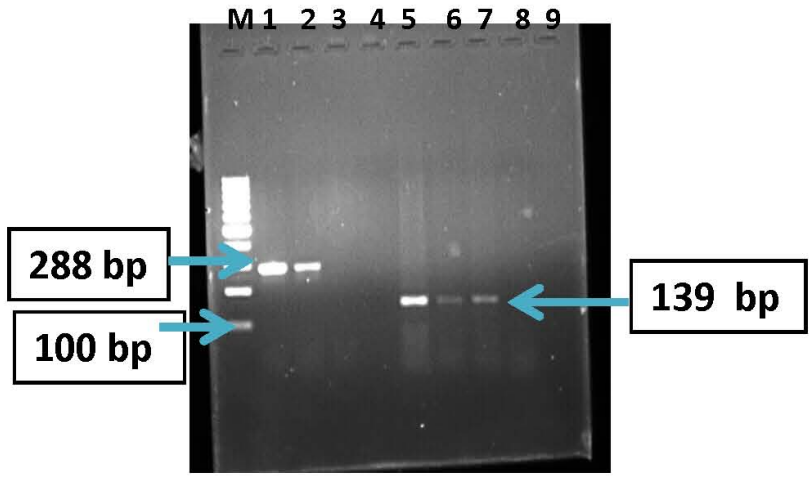


Figure 12. PCR amplification of bla Oxa-48 (288 bp) and IMP (139 bp)
Lane M: 100 bp DNA Ladder, Lane 1-9: (Test Isolates)

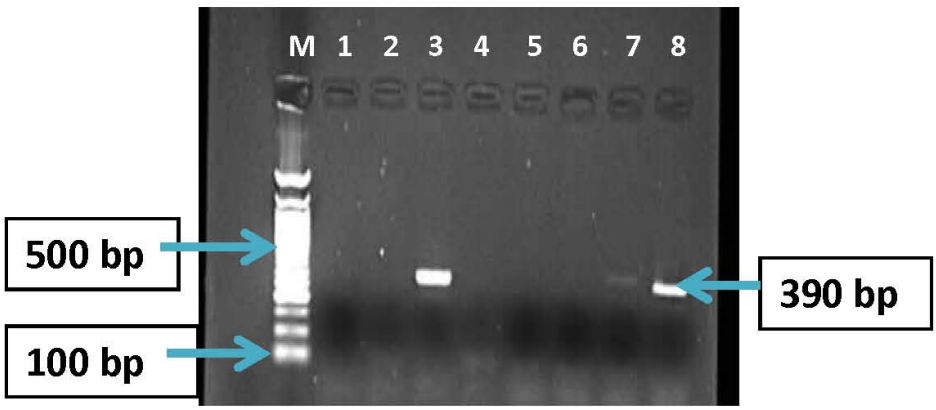


Figure 13. PCR amplification of bla VIM (390 bp)
Lane M: 50 bp DNA Ladder, Lane 1: Neagtive Control *E. coli* ATCC 25922,Lane 2-8: (Test Isolates)

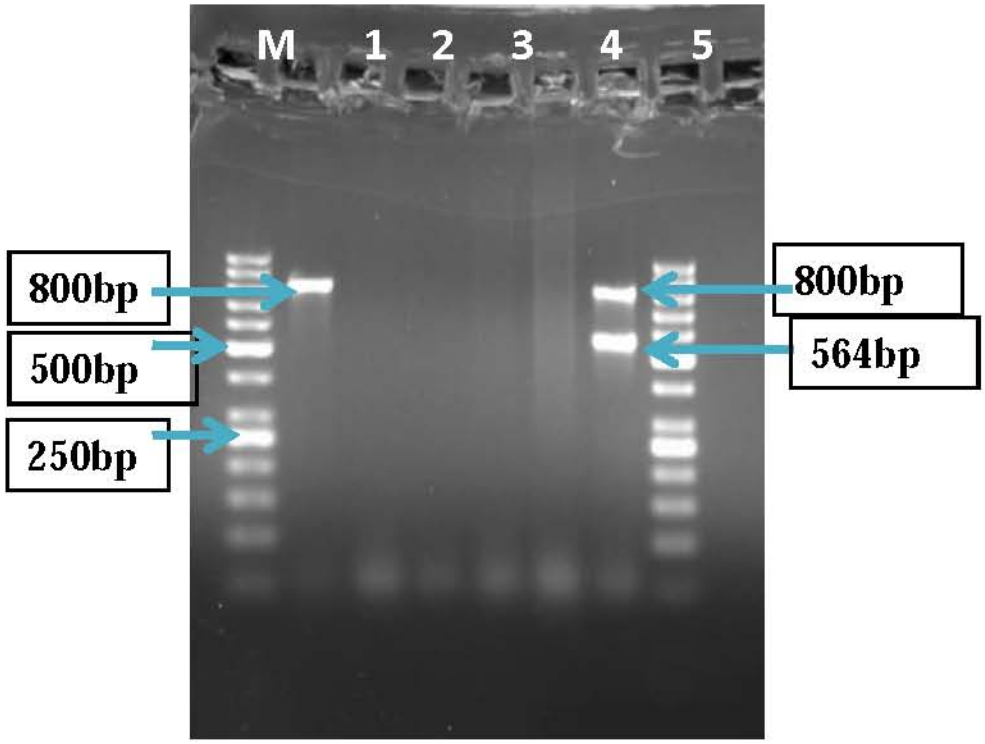


Figure 14. PCR amplification of bla TEM (800bp) and bla Oxa-1 (564 bp) gene

Lane M: 50 bp DNA ladder ,Lane 1 : Positive control, Lane 2: Negative control (*E. coli* ATCC 25922) ,Lane 3- 6: Test Isolates

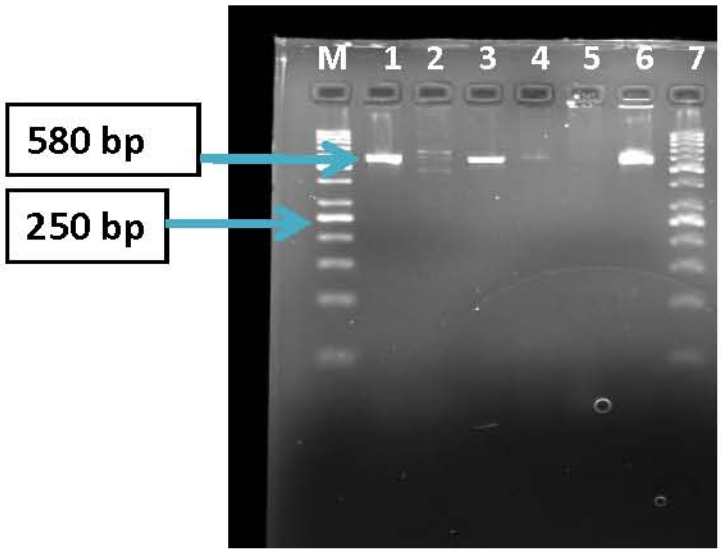


Figure 15. PCR amplification of bla CTX-M gene (580 bp)

Lane M: 50 bp DNA Ladder, Lane 1-4: Test Isolates, Lane: 5 Neagtive Control *E. coli* ATCC 25922, Lane 6: Positive control

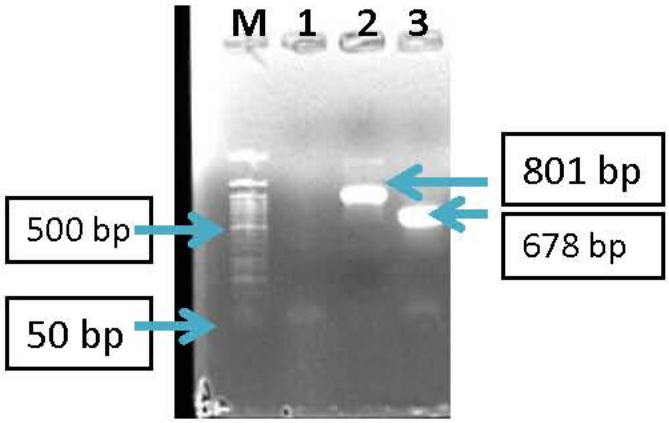


Figure 16. PCR amplification of bla VIM gene (801bp) and IMP gene (678 bp)
Lane M: 50 bp DNA Ladder, Lane: 1 Neagtive Control *E. coli* ATCC 25922
Lane 2,3: Test Isolates

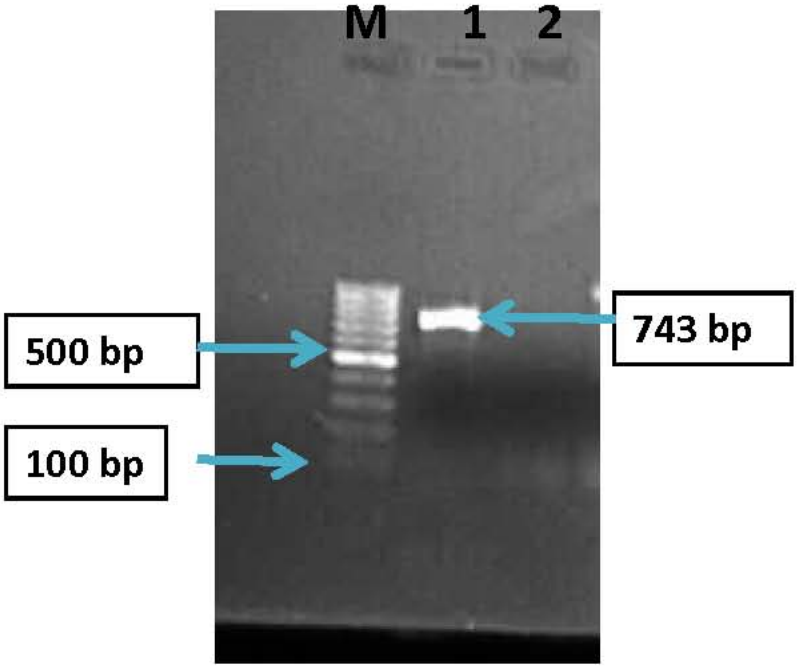


Figure 17. PCR amplification of bla Oxa-48 gene (743 bp)
Lane M: 100 bp DNA Ladder, Lane 1: Test Isolates
Lane: 2 Neagtive Control *E. coli* ATCC 25922

>Seq 1 [Organism=*Escherichia coli*] [Clone=VS-39] metallo-beta-lactamse VIM-2 plasmid unnamed, partial CDS

ATTTGACCGCGTCTATCATGGCTATTGCGAGTCCGCTCGCTTTTTCCGTAGATTCTAGCGGTG
AGTATCCGACAGTCAGCGAAATTCCGGTCGGGGAGGTCCGGCTTTACCAGATTGCCGATGGT
GTTTGGTCGCATATCGCAACGCAGTCGTTTGATGGCGCAGTCTACCCGTCCAATGGTCTCATT
GTCCGTGATGGTGATGAGTTGCTTTTGATTGATACAGCGTGGGGTGCGAAAAACACAGCGGC
ACTTCTCGCGGAGATTGAGAAGCAAATTGGACTTCCTGTAACGCGTGCAGTCTCCACGCACT
TTCATGACGACCGCGTCGGCGGCGTTGATGTCCTTCGGGCGGCTGGGGTGGCAACGTACGCA
TCACCGTCGACACGCCGGCTAGCCGAGGTAGAGGGGAACGAGATTCCCACGCACTCTCTAG
AAGGACTCTCATCGAGCGGGGACGCAGTGCGCTTCGGTCCAGTAGAACTCTTCTATCCTGGT
GCTGCGCATTCGACCGACAACTTAGTTGTGTACGTCCCGTCTGCGAGTGTGCTCTATGGTGGT
TGTGCGATTTATGAGTTGTCACGCACGTCTGCGGGGAACGTGGCCGATGCCGATCTGGCTGA
ATGGCCCACCTCCATTGAGCGGATTCAACAACACTACCCGGAAGCACAGTTCGTCATTCCGG
GGCACGGCCTGCCGGGCGGTCTAGACTTGCTCAAGCACACAACGAATGTTGTA

>Seq 2 [Organism=*Escherichia coli*] [Clone=VU-02] metallo-beta-lactamse IMP-8 plasmid unnamed, partial CDS

AGCGGCTTTGCCTGATTTAAAAATCGAGAAGCTTGAAGAAGGTGTTTATGTTCATACATCGT
TCGAAGAAGTTAACGGTTGGGGTGTTGTTTCTAAACACGGTTTGGTGGTTCTTGTAAACACT
GACGCCTATCTGATTGACACTCCATTTACTGCTACAGATACTGAAAAGTTAGTCAATTGGTTT
GTGGAGCGCGGCTATAAAATCAAAGGCACTATTTCCTCACATTTCCATAGCGACAGCACAGG
GGGAATAGAGTGGCTTAATTCTCAATCTATTCCCACGTATGCATCTGAATTAACAAATGAAC
TTCTTAAAAAAGACGGTAAGGTGCAAGCTAAAAACTCATTTAGCGGAGTTAGTTATTGGCTA
GTTAAAAATAAAATTGAAGTTTTTTATCCCGGCCCGGGGCACACTCAAGATAACGTAGTGGT
TTGGTTACCTGAAAAGAAAATTTTATTCGGTGGTTGTTTTGTTAAACCGGACGGTCTTGGTAA
TTTGGGTGACGCAAATTTAGAAGCTTGGCCAAAGTCCGCCAAAATATTAATGTCTAAATATG
GTAAAGCAAAACTGGTTGTTTCAAGTCATAG

>Seq 3 [Organism=*Escherichia coli*] [Clone=VA-19] blaOxa-181 plasmid unnamed, partial CDS

TGGCAAGAAAACAAAAGTTGGAATGCTCACTTTACTGAACATAAATCACAGGGCGTAGTTG
TGCTCTGGAATGAGAATAAGCAGCAAGGATTTACCAATAATCTTAAACGGGCGAACCAAGC
ATTTTTACCCGCATCTACCTTTAAAATTCCCAATAGCTTGATCGCCCTCGATTTGGGCGTGGT
TAAGGATGAACACCAAGTCTTTAAGTGGGATGGACAGACGCGTGATATCGCCGCTTGGAAT
CGTGACCATGACTTAATTACCGCGATGAAGTACTCAGTTGTGCCTGTTTATCAAGAATTTGCC
CGCCAAATTGGTGAGGCACGTATGAGTAAAATGCTGCACGCCTTCGATTATGGCAATGAGG
ATATCTCGGGCAATGTAGACAGTTTTTGGCTCGATGGTGGTATTCGCATTTCGGCTACCCAGC
AAATCGCTTTTTTACGCAAGCTGTATCACAACAAGCTGCACGTTTCTGAGCGTAGTCAGCGC
ATCGTGAAACAAGCCATGCTGACCGAAGCCAATGGCGACTATATTATTCGGGCTAAAACGG
GATACTCGACTAGAATCGAACCTAAGATTGGCTGGTGGGTTGGTTGGGTTGAACTTGATGAT
AATGTGTGGTTTTTTGCGATGAATATGGATATGCCCACATCGGATGGTTTAGGGCTGCGCCA
AGCCATCACAAAAGAAAGTGCTCTTGA

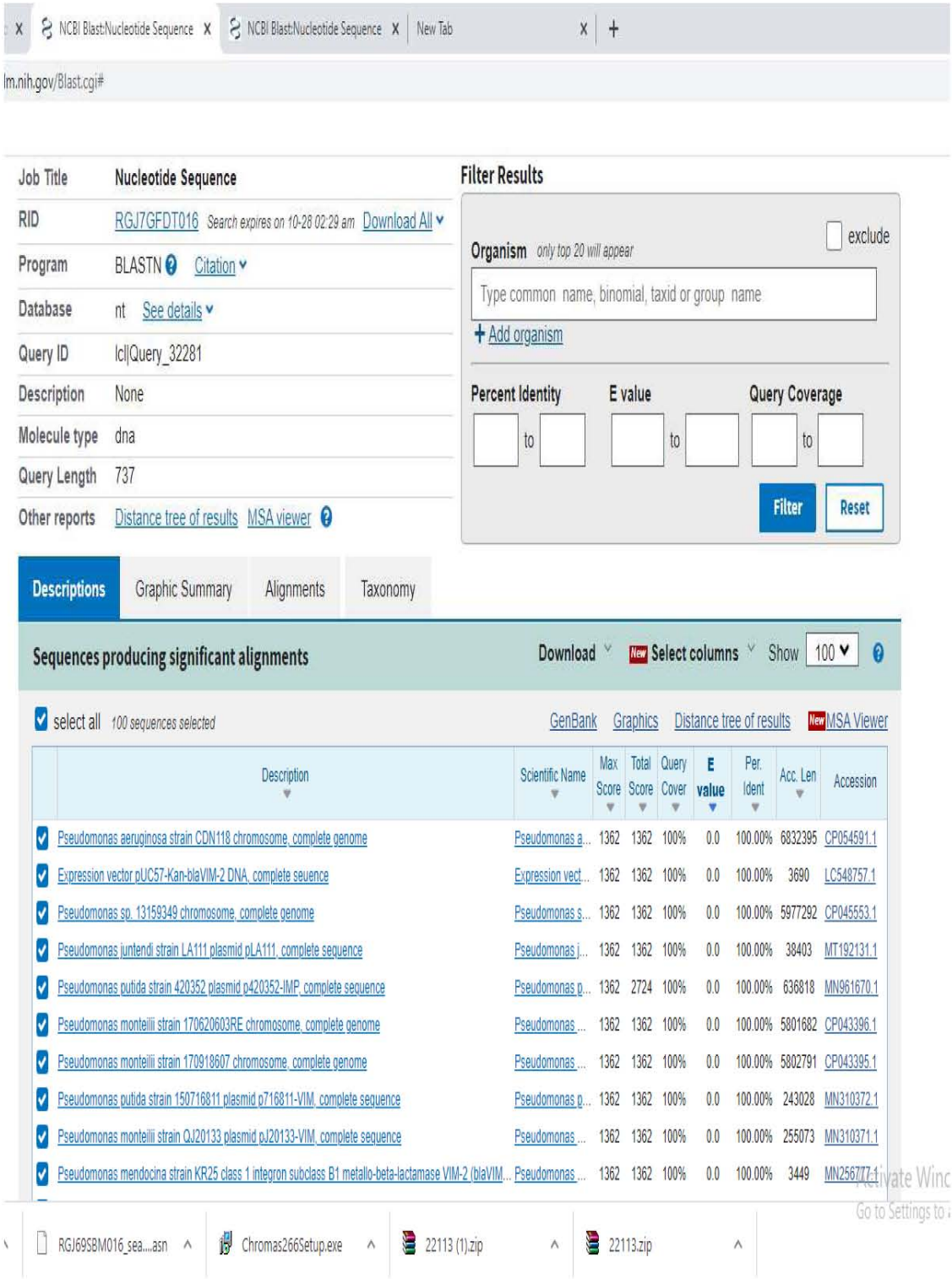


Figure 18. BLAST analysis result of VIM gene sequence

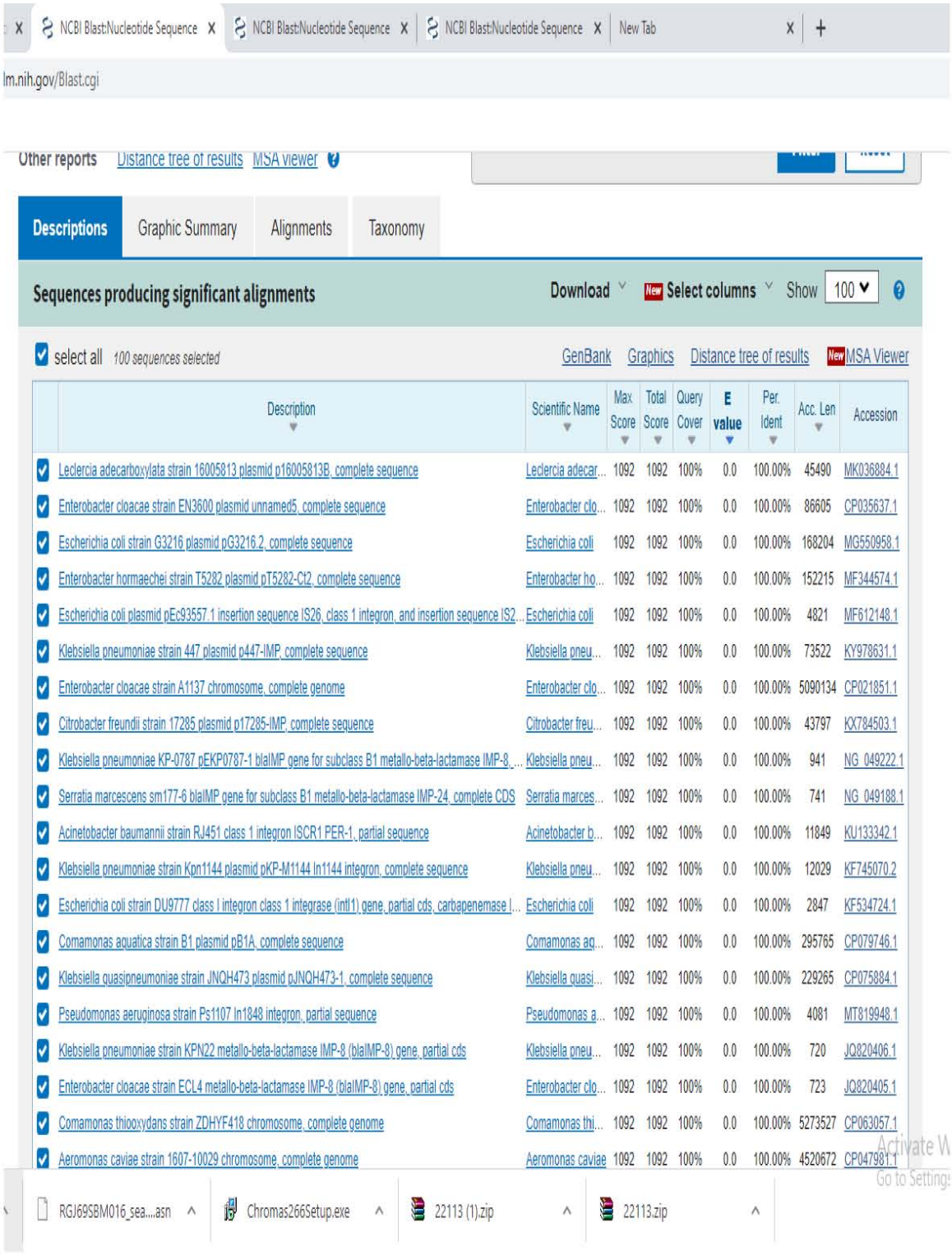


Figure 19. BLAST analysis result of IMP gene sequence

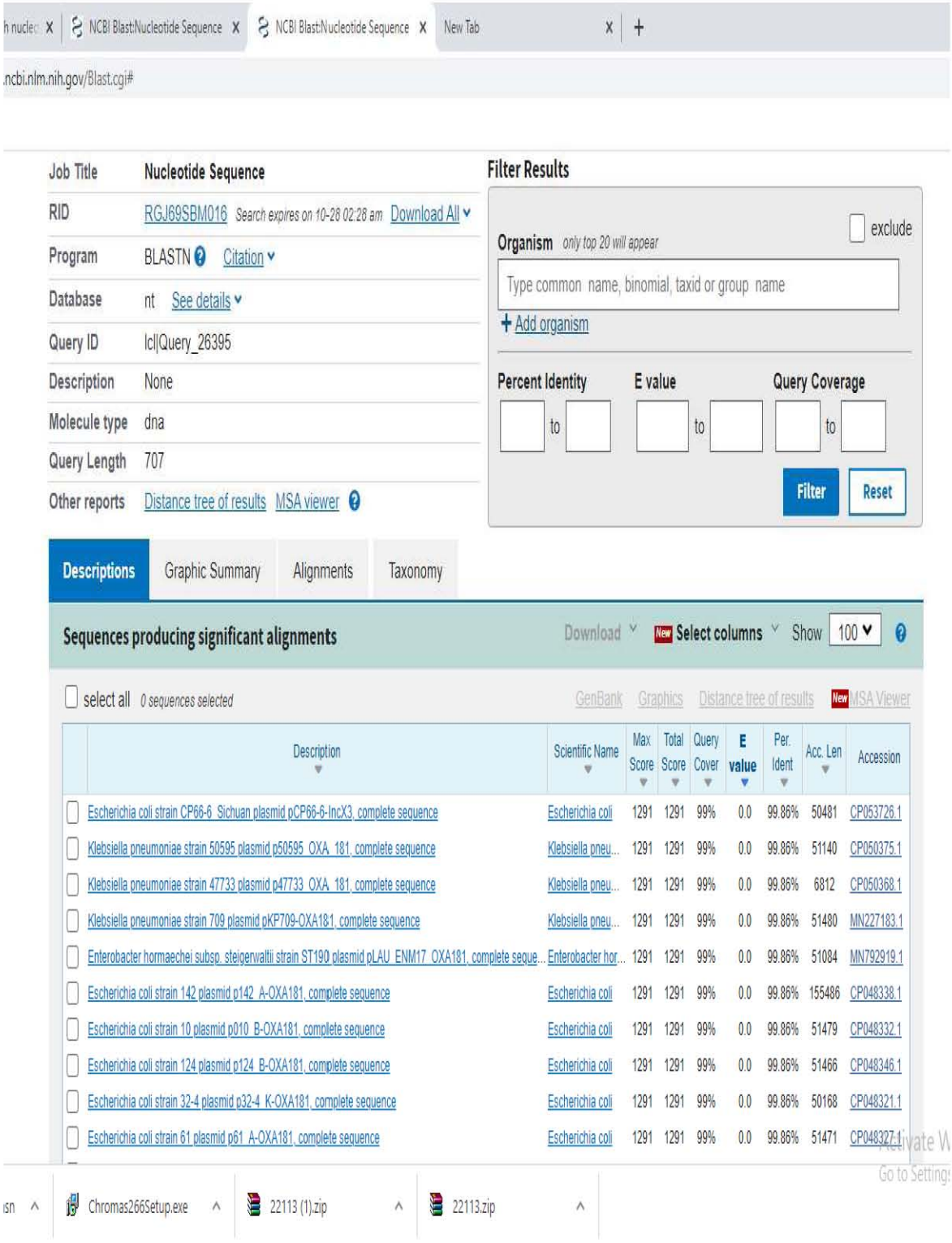


Figure 20. BLAST analysis result of Oxa-48 gene sequence

ranges from 65-175 ng/µl. The details of purity and concentration of purified RNA from each isolate is given in table 13.

4.8.2 Standardisation of PCR condition using gradient PCR

Each primer pair was tested at three different annealing temperatures (52°C, 60°C and 62°C) using gradient PCR to find the most suitable temperature (Figure 21, 22 and 23). The gradient PCR product was subjected to agarose gel electrophoresis. The most optimum temperature was ascertained, if bright intense specific amplification has occurred in absence of non-specific amplification. On the basis of gradient PCR results, it is found that all primer pair uniformly produce specific amplification at 60°C, hence this temperature was used for Q-PCR analysis of genes in further studies.

4.8.3. Relative expression of outer membrane porin gene

The transcriptional response of outer membrane porin genes *ompK35* & *ompK36* of *Klebsiella*, *ompF* & *ompC* of *Citrobacter and Enterobacter* and *E.coli* and *oprD* of *Pseudomonas sp.* was analysed under carbapenem stress and relative to the carbapenem susceptible isolates without carbapenem stress by Q-PCR analysis. The transcriptional expression of EC-*ompF* and EC *ompC* gene exhibited decreased activity of both these genes in all the *E. coli* isolates, when compared with *E. coli* ATCC 25922 (Figure 24 and 25). In order to determine whether the extent of down-regulation of EC-*ompF* and EC-*ompC* genes in any way related to the corresponding increase in MIC, Log2MIC values were co-related with Log2 mRNA fold change. The corresponding decrease in EC-*ompF* and EC *ompC* did not bear any significant role in decrease/increase in MIC value of Imipenam ($p>0.05$). Similarly all but one of the *Enterobacter* and *Cirobacter* isolates, decreased activity of OMP-F and OMP-C was observed (Figure 26). In Isolate VU-03 (*Citrobacter* spp.) increase activity of OMP-F was noticed. In case of *Klebsiella* isolates a corresponding increase in *ompK35* activity was noticed along with down regulation of *ompK36* gene when compared with *Klebsiella pneumoniae* ATCC BAA 1706 (Figure 27).

4.8.4 Relative expression of efflux pump gene

To detect the expression level of AcrAB-tolC system in *E. coli, Klebsiella, Citrobacter,* and *Enterobacter* isolates and MexAB system in *Pseudomonas sp.* with

Q-PCR analysis was performed after imipenem selection pressure. For normalization of Q-PCR result, the expression level of the efflux pump gene *acrA, acrB* and *mexB* gene under normal condition was used in carbapenem susceptible isolates. Over-expression of *acrA* and *acrB* gene was observed in all the isolates including *E. coli, Klebsiella, Citrobacter* and *Enterobacter* (Figure 28, 29, 30 and 31). It was observed that the increased transcriptional expression of *acrA* and *acrB* gene was directly co-related with the increased MIC to imipenem ($P < 0.001$) in all the *E. coli* isolates. In *Pseudomonas* isolate (VS-01) an increase activity of *mexB* gene with corresponding decrease in *oprD* gene was observed (Figure 32).

Table 13. Concentration and purity of purified RNA samples

Isolate No.	260/280	260/230	Concentration(ng/µl)
VS 01	2.15	1.70	62.5
VS 02	2.38	1.40	65.00
VS 39	2.20	2.00	54.5
VS 51	2.39	0.47	35.5
VA 19	1.89	1.60	121.5
VA 52	2.06	1.94	68.5
VA 53	1.96	1.94	62.5
VA 55	2.27	2.34	2.75
VA 66	1.99	1.45	85.3
VA 77	1.83	2.05	77.5
VA 99	1.95	1.43	132.8
VA 100	2.00	1.67	65.1
VU 02	1.96	1.62	62.95
VU 03	1.73	1.46	12.5
VU 08	1.87	1.22	40.5
VU 14	1.99	1.84	174.5
VU 16	1.60	1.14	47.5
VU 17	1.74	1.15	63.25
KPC 1705	1.55	1.47	24.8
KPC 1706	2.1	2.14	67.5

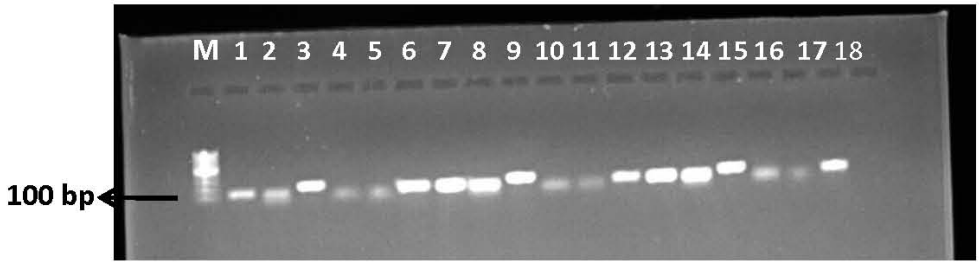


Fig 21: Figure showing gradient PCR standardization of *omp*K35, *omp*K36, *ompF* , *ompC*, *EC-ompF* and *EC-ompC* gene primers , Lane 1,7, 13: OMPK35 gene (124bp), Lane 2,8,14: OMP-F (139 bp), Lane 3,9,15: OMPK36(180 bp), Lane 4,10,16: OMP-C (113 bp), Lane 5, 11,17: EC OMPF (118 bp), Lane 6,12,18 EC OMPC (124 bp), Lanes M: 100 bp Plus DNA ladder

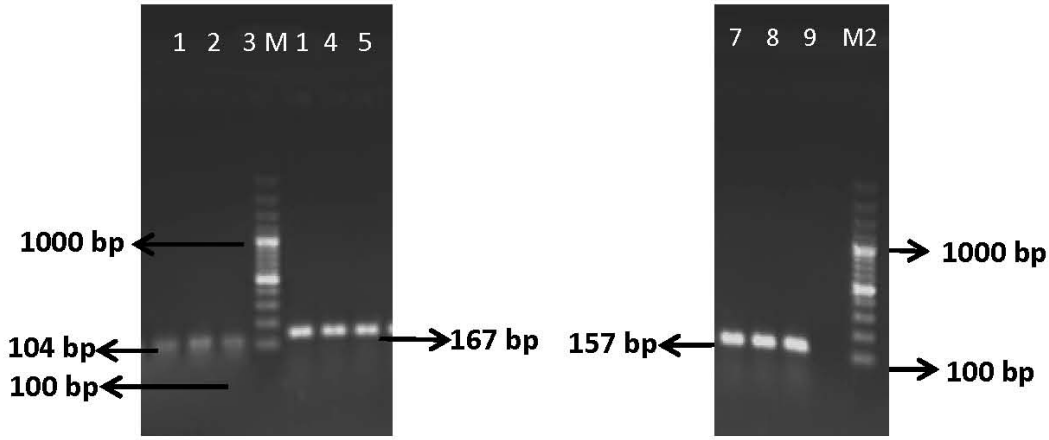


Fig 22: Figure showing gradient PCR standardization of *rspL*, *mexB* and *oprD* gene primers
Lane 1-3: rspL gene (104bp), Lane 4-6: MexB (167 bp), Lane 7-9: OprD gene(157bp), Lanes M1 & M2: 100 bp Plus DNA ladder

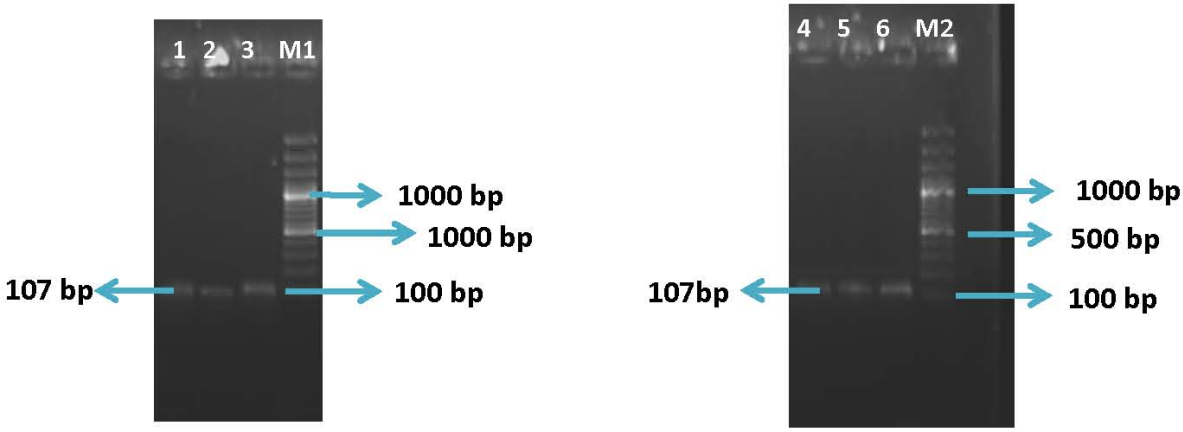
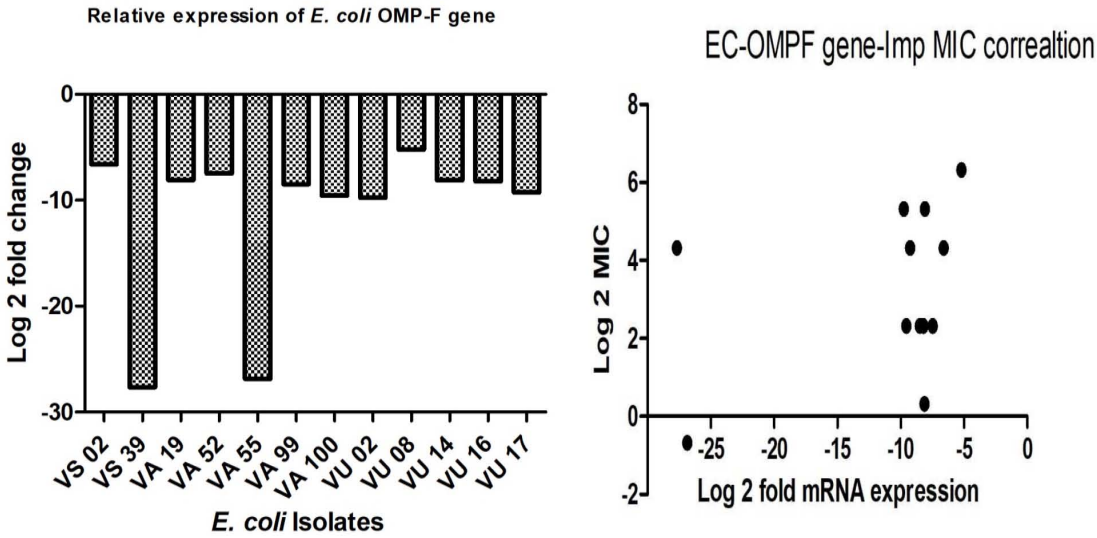
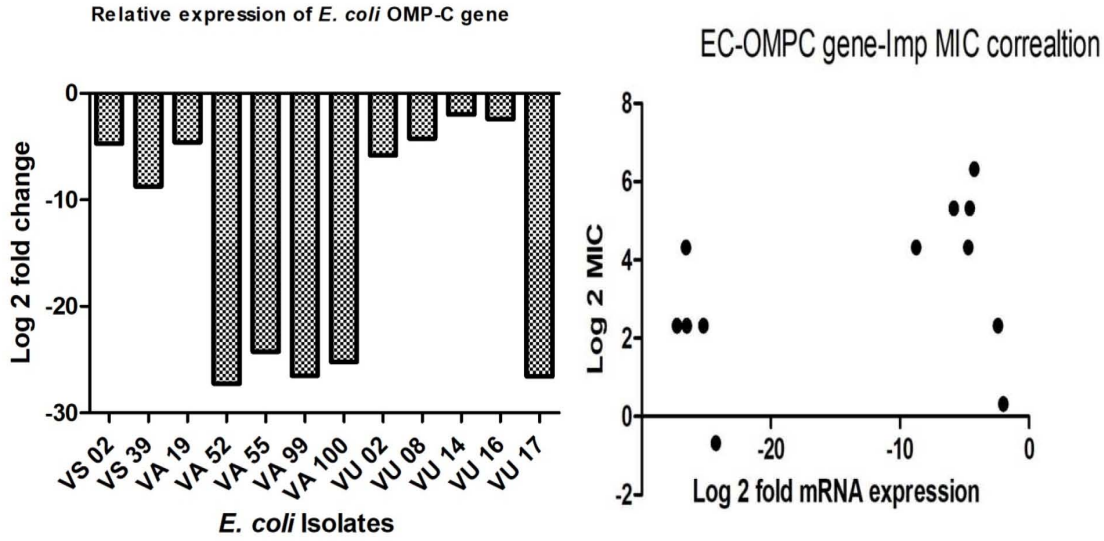


Fig 23: Figure showing gradient PCR standardization of *acrA* and *acrB* gene
Lane 1-3: AcrA gene (107bp), Lane: 4-6: AcrB gene (107bp), Lanes M1 & M2: 100 bp Plus DNA ladder



Correlation		A
		MIC (IMP)
		Y
1	Number of XY Pairs	12
2	Pearson r	0.3381
3	95% confidence interval	-0.2928 to 0.7638
4	P value (two-tailed)	0.2825
5	P value summary	ns
6	Is the correlation significant? (alpha=0.05)	No
7	R square	0.1143

Figure 24. Expression of *E. coli ompF* gene in CNSE isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. The Log 2 MIC value of Imepenem were correlated with Log2 fold expression of *E. coli ompF* mRNA. Down regulation of *E. coli ompF* gene was observed in all the isolates however, no correlation observed between MIC and down regulated gene expression.



Correlation		A
		MIC (IMP)
		Y
1	Number of XY Pairs	12
2	Pearson r	0.3767
3	95% confidence interval	-0.2517 to 0.7817
4	P value (two-tailed)	0.2274
5	P value summary	ns
6	Is the correlation significant? (alpha=0.05)	No
7	R square	0.1419

Figure 25. Expression of *E. coli ompC* gene in CNSE isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. The Log 2 MIC value of Imepenem were correlated with Log2 fold expression of *E. coli ompF* mRNA. Down regulation of *E. coli ompC* gene was observed in all the isolates however, no correlation observed between MIC and down regulated gene expression.

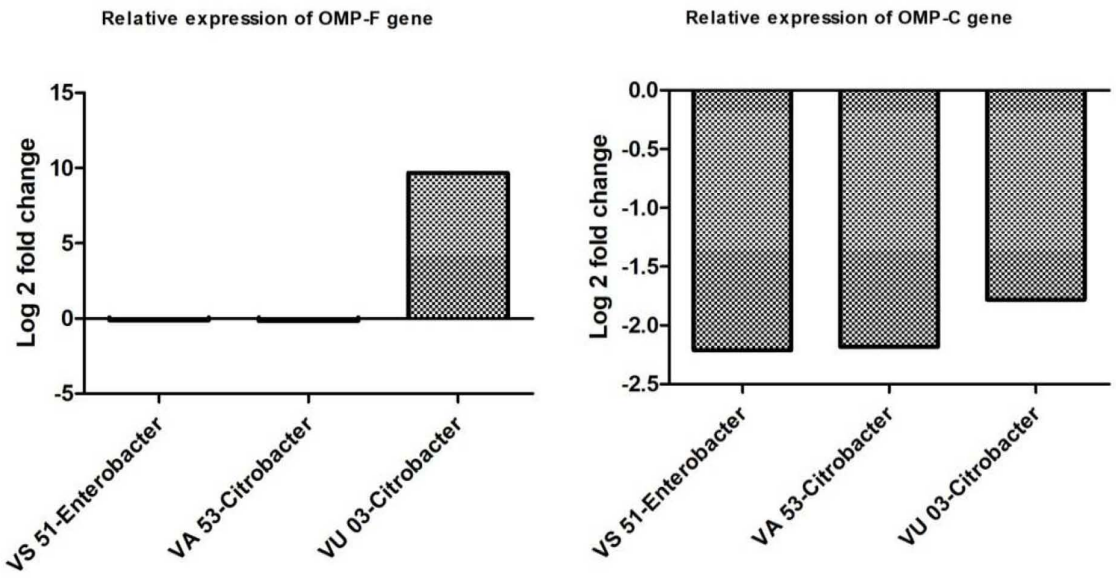


Figure 26. Expression of *ompF* and *ompC* gene in CNSEn and CNSC isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. Down regulation *ompF* and *ompC* gene was observed in all the isolates except VU-03 (Citrobacter) where *ompF* gene is upregulated.

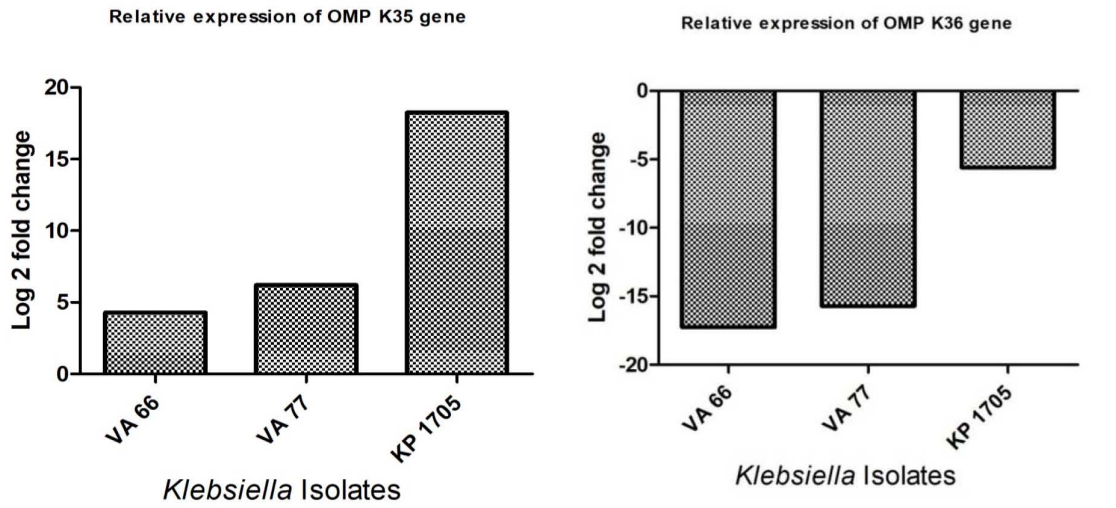
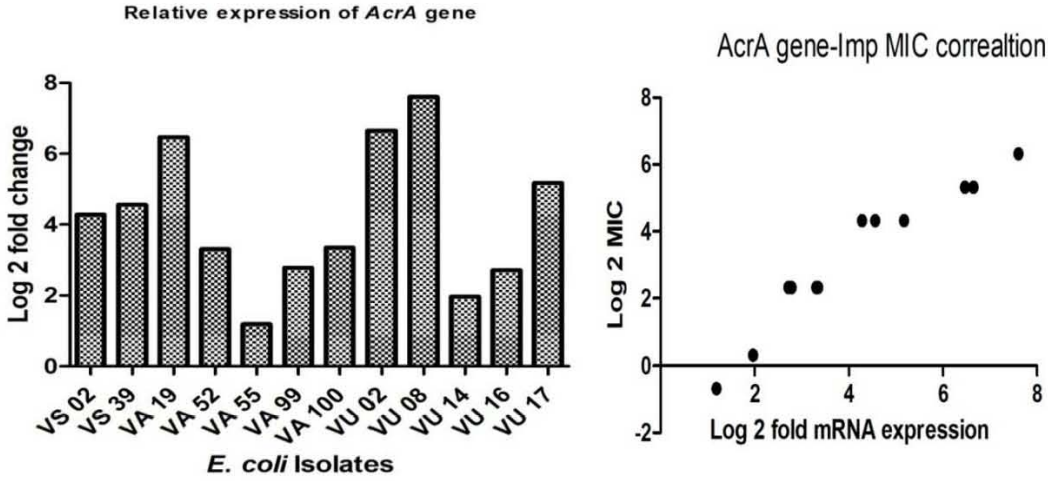
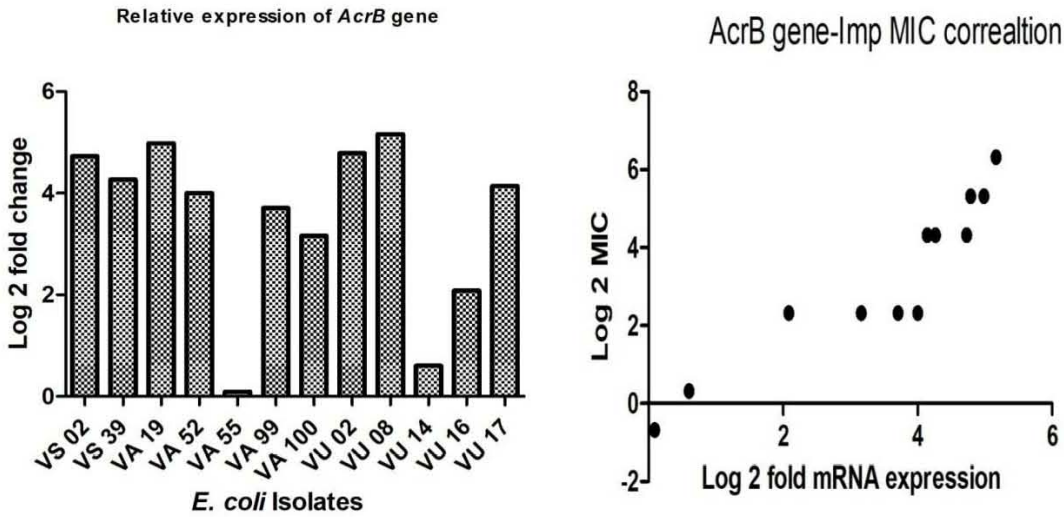


Figure 27. Expression of *ompK35* and *ompK36* gene in CNSK isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. Up regulation of *ompK35* and corresponding down regulation of *ompK36* gene was observed.



Correlation		A
		MIC (IMP)
		Y
1	Number of XY Pairs	12
2	Pearson r	0.9619
3	95% confidence interval	0.8662 to 0.9895
4	P value (two-tailed)	< 0.0001
5	P value summary	***
6	Is the correlation significant? (alpha=0.05)	Yes
7	R square	0.9253

Figure 28. Expression of *acrA* gene in CNSE isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. The Log 2 MIC value of Imepenem were correlated with Log2 fold expression of *acrA* mRNA. Upregulation of *acrA* gene was observed in all the isolate with positive correlation between MIC and upregulated gene expression.



Correlation		A
		MIC (IMP)
		Y
1	Number of XY Pairs	12
2	Pearson r	0.9294
3	95% confidence interval	0.7617 to 0.9804
4	P value (two-tailed)	< 0.0001
5	P value summary	***
6	Is the correlation significant? (alpha=0.05)	Yes
7	R square	0.8637

Figure 29. Expression of *acrB* gene in CNSE isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. The Log 2 MIC value of Imepenem were correlated with Log2 fold expression of *acrB* mRNA. Up regulation of *acrB* gene was observed in all the isolate with positive correlation between MIC and upregulated gene expression.

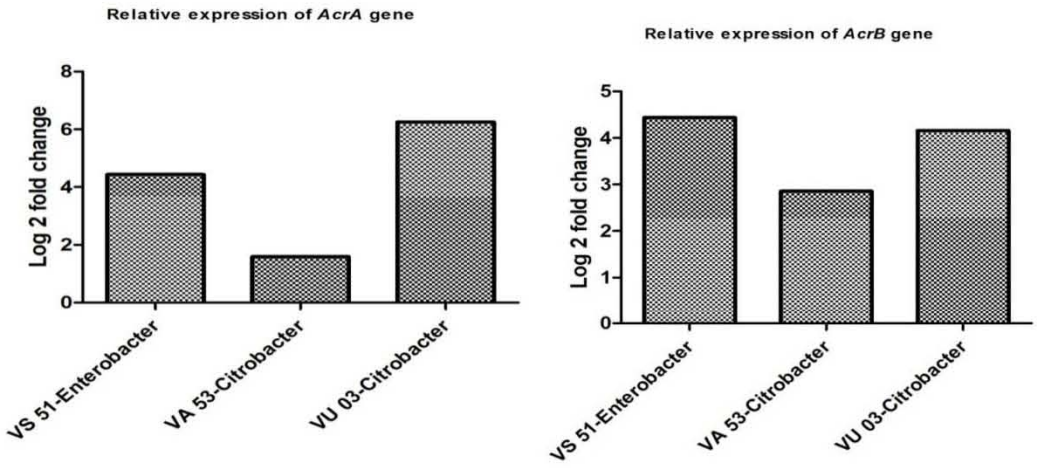


Figure 30. Expression of *acrA* and *acrB* gene in CNSEn and CNSC isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. Up regulation of *acrA* and *acrB* gene was observed in all the isolates.

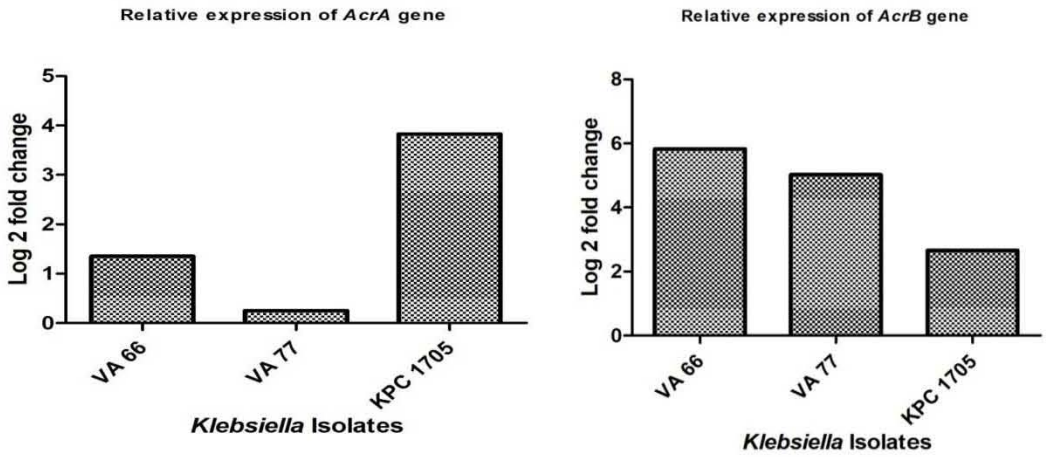


Figure 31. Expression of *acrA* and *acrB* gene in CNSK isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. Up regulation of *acrA* and *acrB* gene was observed in all the isolates.

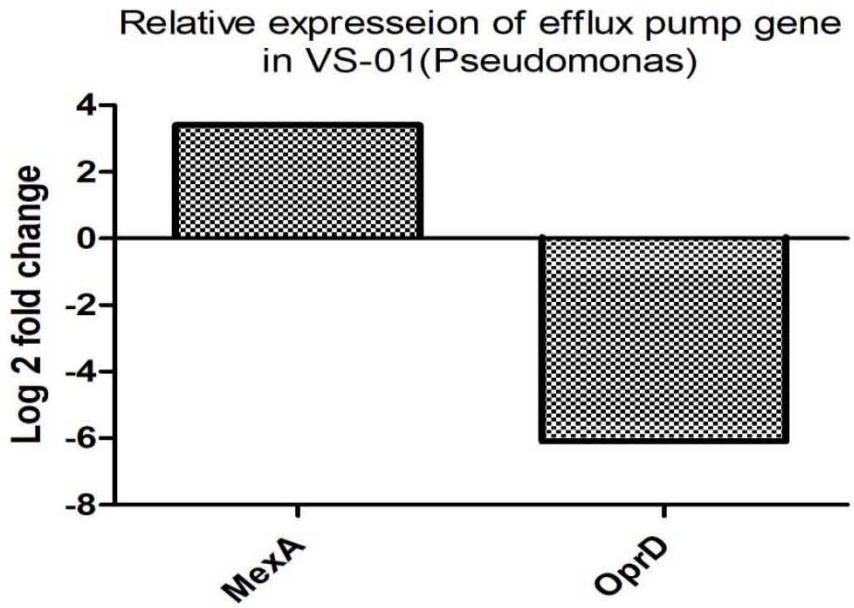


Figure 32. Expression of *mexB* and *oprD* gene in CNSP isolates. Relative changes in the expression of genes were quantified by real-time PCR by comparison with expression levels of the housekeeping gene *rspL* and expressed as the fold-change. Up regulation of *mexB* and Down regulation of *oprD* was observed.

Discussion

CHAPTER-5

DISCUSSION

Dissemination of multi-drug resistant bacteria is a major public health concern. Carbapenems which are considered drugs of last resort have been used quite effectively for decades. Carbapenem-resistant Enterobacteriaceae is a family of bacteria that are very difficult to treat because they are refractory to the application of carbapenems and other beta-lactam antibiotics. The increasing prevalence of CRE infections represents a major threat to human and animal health. CRE utilizes several different mechanisms to survive carbapenem selection pressure viz. porin channel, efflux pump, carbapenemase, AmpC beta-lactamase, mutation, etc. (Yong et al., 2009). The present study was undertaken to elucidate the role played by various factors which contribute and effectively translate into graded response against carbapenem antibiotics in isolates originated from livestock and companion animal sources.

The CRE isolates included in the present study belongs to genera *Pseudomonas, Enterobacter spp. Citrobacter spp., Klebsiella spp.* and *Escherichia coli*. Carbapenem resistance is known to be associated with several enterobacterial isolates recovered from livestock sources viz. *Enterobacter* spp. (Nandi et al., 2013), *E. coli* (Ghatak et al., 2013, Pruthvishree et al., 2017), *Morganella spp., Citrobacter spp., Proteus spp., Providencia* spp. (Mollenkopf et al., 2017), and *Salmonella spp.* (Singh et al., 2012, Naik et al., 2015). Widespread carbapenem resistance has been reported in *Escherichia coli* by various researchers (Ghatak et al., 2013, Pruthvishree et al., 2017, Ibrahim et al., 2016, Ojo et al., 2016). In a study conducted by Xu et al., (2015) based on available PubMed and Embase databases data searches from 2000 through 2012, the rank order of resistance rates to imipenem among Enterobacteriaceae genus was as follows: *Serratia* spp. (1.8%) > *Proteus* spp. (1.6%) > *Klebsiella* spp. (0.8%) = *Citrobacter* spp. (0.8%) > *Enterobacter* spp. (0.7%) > *E. coli* (0.2%).

Phenotypic detection tests like carba-NP and carbapenemase inhibition assay (CIA) could able to recognize twelve and ten isolates respectively as carbapenemase producers. Out of 12 isolates that were producing carbapenemase as observed by

carbaNP test, among them blaImp was detected in 7, blaVIM was detected in 4, blaOXA-48 in 3, whereas in 2 isolates PCR experiment could not fetch any amplification although they were Carba-NP test positive. It is worth mentioning that, one of the isolates is bla CTX-M while the other one is positive for both bla CTX-M and bla TEM. Bush and Jacoby (2010) stated that the well-known hydrolytic spectrum of ESBLs are extended to include expanded-spectrum cephalosporins among other agents (i.e., penicillins, cephalosporins, monobactams). Hence, hydrolysis of carbapenem in presence of extended-spectrum beta-lactamase needs further investigation. In a recent in vitro study ESBL production was associated with reduced susceptibility to carbapenems and higher frequency of emerging carbapenem-resistant subpopulations in *E. coli* (Adler et al., 2013). Whereas, in one isolate which is testes negative for both CarbaNP and CIA test negative, carbapenemase genes were detected using specific primers. Although the carba-NP test has been reported to be 100% sensitive and specific for Enterobacteriaceae, occasional false-negative results were also obtained in several isolates producing GES-5, SME-1, OXA-48-carbapenemase (Tijet et al., 2013). False-negative results were also obtained for isolates with VIMs or OXA-48s (Literacka et al., 2017).

We herein describe the presence of bla-OXA-181 from canine and feline sources. OXA-181, a derivative of OXA48 with the substitution of single amino acid, was first identified in India by Potron et al. (2011), and then has been spread to many different countries. Nigg et al. (2019) described the presence of carbapenemase gene bla OXA-181 in *Escherichia coli* isolates recovered from fecal samples in Switzerland. Sankar et al., 2021, described a high prevalence of carbapenemases among companion animals like dogs (NDM followed by OXA-181, KPC, OXA-48 and VIM).

Among 18 carbapenem non-susceptible isolates five isolates (32.9%) showed CCCP mediated enlargement of the inhibition zones around the IMP disc on the CCCP-IMP disc synergy test. A pattern of two-fold MIC reduction in IMP after adding CCCP was observed in three out of eighteen isolates only. CCCP as a protonophore that reversibly binds protons (H+) and transports them across the cell membrane, leading to membrane depolarization, eradication of the electrochemical concentration gradient (ECG), and reduced ATP production by ATP synthase (Spindler et al., 2011; Yu et al., 2015; Ni et al., 2016). CCCP has been reported to

reduce efflux activity in carbapenem-resistant Gram-negative bacteria such that resistance to carbapenem was reduced or reversed (Huang et al., 2008). However, in the present study, CCCP was not found very active in reversing the imipenem resistance significantly, while no effect was observed when ertapenem was used. Both commensals and pathogenic bacteria have different mechanisms of using their efflux pumps to remove amphipathic or lipophilic substances in and out of the cells. In an earlier study, it was reported that *Pseudomonas aeruginosa* efflux-pump overexpression occurs more regularly when meropenem is used when compared with imipenem. Osei and Amoaka reported no change in carbapenem MIC after adding CCCP except in one isolate (Osei, 2017). Perhaps future studies involving the use of peptidomimetic efflux-pump inhibitor (PaβN) in Gram-negative bacteria, may give better insight to study the in vivo effect of efflux pump inhibitor on carbapenem-resistance.

RND-type multidrug efflux pump AcrAB-TolC, which is a major contributor to intrinsic multidrug resistance in Enterobacteriaceae (Li et al., 2015). It was observed that the transcriptional expression of AcrA and AcrB was increased in all the isolates under study in response to imipenem stress. A quite similar response of AcrA was observed by Chetri et al., (2019). Efflux pumps such as AcrAB-TolC and MexAB-OprM, are essential for bacterial survival and colonization/virulence, especially during infection when the pathogen is attacked by toxic substances or adheres with the host (Zgurskaya and Nikaido, 2000). The AcrAB-TolC is a constitutive system in *Escherichia coli*, and largely has a role in characteristic intrinsic resistance to antimicrobials such as erythromycin and fusidic acid as well as dyes and detergents (Nikaido, 1996). Efflux pump activity was observed in imipenem resistant strains with over-expression of AcrA (Rosenberg et al., 2000).

We observed a strong positive correlation between the leval of MIC and the increased activity of AcrA and AcrB efflux pumps. Isolates with a higher level of expression of these genes also showed high MIC against imipenem. In a similar study conducted by Chetri et al. (2019) on carbapenem-resistant *E. coli*, a strong correlation between ertapenem resistance and AcrA over-expression was observed. However, efflux pump gene AcrB has also been reported to repress the expression of the porin OmpF gene (Davin-Regli et al., 2008). In this study carbapenem-resistant *E. coli* isolates were studied of which a decrease in the expression of OmpF and OmpC genes

were observed in AcrAB overexpressed isolates which is in agreement with previous studies (Koyano et al., 2013, Philippe et al., 2015, Jaskulski et al., 2013). Several studies have demonstrated that inactivation of the efflux pump AcrAB-TolC by the lack of one of its structural components directly affects the virulence of the bacteria, indicating that this system is required for the bacteria to be pathogenic (Martinez et al., 2009).

Bacterial outer membrane porins mediate the passive diffusion of antibiotics across the outer membrane, hence they are closely associated with antibiotic resistance in Gram-negative bacteria. Porin loss/mutation and increased efflux pump activity is major contributor to an innate resistance mechanism. Gram-negative pathogens, including *Escherichia coli*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, β-lactams, and fluoroquinolones were known to penetrate the OM through the non-specific porin OmpF (Mach et al., 2008; Delcour, 2009). OmpF and OmpC seem to be associated with the transport of carbapenem, which is consistent with previous results (Nikaido et al., 1983; Lou et al., 2011). Yigit et al. (2002), suggested that alteration of both the OmpF and OmpC porin analogs is primarily responsible for the imipenem-resistant phenotype of *E. aerogenes*.

Non-specific porins (OmpF, OmpA, and OmpC) play a distinct role in antibiotic resistance. In our study, we observed decreased expression of *OmpF and OmpC* in all *E. coli* isolates. Larkin and co-workers describe carbapenem resistance in *E. coli* isolated from urine and identified that ompC porin loss plays an important role in resistance (Larkin et al., 2020). Several researchers have highlighted that reduced expression of *ompF* and *ompC* in *E. coli* was frequent in resistant strains (Yoshida et al., 2006). Chetri et al., 2019. After, exposure to sub-inhibitory concentration of imipenem down-regulation of *ompC* gene was also observed which is in support of the present study. Previous studies have shown that a negative correlation with decrease *ompK35* and/or o*mpK36* activity, with increased carbapenem MICs. However, we could not observe any correlation with increased imipenem MIC in the isolates with the level of decreased activity of these porins. Similar observations were made by Netikul and coworkers, who demonstrated that a reduction of ompK35 expression did not show a significant difference among isolates resistant to carbapenems, and did not correlate with levels of increased MIC values of each carbapenem among these isolates (Netikul et al., 2015). Several specific porins are

associated with the passive transport of some antibiotics. However, most specific porins did not affect the MICs of the tested antibiotics (Choi and Lee, 2019). Most of the studies show showed various degrees of decreased expression of either the o*mpK35* or o*mpK36* gene or both in Klebsiella isolates resistant to carbapenems. However, contrary to general findings by another researcher we observed a generalized increase in *ompK35* activity in corresponding isolates where *ompK36* expression was declining.

Summary and Conclusions

CHAPTER-6

SUMMERY AND CONCLUSION

The present study was undertaken to elucidate the role played by various factors which contribute and effectively translate in to gradede response against carbapenam antibiotic in isolates originated from livestock and companioun animal sources. The CRE isolates maintained in the department were used for the detail molecular characterization. The isolates were identified by biochemical tests. The antibiotic sensitivity test was performed to evelauate its response against antibiotic of other classes. The phenotypic tests were performed to detect carbapenemsae activity which was further presence/absence of beta-lactamase gene was confirmed by PCR based analysis. Effect of efflux pump activity was evaluated by varius phenotypic tests in presence of selective efflux pump inhibitor. Lastely quantitative gene expression analysis was done to establish role of porin channel and efflucx pump in carbapenam resistance.

The CRE isolates included in present study, belongs to genera *Pseudomonas, Enterobacter spp. Citrobacter spp., Klebsiella spp.* and *Escherichia coli*. Phenotypic detection tests like carba-NP and carbapeneamse inhibition assay (CIA) could able to recognizes twelve and ten isolates respctively as carbapenemase producers. Out of 12 isolates that were producing carbapenemase as observed by carba NP test, among them blaImp was detected in 7, bla VIM was detected in 4, bla OXA-48 in 3, whereas in 2 isolates PCR experiment could not fetch any amplification although they were Carba NP test positive. It is worth mentionaing that, one of the isolates is bla CTX-M while the other one is positive for both bla CTX-M and bla TEM

Among 18 carbapenem non-susceptible isolates five isolates (32.9%) showed CCCP mediated enlargment of the inhibition zones around the IMP disc on the CCCP-IMP disc synergy test. A pattern of two-fold MIC reduction in IMP after adding CCCP was observed in three out of eighteen isolates only.

It was observed that the transcriptional expression of *acrA* and *acrB* was increased in all the isolates under study in response to imipenam stress. We observed a strong positive correlation between leval of MIC and increased activity of AcrA and

AcrB efflux pump. Isolates with higher level of expression of these genes also showed high MIC against imipenam.

In our study we observed decreased expression of *ompF* and *ompC* in all *E.coli* isolates. However, we could not observe any correlation with increased imipenam MIC in the isolates with level of decreased activity of these porins. A generalized increase in *ompK35* activity in corresponding *Klebsiella* isolates was observed, where *ompK36* expression was declining.

Based on the above results following conclusion can be drawn out:

1) Majority of isolates with Carbapeneam resistant phenotype and genotype are also multidrug resistance. This can be explained by co-selection and co-transmission of multiple drug resistant gene between the isolates mediated by Integrons.

2) Carba NP test and Carbapenemase inactivation assay (CIA) are cheap, eassy to perform and reliable phenotypic tests to identify Carbapenem resistant phenotypes.

3) Carbapenem resistant in some isolates (VA-66, VA-99, VU-08, VU-16 and VU-17) despite the absence of carbapenemase gene indicate importance of other contributory factors like decrease of porin activity and/or increase in efflux pump activity. Furthermore role of AmpC β-lactamse in carbapenem resistant in Carbapenemse negative phenotypes needs to be investigated in future.

4) Phenotypic tests to establish the role of efflux pump in carbapenem resistance does not provide clear-cut indication. Probably a more suitable choice of efflux pump inhibitor other than CCCP may have given better insight.

5) The isolates were harbouring metallo-beta-lactamase VIM, IMP and Oxa-48 in the genome. The co-selection of extended spectrum beta lactamase in some of the isolates explains the multiple drug resistant phenotypes of the isolates under study.

6) The sequence analysis of representative isolates reveled presence of VIM-2, IMP-8 and Oxa-181, which are widely prevalent in human infection in India.

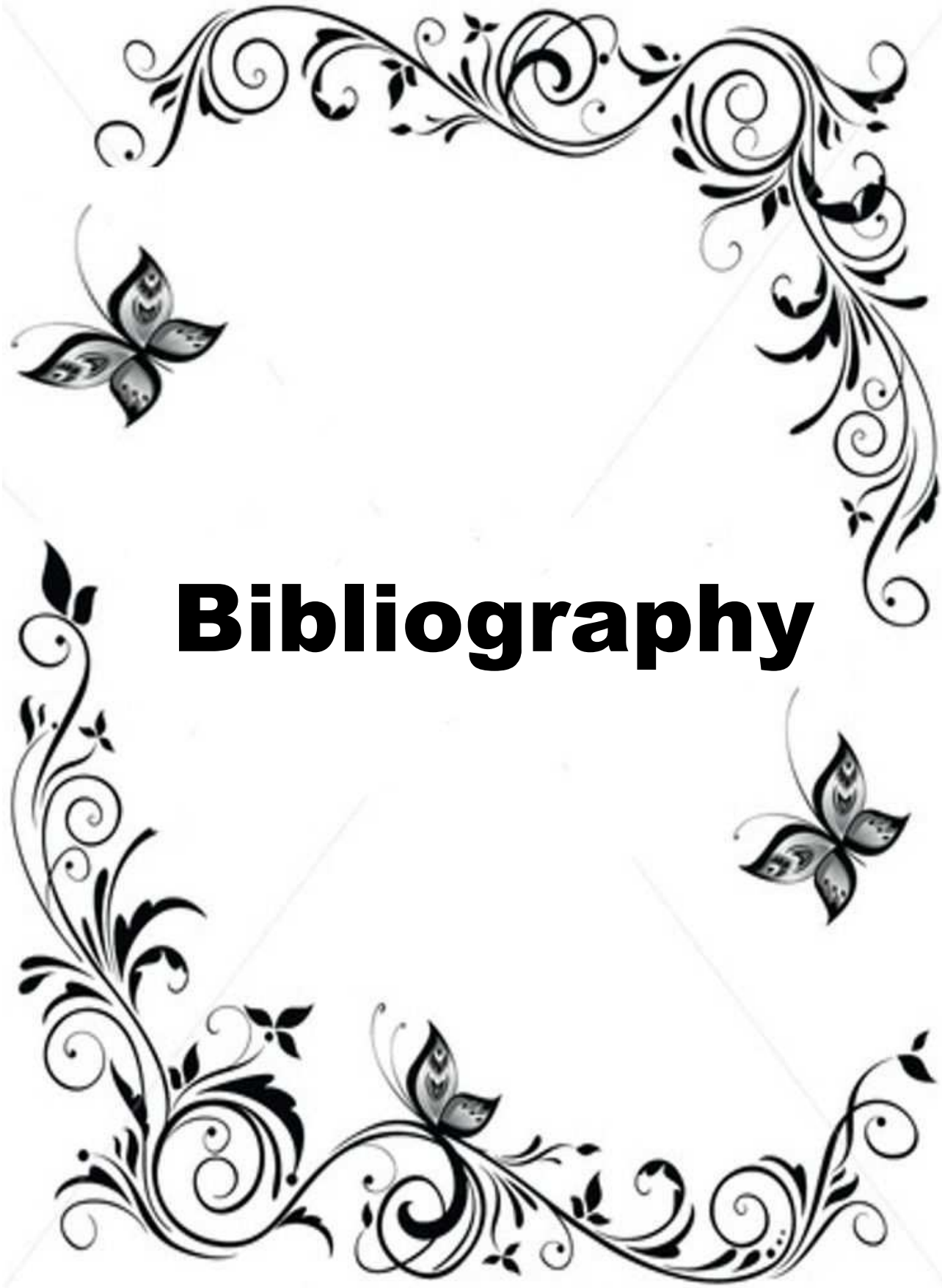
7) Decreased or loss of expression of Outer membrane porins plays an important role in carbapenam resistant in gram negative bacilli (GNB) . However, a clear-cut correlation between degree of loss of porins and MIC levels of isolates could

not be established which underlines role of other factors.

8) The loss of OMPK-36 porins in Klebsiella isolates with corresponding rise in OMP-K35 highlights importance of OMP-K36 in resistance in Klebsiella isolates with reference to carbapenem group of antibiotics.

9) In Enterobacteriace AcrAB-TolC efflux pump plays an important role in carbapenem resistant. Increase activity of AcrAB-TolC efflux pump is directly correlated with increased level of resistance to Imipenam .

10) Imipenem resistance in *Pseudomonas* species is associated with loss of porin OprD, and increase transcription of *mexB* efflux pump gene.



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REAGENTS USED FOR BACTERIOLOGICAL MEDIA

1. Buffer peptone water:

	Grams/Litre
Peptone	10.0
Sodium chloride	5.0
Disodium phosphate	3.5
Potassium dihydrogen phosphate	1.5

pH 7.2 ± 0.2 @ 25°C

2. Glucose phosphate peptone (GPP) water

Peptone	0.5 g
Di- potassium hydrogen phosphate	100 ml

3. Mac Conkey Agar:

Ingredients	**Grams/Litre**
Peptone	20.0
Lactose	10.0
Bile salts	5.0
Sodium chloride	5.0
Neutral red	0.075
Agar	12.0

Final pH 7.4 +/- 0.2 at 25°C

4. EMB Agar

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	10.000
Dipotassium phosphate	2.000
Lactose	5.000
Sucrose	5.000
Eosin - Y	0.400
Methylene blue	0.065
Agar	13.500

5. BHI Agar (Brain Heart Infusion Agar)

Ingredients	**Gms / Litre**
HM infusion powder	12.500
BHI powder	5.000
Proteose peptone	10.000
Dextrose (Glucose)	2.000
Sodium chloride	5.000
Disodium phosphate	2.500
Agar	15.000
Final pH (at 25°C)	

REAGENTS USED FOR BACTERIOLOGICAL STAINING

Ammonium oxalate	0.8 gm
Dissolve into 80.0 ml distilled water	
Crystal violet	2.0 gm
Dissolve into 20.0 ml of 95% ethyl alcohol.	

1. Crystal violet

- Mix the two solutions together and allow them to stand overnight at room temperature (25°C).
- Filter through coarse filter paper before use.
- Store at room temperature (25°C).

2. Gram's iodine

Iodine (crystalline)	1.0 g
Potassium iodide	2.0 g
Grind both well with mortar	
Distilled water	300.0 ml

Store at room temperature (25°C)

Covered the bottle with foil- (to protect solution from light).

3. Decolorizer

Ethyl alcohol (95%)	50 ml
Acetone	50 ml

4. Preparation of Safranin

1. Safranin-O	2.5 g

Dissolve into 100.0 ml 95% ethyl alcohol.

Safranin (from step 1)	10.0 ml
Distilled water.90.0 ml	

Store at room temperature (25°C).

REAGENTS USED FOR PHENOTYPIC DETECTION OF CARBAPENEMASE PRODUCTION

1. Carba NP test reagents:

Solution A

Phenol red (0.5%)	50gm
Distilled water	10ml
$ZnSO_4.7H_2O$ (10mM)	28.8 mg
Distilled water	10ml
NaOH (0.1N)	40 mg
Distilled water	10ml

Solution B

Solution A and Imipenam (6 mg/ml)

2. Normal saline (0.9%)

NaCl	0.9gm
Distilled water	100 ml

REAGENTS FOR AGAROSE GEL ELECTROPHORESIS

1. TE buffer (100x)

Tris base	157.6 gm
0.5 M EDTA (pH 8.0)	37.6 gm

2. Tris-acetate- EDTA (TAE) buffer (10X)

Tris base	48.4 gm
Glacial acetic acid	11.42 ml
0.5 M EDTA (pH 8.0)	7.44 gm
Distilled water was added to make the final volume	1000 ml

A working solution of 1X was used.

3. Tris-boric- EDTA (TBE) buffer (10X)

Tris base	108 gm
Boric acid	55 gm
0.5 M EDTA (pH 8.0)	40 ml
Distilled water was added to make the final volume	1000 ml

4. Ethidium bromide stock solution (10mg/ml)

- Ethidium bromide (100mg)
- Distilled water (10ml)
- The solution was mixed and stored at 4ºC. A concentration of 0.5 µg/ml was used in preparing agarose gel.

5. DNA ladder marker (Working solution)

- DNA ladder marker 1 part
- 6X loading dye 1 part
- Nuclease free water 4 part

CV OF STUDENT

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Place: mathura

Usha 08/11/21

Signature

UNDERTAKING OF COPY RIGHT

I **Dr. Usha Jaiswal,** Enrolment No **V-2116/19** undertake that I give copy right to the DUVASU, Mathura of my thesis entitled **"Molecular characterization of Carbapenem - nonsusceptible Enterobacterial isolates of animal origin".**

I also undertake that patent, if any, arising out of research work conducted during the programme shall be filed by me only with due permission of the competent authority of DUVASU, Mathura (UP).

Usha 20/11/21

Signature of Student