STUDY OF COLISTIN SENSITIVITY AMONG CLINICAL ISOLATES OF EXTENSIVELY DRUG RESISTANT GRAM NEGATIVE BACILLI

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University

In partial fulfillment of the regulations

For the award of the degree of

M.D. MICROBIOLOGY

Branch - IV

DEPARTMENT OF MICROBIOLOGY
PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH,
PEELAMEDU, COIMBATORE, TAMILNADU, INDIA

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CERTIFICATE

PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH

COIMBATORE

CERTIFICATE

This is to certify that the dissertation work entitled **"study of colistin sensitivity among clinical isolates of extensively drug resistant gram negative bacilli"** Submitted by **Dr. T.kalaiselvi** and this work were done by her during the period of study in this department from March2015 to May 2016. This work was done under direct guidance of **Dr. J.Jayalakshmi,** Professor, Department of Microbiology,PSG IMS& R.

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T.Kalaiselvi.

TABLE OF CONTENTS

TABLE OF CONTENTS: **PAGE NO:**

INTRODUCTION

INTRODUCTION:

Colistin belongs to the group of polymyxinswith the bactericidal activity and wide spectrum of activity against most strains of Enterobacteriaceae, *Acinetobacter spp* and *Pseudomonas aeruginosa*. Therapeutic usage of parenteral administration of colistin was less minimal due to concerns about the high incidence of side effects, particularly nephrotoxicity. Recent increase in the multidrug resistant (MDR) and extensively drug resistant (XDR) strains of Gram-negative bacilli, predominantly*P. aeruginosa,E.coliKlebsiella spp,Enterobacter spp* and *Acinetobacter* spp., hadprovoked and renewed interest in the usage of colistin as a last resort drug in patients who are critically ill.[1]Extensive antibiotic resistance has been developed in Gram-negative bacteria, due to both innate resistance in some species and the fact is, that they are highly adaptive in acquiring antibiotic-resistant determinants from each other. Resistant pattern increases right from beta lactamase, aminoglycosides, quinolones and carbapenems. Now in most of the cases, colistin is used as a last high end antibiotic option used as a therapeutic option in bloodstream infections.

Due to over usage of colistin there is emergence of resistant development to this last life saving agent .[2]Resistant to colistin is due to the modification in the lipopolysaccharide the main site of colistin action.[3]Recently plasmid mediated resistance has been identified. MCR-1 gene, a plasmid mediated resistant gene has been reported [4]. This study plans to identify the increasing MIC for colistin by reference methods agardilution method and microbroth dilution method,and to detect the genes responsible for colistin resistance .

AIMS AND OBJECTIVES

AIM:

To know the sensitivity of colistin against extensively drug resistant (XDR) gram negative clinical isolatesin tertiary care hospital.

OBJECTIVES:

- To determine the Minimum inhibitory concentration(MIC)of colistin against extensively drug resistant gram negative bacill by Broth dilution method(gold standard) ,agar dilution method.
- To compare various phenotypic detection methods for colistin resistance
- To look for increasing MIC values in extensively drug resistant gram negative bacilli.
- To detect MCR-1 gene responsible for colistin resistance in various clinical isolates by molecular method.

REVIEW OF LITERATURE

REVIEW OF LITERATURE:

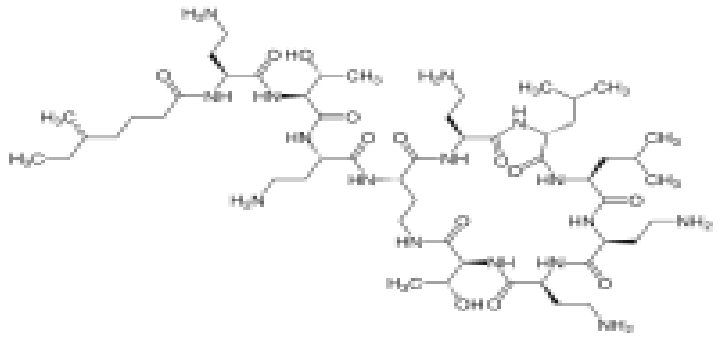
DISCOVERY AND HISTORY OF COLISTIN:

Polymyxin belongs to the classification of cyclic polypeptide group of antimicrobials .It was discovered in 1947 from the bacteria *paenibacillus polymyxa*.Polymyxins have five different compounds consist of polymyxin A-E .Polymyxin B and polymyxin E(colistin) are in clinical use colistin belongs to Polymyxin E group. .Polymyxin B differs from colistin by single amino acid .Isolation of the drug colistin was originally from *Bacillus polymymyxa var colistinus*which is aflask of fermenting bacteria by koyama Y in Japan in 1949[5].usage of colistin was formerlyseen in Japan and later ineurope during 1950's ,followed by united states. Availability of colistin as intravenous formulations as colistimethate sodium for treatment purposes came into use during the period of 1959 .[6] Colistin was used as treatment option in1960-1970 for gram-negative infections;but later in 1970's its usage was largely replaced with aminoglycosides and declined due to its toxicities.In1990-2000, for MDR gram-negative pathogens associated with lung infections the mainstay of treatment was colistin .From 2000 onwards colistin was used for treating treat healthcare associated infection caused by MDR gram negative infection.[7]Inhaled colistin was proposed as an alternative or adjunction for the treatment of lung infections with MDR gram- negative bacteria.[8]

CHEMICAL STRUCTURE OF COLISTIN:

Colistin(PolymyxinE1) is a multicomponent polypeptide antibiotic .It is a secondary metabolite non ribosomal peptides . Primary fatty acid structure with, γ-diaminobutyric acid(Dab), threonine(thr) ,leucine(Leu) shown in **figure(1)** .Mainly composed of colistin A(polymyxin E1)which is acylated by (s)-6-methyloctanoic acid and colistin B (polymyxin E2)) which is acylated by (s)-6-methylheptanoic acid. A single molecule witha lipophilic fatty acid chain andhas a cationic polypeptide ring. The common differences between polymyxin E(colistin) and polymyxin B are shown in **table 1**.

Fig-1 Representing the Chemical structure of colistin



N-(4-amino-1-(1-(4-amino-1-oxo-1-(3,12,23-tris(2-aminoethyl)-20-(1-hydroxyethyl)-6,9-diisobutyl-2,5,8,11,14,19,22-heptaoxo-1,4,7,10,13,18-hexaazacyclotricosan-15-ylamino)butan-2-ylamino)-3-hydroxybutan-2-ylamino)-1-oxobutan-2-yl)-*N*,5-dimethylheptanamide

Table 1: showing differences between polymyxin B and colistin(polymyxinE)

	Colistin	Polymyxin B
CLASSIFICATION OF DRUG	Belongs to group of Polymyxin E	Belongs to group of Polymyxin B
CHEMICAL STRUCTURE	6methyloctanoic acid with residue phenyalanine	6methyloctanoic acid with residue leucine
ADMINISTRATION OF DRUG	Prodrug colistimethate sulphonate is administered	Active drug polymyxin B is given
MECHANISM OF ACTION	Acts on the outermembrane of bacterial cell wall	Similar to action of colistin
SPECTRUM OF ACTIVITY	Active against gram negative infections	Active against gram negative infections
ROUTE OF CLEARENCE	Invitro prodrug form changes to active form and gets excreted in urine.[9]	Active form gets excreted in urine
TOXICITY	Nephrotoxicity is high.[10]	Nephrotoxicity is low

AVAILABLE FORMS OF COLISTIN:

Colistin sulphate and colistimethate sodium(CMS) also called as,(sodium colistin methane sulphate,colistin methane sulphonate) are the two available forms of colistin. Colistin sulphate is commonly used as oral,topical and inhalational forms chemically it is polycationic.It is more stable.colistimethate sodium which is chemically modified is formed by the reaction of formaldehyde with colistin and sodium bisufite,in addition of a sulfomethyl group to primary amines of colistin.[12]It is polyanionic.CMS is administered parenterally,it is commonlyused because it is less toxic when compared to colistin sulphate.CMS is available in powder form .It should be reconstituted before administration.They label the content by international unit IU,(they contain0.5,1,or 2 million international units(MIU) per vialrespectively,there arefew brand names such as

(xylistin ,coly-monas,colomycin) . For colistin base activity (coly-Mycin M) the contents are labelled in (milligram)mg of colistin base activity (150 mg per vial).The dose content per vial may vary from different brands and description /prescription,so the clinicians must be careful about this product and they should be very much clear in dosing the colistin base activity or the CMS and the units are milligram(mg) or International units.[13]

1mg of colistimethate sodium=12,500 IU=0.375 mg of colistin base activity.[14]

1mg of colistin base activity =32,500 IU.=2.6 mg of colistimethate sodium

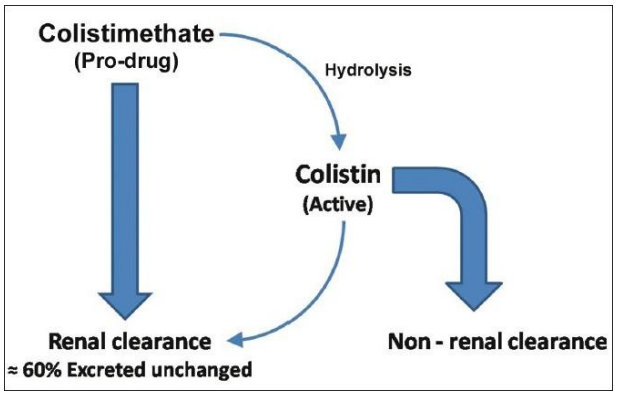
DOSAGE OF COLISTIN:

Dosage of intravenous colistimethate sodium as suggested by the manufacturers of united states they are given in two or four divided doses (31,250-62,500 IU/Kg)2.5-5mg/kg per day. Dosage adjustments are required for the patients when creatinine level is1.6 or >2.6mg/dl, mild to moderate renal dysfunction .In case of serious infections the dosage adjustment of intravenous colistin is 2 million international units every 12,24,36 hours respectively.[15]Dosage recommendations by manufacturers in UK is (50,000 -75,000 IU /kg) For adults and children with body weight of less than or equal to 60kg and the dosageis 4-6mg/kg per day into three divided doses .The dosage administered (1-2 million IU) 80-160mg every 8hours are recommended in patient'of more than 60kg s. Both colistin sulphate and colisti sulphate are available in inhalational forms. When colistin is administered by inhalational route, the dose that is recommended by manufacturers of UK is 40mg(50,000 IU) every 12 hours for the patients with body weight of less than 40 kg and patient's body weight of more than 40 kilogram the recommended dose is 80mg (1 million IU) every 12hours .[16] Colistimethate sodium is generally admistered for 10-14 days.Doses are commonly adjusted depending upon the renal function.CMS generally has a 12,500 IU/mg as a drug potency,where as pure colistin base the drug potency is of 30,000 IU/mg.so colistin sulphate has a high drug potency rate when compared to colistimethate sodium.Modifications of the daily dosing regimen may vary in total only in the case of patient's in renal impairment and the dosage are followed as guided by the manufacturer's instructions(coly-mycin2002)

PHARMACOKINETICS OF COLISTIN:

Colistin sulphate has an active antimicrobial activity.Half life for colistin sulphate is 4 hours .It is highly stable. Colisti methate sodium(CMS) is an non active pro drug(**fig2**).Half life for colisti methate sodium is 2 hours. CMS not being constant both in vitro and vivo because it is hydrolysed by chemical derivatives.pharmacokinetic ,pharmacodynamic properties of colistin that have been reported in the old studies were mostly used for measuring the concentration of the drug and the derivatives of the drug.[17]colistin gets tightly bounded to the lipidmembrane of cells of many body tissues including lung,liver , brain, muscles and heart .[18].Both colisti methate sodium and the colistin sulphate exhibit their bactericidal activity in a dependent concentration .Recent studies in meningitis caused by multi drug resistant *Acinetobacter baumanii* where it was susceptible only to colistin,and it was treated successfully by administering with intravenous colistimethate sodium.It was found that the penetration of colistin in cerebrospinal fluid was 25% of serum concentration reached the sustained level of bactericidal concentration.[19]About 60% of colistimethate sodium are being excreted as a unchanged drug in urine during the first 24 hour after dosing. The mode of excretion primarly is mainly through the glomerular filtration. No biliary excretion is reported in humans.

FIG:2 Colistimethate prodrug,colistinsulphate is an active drug



TOXICITY AND ADVERSE EFFECTS:

Two types of toxicity have been reported with usage of colistin ,nephrotoxicity and neurotoxicity. On Intra venous administration of colistin,Harmfulness is mainly dose dependent and it is rescindable on discontinuation of treatment .Though strict molecular mechanism of toxicity is not known, the polymyxins were highly known to cause nephrotoxicity.[20]Fatty acid and D-amino acid contents that are the components present in the colistin drug causes renal toxicity which in turn leads to acute tubular necrosis. On treatment with colistin it has a significant risk particularly in age group of more than 60 years it is known to cause drug induced nephrotoxicity.[21] Aerosol inhalation therapy of polymyxin E is known to tolerate with adverse effects like cough, bronchospasm ,it is due to the presence of excipients or preservatives.In Early period while experience in use of colistin revealed a high toxicity mainly

nephrotoxicity.[22]Now in recent data the studies designate that the colistin drug related toxicity, nephrotoxicity found to be less.Mainly two studies conducted exclusively among patients admitted in intensive care unit who received 3million IU of colistin administered intravenously every 8th hourly,the incidence of nephrotoxicity were 18.6%, and 14.3% respectively.[23]Excessive daily dosing will lead to more frequent development of nephrotoxicity.so there must be close monitoring of renal parameter.

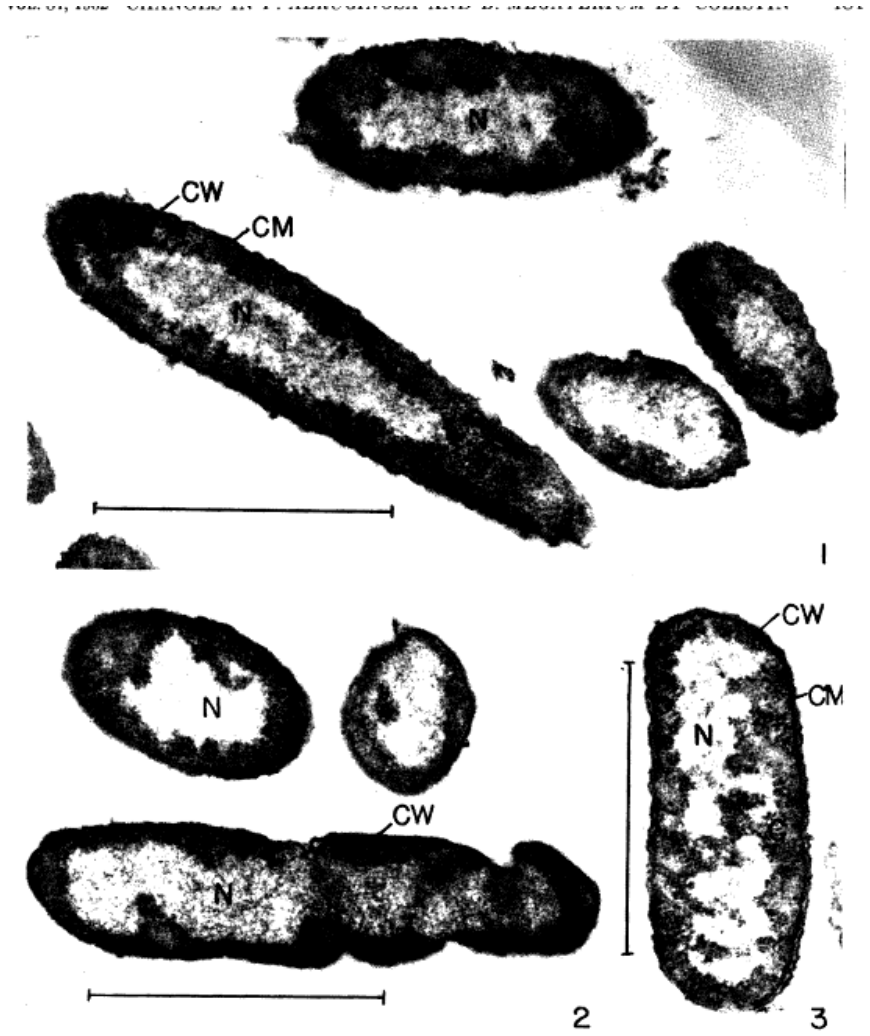
MECHANISM OF ACTION:

Initial site of mechanism of action of colistin is mainly on the bacterial cell membrane.It mainly acts on gram negative bacteria. It acts on the lipopolysaccharide(LPS)molecules present in the outer membrane of gram negative bacteria cellwall where the cationic and anionic polypeptide interacts each other by the electrostatic interactions,the colistin drug gets binded , leading to derangement of the cell membrane.colistin competitively displaces the divalent cations such as magnesium (mg+2)and calcium(ca+2)ions from the phosphate groups of membrane lipids which normally function as a stabilizing agent for the lipopolysaccharide molecules.As a consequence of this process the permeability towards the cell envelope gets increased and results in disruption of cellwall outer membrane leading to leakage of intracellular contents and consequently resulting in bacterial cell death. There are numerous projections appearing on the gram negative bacterial cell wall on exposing to colistin are shown in the electronmicroscope**(fig3a).**It shows

that the bacterial cytoplasmic membrane gets incompletely impaired and a part of the cytoplasmic material gets released in forms of fibrous material through the cracks present in the cell wall.[24]Polymyxin helps in preventing the lethal endotoxic activity of the bacterial lipopolysaccharide.In1962 chapman demonstrated the cytological changes on exposure of *E.coli* and *pseudomonasaeruginos*a on exposure to colistinsulphate. Broth cultures of *Pseudomonas aeruginosa* and *E.coli* were been exposed to the antibiotic colistin sulfate. Control (unexposed) and the exposed cells were fixed, dehydrated, and embedded in methacrylate. Ultrathin sections were examined in an RCA EMU2-D electron microscope. Two conspicuous cytological changes were noted in *E.coli* and *P.aeruginosa*.[25] First it was found to be that thereremained no longer demonstrable of nuclear material in its normal sites, leaving an empty space, and next the cytoplasm lost its granularity, and became homogeneous. cells which showed these morphological changes were found to be nonviable **(fig3b).**

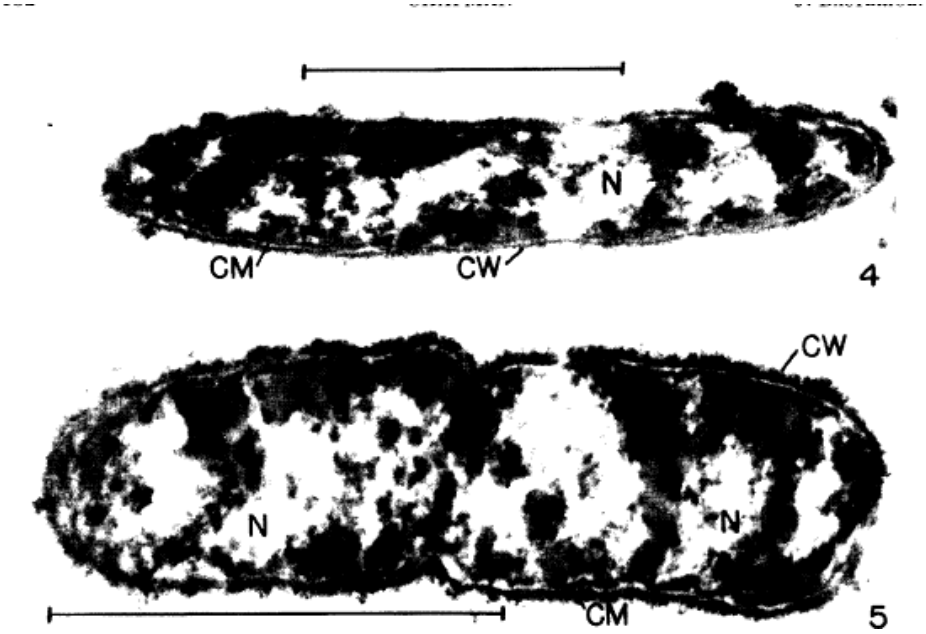
FIG 3a&3b: Shows Morphological changes of *E.coli* and *pseudomonas aeruginosa* before unexposure and after exposure to colistin sulphate under Electron microscope.

Fig 3a:Unexposed cell control showing no changes



Cell wall CW,Cytoplasmic membraneCM,Nucleus

Fig3b :Appearance of control cells after exposure to colistin sulphate the nucleus area appears to be empty and the cytoplasm has lost its cellularity



SPECTRUM OF ACTIVITY OF COLISTIN:

Colistin is mostly active against wide variety of Gram-negative organisms. Most Common clinical isolates such as Escherichia coli ,*klebsiella spp;,,Enterobacter spp, Acinetobacterspp Pseudomonas aeruginosa,* these organisms are mainly responsible for nosocomial infections.In addition to that, colistin have significant activity existing against *Salmonella spp;Hemophilus spp ,Shigella spp;* and *Pasteurella spp*.It has a outstanding bactericidal activity to most of the aerobic gram negative organisms .colistin has also been reported that it has an potential activity against several species of mycobacteria ,that includes Mycobacterium tuberculosis ,*Mycobacterium xenopi ,Mycobacterium intracellulare M.fortuitum, Mycobacterium intracellulare,*.[26]

EFFECT OF COLISTIN ACTIVITY IN MULTIDRUG RESISTANT *ACINETOBACTER* INFECTIONS :

Acinetobacter baumannii is a nonfermentative, gram negative,aerobic coccobacillus which is extensively found in natural environment, has been increased to cause nosocomial infections.[27]Particularly this microorganism has the characterisation of rapid development of resistance to the majority of antimicrobials ,that includes fluoroquinolones,aminoglycosides,carbapenems(manikal2000). Colistin is widely used in multidrug resistant *Acinetobacterbaumannii* ventilator associated pneumonia(VAP),Intravenous colistin found to be effective.In case of carbapenemase resistant strains colistin was found to be safe and effective for the management of ventilator associated pneumonia. colistin mainly acts on the cell membrane disturbing the cell membrane,leading to increase in permeability and thus resulting in cell death.colistin has bactericidal activity against *Acinetobacter species* and its effect is mostly concentration dependent.(95).some observational studies have reported cure rates or improvement for colistin of 57%-77% among severely ill patients with multidrug resistant *Acinetobacter* infections including bacteremia,intra abdominal infections,pneumonia,sepsis, and meningitis.(100) Recently the use of colistin has been increased due to the lower rates of renal toxicity.Colistin has been suggested as the therapeutic option for treating ventilator –associated pneumonia(VAP) caused by multidrug resistant Gram- negative organisms .[28]For the patients with multidrug resistant *Acinetobacter baumannii*

pneumonia, they are treated with parenteral colistin,and the response rates ranges from 25 to 71%.[29]There are few more reports that add on, indicating that the aerosolized colistin may also be beneficial and additional safe therapeutic intervention for the management of *Acinetobacter baumannii* ventilator-associated pneumonia(VAP).[30]

EFFECT OF COLISTIN ON *PSEUDOMONAS* INFECTIONS:

Pseudomonas aeruginosa is an aerobic,nonsporing, gram negative bacteria,and it is motile.It is widely distributed in soil and water.It is an opportunistic pathogen implicated to cause respiratory tract infections,urinary tract infections,gastrointestinal tract infections,otits media and bacteremia,burns and cystic fibrosis.It also plays a prominent role in nosocomial infections. Mode of activity of colistin in pseudomonas infections is similar to that of *Acinetobacter*. Colistin has the ability to bind and it neutralises the bacterial endotoxin activity and all the endotoxins gets released during infective exacerbations. Isolates from cystic fibrosis have abundance of extra cellular polysaccharides composed of alginate polymers.It has been widely used for the treatment and eradication of pseudomonas infections in people affected with cystic fibrosis.In some case of cystic fibrosis it has been used in aerosolformulations.[31] The polymyxin adaptive response systemare generally regulated by thetwo component regulatory system.[32,33] Parenteral use of colistin in multidrug resistant *P.aeruginosa* ,has shown a great outcome in patients with nosocomial acquired pneumonia.[34]

EFFECT OF COLISTIN ON*ENTEROBACTERCIAE* INFECTIONS:

Colistin shows invitro activity against *Enterobactericeae* with the exceptions of *Serratia* spp, P*rovidentia spp*, *Proteusspp*,which are naturally or intrinsically resistant to colistin.It is also not active against *vibriospp*.[35]*Enterobactericeae* shows carbapenem resistant in which almost all classes of antimicrobials develops resistance resulting in high rates of mortality and morbidity.[36]So at this moment colistin has also been used as the therapeutic option to treat carbapenem resistant *Enterobactericeae*. So far there is no therapeutic availabilities. Colistin is a long back tracked drug used to treat severe invasive infections due to Gram -negative bacilli . In most of the cases colistin is the preferred worthwhile therapeutic alternative for the treatment of invasive blood stream infections that aredue to carbapenem -resistant *Enterobactericea* .[37]usage of parenteral colistin has been found to be harmless and in effect in treating the infections such as bone and joint infections, urinary tract infections(UTIs),meningitis and the bacteremia produced by MDR gram-negative bacterial isolates[38.] Inhalational form of colistin has been used as an adjuvant therapy for treating pneumonia or chronic lung infection with multi drug resistant gram negative bacteria .[39]

NATURAL OR INTRINSIC RESISTANCE OF COLISTIN:

Intrinsic or natural resistance of colistin to some organisms, mainly they have alterations in lipid A which accounts for reduced binding of polymyxin.Intrinsically resistant organisms to colistin are 4'-phosphate moiety of lipopolysaccharide *Neisseria spp;Moraxella catarrhalis,Helicobacter pylori,Serratia marcescens,Proteus spp;Providencia spp; Vibriospp; Aeromonasspp; Burkholderia cepaciacomplex ,chromobacteriumviolaceum,; Morganella spp, Yersiniaenterocolictica,Edwarsiella, Brucella, Francisellatularensis, Elizabethkingiameningoseptica.*In *Proteus spp* the intrinsic mechanism of colistin is mainly in the modification of lipid A,It is found that resistance has been related with the 4-amino-4-deoxy-l-arabinopyranose (L-Ara4N) that is being linked with the 4'-phosphate moiety of lipopolysaccharide.[40]In other burkholderia cepacia complex, chromobacterium spp the intrinsic resistance to colistin has been noted similar changes , that it is due to ineffectual binding to the outer membrane, number of low phosphate in low consequence and the carboxylate groups in the lipopolysaccharide and are due to the presence of the aminodeoxypentose which is protonated[41].*Burkholderia cepacia* complex and the *burkholderia spp* are innately resistant to colistin ,the reason behind this is that L-Ara4N plays a main component and it is a part of both the kdo and lipidAmoieties of lipopolysacharides of *Burkholderia* spp.[42]In addition,to that, colistin is not active against gram-negative and gram-positive cocci,gram-positive aerobic bacilli,fungi and parasites

Colistin resistant in *Acinetobacter baumannii*:

Acinetobacter baumannii is now a days known to be the major hospital associated pathogen, that is responsible for major spectrum of disease that includes respiratory,surgicalsite ,blood stream infections,ventilator associated infections .[43]Recently resistance to colistin have been reported in *Acinetobacter baumannii* clinical strains .[44]In united states surveillance study was conducted and found that 5.3% of all strainsof *Acinetobacter baumannii* were resistant to colistin. . [45]The mutations in the genes that encodes the two-component signaling proteins (TCS) that is PmrA and PmrB are being linked to colistin resistance in *A. Baumannii*.Recent studies indicates that resistant to colistin is generally due to the alteration in the two component system that is the pmrAB system.[46]In vitro clinical studies were done for the colistin resistant strains,that is the MDR A. baumannii isolates.Mutatioins and alterations and the genes encoded in the two component system proteins of pmrAB was performed by using a DNA sequencing method.

Colistin susceptibility testing :

As far as concern antimicrobial susceptibility testing for colistin by disc diffusion method(DD) Kirby-Bauer disc method is not recommended by any of the antibiotic sensitivity testing guidelines.According to The clinical and Laboratory standards Institute (CLSI), It provides the disc diffusion breakpoints only for the *Pseudomonas aeruginosa* .But not for any other gram negative bacilli.The European Committee on Antimicrobial Susceptibility Testing(EUCAST) also does not provide any colistin disc diffusion breakpoints in their breakpoint tables. It has long been stated that colistin disc diffusion method has been found to be problematic for gram negative isolates except *Pseudomonas* and it also yields a high rate of very major errors(VME) that is false susceptibility when compared with reference agar and broth dilution method.[47]Studies have said that colistin susceptibility testing by disc diffusion method have been found to be upto 32% very major errors when compared with the reference Minimum Inhibitory Concentration method like agar dilution method or broth dilution method.[48]

Disc diffusion susceptibility testing:

Disc diffusion susceptibility testing for colistin were generally done by using the colistin discs(Bio-RadMarnes la coquette,France) which contains the disc content of 10µg of colistin and 50 µg of colistin disc content. According to CLSI(Clinical laboratory standard institute) the disc content of colistin used is 10µg, the disc diffusion zone diameter for pseudomonas aeruginosa ≥11mm is

susceptible,≤10mm is resistant.As per the clsi guidelines there are no interpretive disc diffusion zone diameters for the*Enterobactericeae* and also the*Acinetobacter isolates* (clsi2016). The disc diffusion zone diameters for 10 µg of colistin disc content were interpreted according to the National committee for clinical laboratory standards 1981 guidelines.The medium used was Mueller hinton agar ,0.5Mcfarland,the zone diameter for resistance was considered as <8mm,susceptible >11mm according to manufactures instruction. According to gales et al,BD Mueller hinton agar were used as the testing medium with the inoculum of 0.5Mc farland,the zone size for resistant <11mm,susceptible is >14mm(criteria of .[49]The british society of antimicrobial chemotheraphy (BSAC)used Iso-sensitest agar medium for antibiotic susceptibility testing of colistin(oxoid UK),the disc content of colistin used was 25µg(oxoid,UK),and the inoculum was 1:100 dilution from the prepared 0.5Mcfarland suspension. breakpoints used for the *Enterobacteriaceae* and the *Acinetobacter* spp. (resistant ≤ 14 mm)and*P. aeruginosa* (resistant ≤ 13 mm).Itwas found that 11(5%) very major errors were found.[50] In certain studies disc diffusion susceptibility testing using the Kirby bauer disc diffusion methods in three different methods have been performed. The microbial inoculum were first matched to the turbidity that is corresponding to that of the 0.5McFarland using a standard calibrated turbidometer ,then it is further inoculated in the recommended media.when disc diffusion method were interpreted according to manufactures instruction it was found that there were 22 (10%) very major error have been found.when disc diffusion interpreted

according to gales et al .The number of very major errors were found to be decreased to 12(44%).[51] The three disc diffusion methods that have been changed in numerousfactors that are notorious to mark the antibiotic susceptibility testing which includes the concentration of the colistin, disc content,inoculum size ,and the form of medium that is being used.However, the resistant pattern of colistin by disc diffusion procedures based on all the three techniques showed a high rate of very major errors(5-11%) which wasunacceptable .Due to some essential characteristic properties of the polymyxins, the agar based susceptibility testing becomes challenging because the polymyxin groups have poor diffusion properties in the agar which results in poor zone of inhibition.[52]This may result categorical difference in the antibiotic susceptibility pattern of theclinical isolates.usage of higher concentrations of colistin disc content doesn't seems to be accurate for test results .Due to the invitro mechnism of the polymyxin groups it may be exaggerated by the cation levels in the agar.[53]For intravenous formulations colistin methate sodium preparation are usedbut for the susceptibility testing colistin sulphate is used ,it iseight times more potent than colistin methate sodium. The performance of Iso- sensitest medium shows little variationhas also been shown that it affects the optimum invitro susceptibility testing to polymyxins remains unsettled.[54]

VARIOUS METHODS FOR DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION (MIC) FOR COLISTIN (POLMYXIN-E)

Colistin susceptibility testing on VITEK 2:

Automated systems like VITEK2 COMPACT (bioMérieux, Hazelwood, MO), have been recently used for the evaluation of colistin susceptibility testing. Reliability of these automated systems when compared with reference method like agar dilution or microbroth dilution it holds good for determining susceptibility of strains of colistinwith genera that have been known not to display resistant subpopulations and found to be reliable with high level of agreement. . In some isolates of genera that are occasionally known to exhibit heteroresistance, an alternative susceptibility testing method should be preferred that is capable of detecting heteroresistance .[55]Vitek 2 automated systems showed no major errors when compared with agardilution method.they showed 100% categorical agreement .[56]In Vitek 2 automated systems the procedure is done according to manufacturers instructions,the bacterial isolates should be freshly isolated ,it should be single type of organism,freshly subcultured,matched with MC farland,(Gram-negative)GN280,GN281 Vitek 2 antibiotic susceptibility testing cards are used.The colistin MIC values ≤0.5 µg to ≥16µg/ml, contains a Interpretative breakpoints of(MIC 2 µg /ml as susceptible, and MIC of 4 µg /ml, resistant) were used for the VITEK 2which have been employed according to the manufacturers instructions .Datas suggest that VITEK2 automated systems appears to be a very useful method for

rapid detection of colistin sensitive and resistance as it exhibits a good categorical agreement with microbroth dilution methods.[57]In routine *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 are routinely used for quality control.[58]

Colistin susceptibility testing on E-Test(Episilometer Test):

Episilometer test(E test) is executed by using the colistin E test(AB Biodisk;Biome'rieux,Sweden).It is done on Mueller-Hinton agar plates according to the manufacturer's procedure instructions. E -test strips are applied after the agar surface are completely dried.Then they are incubated at 35c for 18hours.The minimum inhibitory concentration is determined at the point where the inhibition of growth is intersected with E-test strip.The susceptibility breakpoints are followed by clinical laboratory standard institute and the breakpoint are assessed only for *pseudomonas aeruginosa* and *Acinetobacter baumannii.*[59]Some studies have shown good concordance when E-test is compared with standard reference method such as agar dilution and microbroth dilution. As the reference methods are little cumbersome, Etest are simple and they are used for susceptibility testing of colistin as an alternative method . In ceratin studies false susceptibility of One to four percentage of results were found , but there was no false-resistant results .[60]E-test has been validated in certain studies with a good correlation when compared with agar dilution showing 87% of categorical agreement in the isolates of *Acinetobacter spp Pseudomonas aeruginosa*.[61]E-test for colistin is useful in clinical cases

associated with severe infections caused by multidrug resistant strains or in clinical failure and E-test can also detect heteroresistant isolates.[62]

REFERENCE METHODS FOR DETERMINATION OF MIC FOR COLISTIN:

There are two standard reference methods that has been widely used for determining minimum inhibitory concentration for colistin .Agar dilution method and microbroth dilution method are the standard reference methods for determining MIC's for colistin.Early experiences with the clinical laboratory standard institute (CLSI) has mentioned that the different batches of Mueller-hinton agar(MHA) affected the interpretation of susceptibility. There was a significant difference in the medium performance which was been noted for the drugs such as colistin ,imipenem, aminoglycosides[63]. There are no CLSI recommended guidelines for colistin susceptible minimum inhibitory concentration (MIC) breakpoints against Enterobactericeae .According to the BSAC (British society for antimicrobial chemotherapy the susceptible break points for colistin against Enterobacteriaceae if ≦4 μg/mL it is considered as susceptible, if MIC >4 μg/mL it is considered as resistant . Isosensitest agar medium is a semidefined medium which has been standardised medium that has been used by BSAC(British society for antimicrobial chemotherapy). But Isosensitest agar from different manufacturers has also shown significant variation.[64]The reference method for testing colistin sucsceptible remains to be still to be defined well. Good concordant results was been observed between agar dilution method and microbroth dilution method which has been

mentioned in the data.[65]Minimum inhibitory concentration of colistin by agar dilution method had been performed by using colistin sulphate powder(sigma-Aldrich,Singapore).The drug has been added to molten cation adjusted Mueller- Hinton agar (Becton-Dickinson, Franklin Lakes, MD, USA) to provide a two- fold dilution ranging from 0.25 to 128µg/ml.Mc farland matched bacterial suspensions are added to the agar plates around 10^4colony forming unit/ml by using a multipoint inoculator. Results are interpretated following incubation at 35°c for 16-24 hours for *Enterobactericeae*,and for *Acinetobacter spp* incubation period around 20-24 hours.[66]MIC break points for *pseudomonas aeruginosa* according to CLSI susceptible≤2µg/ml ,intermediate4µg/ml , resistant≥8µg/ml.MIC breakpoints for *Acinetobacter spp* susceptible is ≤2,resistant is ≥4.

Combination therapy with colistin:

The antimicrobial agent that are combined with colistin are rifampicin[67],ciprofloxacin,Ceftazidime,gentamicin,imipenem,meropenem,piperacillin.However the antimicrobials that are commonly combined with colistin are rifampicin and carbapenems. In multidrug resistant *Pseudomonas aeruginosa* diabetic foot infections colistin either alone or in combination with other antimicrobials found to be safe and effective .[68]In case of multidrug resistant *Acinetobacterbaumannii* infections combination of imipenem with colistin was found to be superior.Carbapenem based combinations offers promising alternatives in treating the infections due to multidrug resistant *Acinetobacter baumannii* infections .[69]

MALDI-TOF APPLICATIONS:

Matrix-assisted laser desorption ionization time of flight(MALDI-TOF)works under the principle of mass spectrometry which has been recently entered into the microbiological laboratories for regular identification of fungi and bacteria.It identifies the antibiotic resistance in the cell wall and cell envelope.It is not limited to the cellwall alone it also detects the cell wall outer membrane structural changes in lipopolysaccharides.This is associated with resistance to the cationic peptides andloss of negative charge of lipid Adue to the post synthetic modifications. For analysis of lipopolysaccharide in MALDI-TOF ,there is some special procedure known as hot phenol method for extraction of llipopolysaccharide.In this the lipopolysaccharides are solubilised by water,then saturated by the phenol and then it is precipitated. Then the samples are applied with the proper matrix on to the mass spectrometry target and analysed .The changes in the lipidA that is caused by 4' phosphatise in colistin resistant porphyromonas gingivalis had been observed by coats et al.Then the change in the molecular weight and the modification of phosphoethanolamine in lipidA associated with colistin resistance in a *Acinetobacter baumanni I* was reported by beciero et al.[70]

MCR-1:

MCR-1 gene is responsible for plasmid mediated colistin resistance. Isolate *E.coli*SHP45 which was isolated in a pig farm in shanghai in July 2013Liu, Yi-Yun et al.[4] The MIC value for this colistin resistant strain was 8µg/ml.This MCR-1 gene is associated with the transposable element which is located on a Inc12 plasmid p HNSHP 45.This MCR-1 was detected in 20% 0f *E.coli* which is isolated from the pigs at the slaughter and 15% from the raw meat in china in Nov 2015.This gene was also detected in clinical isolates from the inpatients of Chinese hospitals and the organisms harbouring this MCR-1 gene were *E.coli* of 1.4% and *Klebsiella pneumoniae* of 0.7%.MCR-1 gene MCR-1 gene belongs to the member of the phosphoethanolamine transferase enzyme super family, with expression in*E. coli* resulting in modification of phosphoethanolamine to lipid A. MCR-1 gene was again reported in nov2015 in china.MCR-1 is a plasmid mediated resistant gene which was found in porcine and bovine colistin resistant *E.coli*. It was isolated in animals, it was about 12.4% .

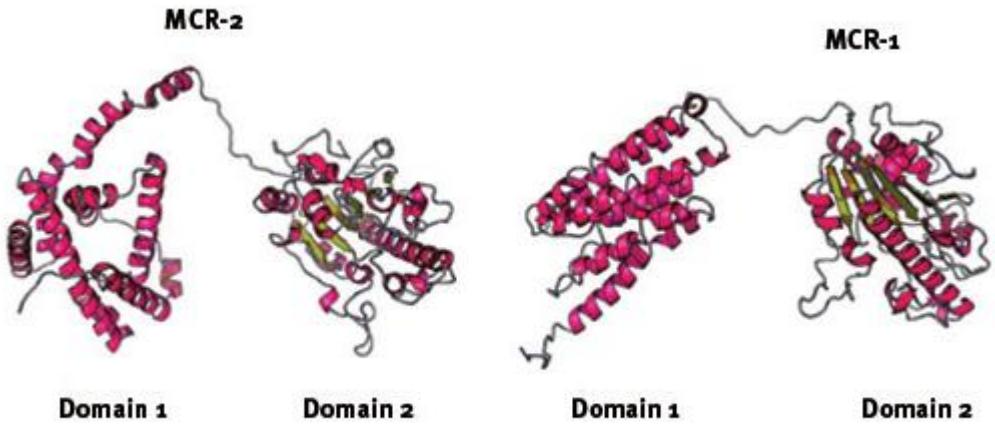
ARRIVAL OF NEW GENE mcr-2

Recently colistin resistant plasmid mediated another gene was identified in porcine and bovine animals, it was found that colistin resistant was not due to MCR-1,it was due to MCR-2 gene .This MCR-2 gene was identified in Belgium June 2016,actually they were screening for MCR-1 in *E.coli* colistin resistant strains and it was found to be negative for MCR-1.*E.coli* colistin resistant strain contained MCR-2.The Prevalence of MCR-2 was11/53 in porcine resistant colistin strain which was found to be higher than MCR-1(7/53).MCR-2 gene is 1617 base pair long,it is nine bases shorter than MCR-1(1626 BP).This MCR-2 gene shows 76.75% identity to that of MCR-1.The mobile element that harbours MCR-2 was identified as internal sequence IS1595 which is distinguished by the presence of ISXO2-like transpose domain.Studies have identified that the open reading frame on MCR-2 encoding lipid phosphatase is similar to that of *Moraxella osloensis*, it is shows identity of 41%.This MCR-2 gene is found to be lipid that is coharboured which is the lipid phosphatase gene showing a higher homology with the phosphatise enzyme of *Moraxella spp(*Xavier BB,).Though this *Moraxella spp*is found to be intrinsically resistant to colistin, intergeneric transfer of MCR-2 from animal or human or environmental origin would be responsible for similarity of the gene. House keeping enzymes are the phosphoethanolaminetransferases which on adding the phosphoethanolamine moiety to the outer 3-deoxy-Dmanno-octulosonic acid(kdo)fatty acid residue of a kdolipidA has been catalysed.The prevalence of MCR-2 is very high when

compared to MCR-1 is due to the carrier plasmid $Incx_4$ which is highely transmissible responsible for rapid transfer of gene. The complete genome of*mcr-2* gene was been amplified (PCR primers are:For MCR2-Forwar primer is 5′ TGGTACAGCCCCTTTATT 3′; MCR2-R 5′ GCTTGAGATTGGGTTATGA 3′), cloned (vector pCR 2.1, TOPO TA Cloning kit, Invitrogen) and electroporated into DH-5 α *E. coli* . This was again reconfirmed by broth dilution method based on EUCAST guidelines.An overall prevalence of MCR-2 was 11.4%

MCR-2 protein consists of two domains one act as a transporter and the other act as a transferase domain with the residue of domain 1 which contains (1 to 229 residues) and domain 2 contains(230 to 538 residues) as shown in Figure4

Figure 4:Showing the tertiary structures ofMCR-2 and MCR-1 .



raptor x (Källberg M,)[72]was used for generating the tertiary structures. MCR-1 and MCR-2 contains domain 1 and domain 2 .Domain 1 was predicted as a transporter and domain2phosphoethanolamine transferase(sulphatase).

GLOBAL PREVALENCE OF COLISTIN RESISTANCE :

Various parts of the world suggest that there is development of resistance towards colistin among gram negative bacteria though the resistance of colistin is not fully understood.

World wide resistance to colistin was uncommon during 90's.The first case was reported in Czech republic in 1999.surveillance in USA hospitals had revealed that5.3% of acinetobacter strains were resistant to colistin. Isolates that were developing resistane were from sputum, nasal aspirate, wound, urine; however, blood remained predominant source of isolation. In several countries slowly there is emergence of colistin resistance has been developed due to improper drug usage. Although resistance to polymyxin are generally less than 10%,but it is found to be higher in Korea, china and mediterrean countries.[73]In Italy colistin resistant strains was first reported in 1999..More hospital outbreaks has been noticed and it has been described in Greece,the increased proportion rate of colistin resistant strains were noted in rectal swabs surveillanceAn overall resistance in colistin was reported at a rate of43%.[74]In cases of carbapenemase producing *Klebsiella pneumoniae* due to overusage of colistin has lead to the emergence of colistin resistance[75].For *Acinetobacter baumannii* resistant colistin isolates in korea were around 214 isolates(27.9%).*For K.pneumoniae* the studies indicate that 55 clinical

isolates(6.8%) from south korea were found to be resistant to colistin.Patients with cystic fibrosis mostly pseudomonas have been isolated which was found to be resistant to colistin.

In Indian studies recent usage of this last resort drug has lead to increase resistance of this drug. About 16% of carbapenem resistant strain were found to be pan drug resistant,In total 3.5% were found to be resistant to both tigicycline and colistin. this shows a significance to this high end antibiotic were there are limited therapeutic option.[76]

COLISTIN RESISTANCE IN *KLEBSIELLA PNEUMONIAE:*

Reports have been stated that the development of resistance to *Klebsiella pneumoniae* isolates is due to the usage of colistin extensively resulting in increased mortality and morbidity.[77]Presence of capsule may be the reason for colistin resistance. .It has the ability to shed capsular polysacharrides ,these released capsular polysaccharides have the ability to bind or trap to the polymyxin and the quantity of the drug is reduced that extents tothe bacterial cellsurface.[78]Resistance to colistin have been reported in various numerous regions,including North America, Europe,Asia,southamerica.The highest overall resistance to the colistin in clinical isolates have also been reported in Greece at 10.5-20% .[79]In*Klebsiella pneumoniae* the colistin resistance is due to the phosphate groups of lipid A contains five times more L-Ara4Nthan those of susceptible strains.Alteration in the compositions ofouter membrane leads to subordinate the negative charges of the cell wall outer membrane of the

Klebsiella.pneumoniae[80] .structural alteration in the lipopolysaccharides similar modifications have been shown in the two component systempmrB/pmrAand phop/phoQ .[81]subsequent upregulation synthesis of arnBCADTEF operon and thepmrC is due to the constitutive activation of the pmrA/pmrB system which is caused by missesense mutation in the outermembrane pmrB or pmrA [82] .Such occurence of pmrA/pmrB genes mutations have been observed in various clinical isolates and isolates of non clinical colistin resistant isoltes of *K.pneumoniae*[83]. Recently colistin resistant,*K.pneumoniae* has shown a profound mechanism that there is mutation or inactivation of mgrB gene,encodes a short ,47 aminoacid transmembrane proteinwith the conserved gene of 141 nucleotides in length,(the length may vary in the naturally colistin –resistant *Enterobactericeae)* which applies negative feedback of the regulatory system(phoP/phoq) .It also shows the feedback mechanism by preventing the kinase activity of phoQ or enhancing its phosphatase activity, which automatically leads to repression of phoP genes overwhelming phoP phosphorylation.[84]Further moremodifications have also being mentioned that mgrB includes a nonsense mutation which leads to the premature termination of MgrB missense mutations and the transmembrane protein which results in the aminoacid substitutionsPoirel et al., 2014.

Mechanism Of Resistance Of Colistin in *Pseudomonas aeruginosa*

In*Pseudomonas aeruginosa*the resistant pattern of colistin is related to that of the enteric bacteria.It has both thepmrA/pmrB two component systems(TCSs)and phoP/phoQ ,each of these components which can regulate the arnBCADTEF operons seperately.Recently colistin resistance in *pseudomonas aeruginosa* Two component system are five Tcss ,they are as follows:pmrA/pmrB [85], 2,phoP/phoQMiller et al., 2011,[86]parR/ParS[87],colR/colS[88].According to Mc phee etal pmrA/pmrB two component system regulates resistance to cationic polypeptides which results in lipopolysaccharide modifications.polymyxin E resistance is associated with modification in the (L-Ara4N) to phosphate groups within the lipid A core moieties of LPS. [89]According to miller et al in addition to lipidA modification ,there is disruption of chromosomal *phoQ* due to the prescence of an intact *phoP* allele stimulated by 4-amino-l-arabinose.with these modifications it was found that there is loss of *phoQ* function mutation which contributed to high level of colistin resistance in clinical isolates. According to Gutu et al *Pseudomonas aeruginosa* develop resistance to colistin by a consequence of mutations in the phoPQ regulatory system,which is mediated by the covalent lipid A modification .Individual deletion of colRS genes in a phoQ mutant are also responsible for colistin resistance.High polymyxin –resistant phoQ mutants of *pseudomonas aeruginosa* isolated from patients treated with colistin in case of cystic fibrosis have shown the mutant alleles of colRS.

Combination of antimicrobial therapies described for extensively drug-resistant (XDR) Gram-negative bacilli infections.

In case ofXDR *Enterobacteriaceae* there are two drug combinations and three drug combinations.In two drug combinations polymyxins are combined with carbapenes,tigecycline and fosfomycin.There are tigecycline based combinations,it is combined with polymyxins,fosfomycin and carbapenems.The three drug combination are Tigecycline,polymyxin and carbapenem. [90,91]

In case of XDR *Acinetobacter baumannii* infections the common two drug combinations are combinations based on polymyxin and combination based on sulbactam.Along with polymyxin tigecycline and carbapenem are used.For sulbactam combination carbapenems,tigecycline and carbapenem are used.The three drug combinations are cefperazone sulbactam +tigecycline + carbapenem are used. Imipenem + rifa Polymyxin-based combinations.[92,93]

In case of XDR *pseudomonas* infections in two drug combination polymyxin with ciprofloxacin,polymyxin with rifampicin ,polymyxin +fosfomycin are used.ciprofloxacin combinations are antipseudomonal betalactams that include the β-lactams active against P. aeruginosa, such as carbapenems (meropenem, imipenem), ceftazidime, aztreonam, piperacillin–tazobactam andcefoperazone–sulbactam.In three drug combinations Aztreonam + ceftazidime + amikacin are used.[94,95]

Prevention and control:

For extensively drug resistant gram negative organisms the treatment becomes limited ,there is no much of data for randomised study of this XDR. This is an upcoming emergence next to MDR. In case of Extensively drug resistant isolates ,mono therapy doesn't help a lot in outcome of the patient including the polymyxins. combination of antimicrobial therapy shows a good outcome of the patient in case of XDR.Risk factors must be properly assessed. The most common risk factors are associated with long term usage with antimicrobial therapy, indwelling catheters,solid organ transplantation,mechanical ventilation,longer stay in hospitalisation. Common XDR Enterobactericeae are *Klebsiellapneumoniae,E.coli*.Principles to be followed when the there is XDR infection.

- First to rule out whether XDR is due to infection or colonisation
- Appropriate usage of antimicrobials,proper susceptibility testing to be followed,in case of any intermediate zone ,MIC Values to be interpretated.,which helps for increasing the dosage.
- Combination therapy to be followed rather than monotherapy in XDR infections
- The dosage regimens should be adjusted according to the pharmacoproperty of the drug.

Primary disease should be addressed properly ,efforts should be taken to eliminate the risk factors. [96]

CONTROL OF XDR-GNB INFECTIONS :

Due to increased infections ,the usage of drug combination increased.Proper antibiotic policy to be followed.Infection control measures to be followed properly. The following measures that helps to prevent XDR-GNB infections is so simple and effective.

Proper hand hygiene:

This is the most simple and cost effective step where each an every individual can follow By hand washing we can prevent cross infection ,in critical wards the staffs those who are handling the ill patients by this simple measure they can prevent the spread of this XDR and reduce the infection.[97]

Proper Isolation:

As soon as the microbiology laboratory gives an intimation that patient is infected with XDR GNB ,the patient shoud be properly isolated,clinicians should instruct that the separate rooms to be provided,atleast partial separation of patient's bed for 1meter,usage of separate devices like sphygmomanometer,thermometer,stethoscope should be used.when the patient is getting discharged or leaving to someother hospital,proper intimation to be given that the patient is affected with XDR infection.[98]

Surrounding Surface Disinfection:

The health care workers . Should be properly advised to clear the surfaces and the floors in the XDR infected patient room. Fluorescence labelling or hygiene monitoring would be effective to ensure that the XDR resistant strains are effectively blocked.[100]

Decolonisation:

Regular chlorhexidine bath for the patients colonised with XDR.It helps in reducing the invasion of infections.[98]

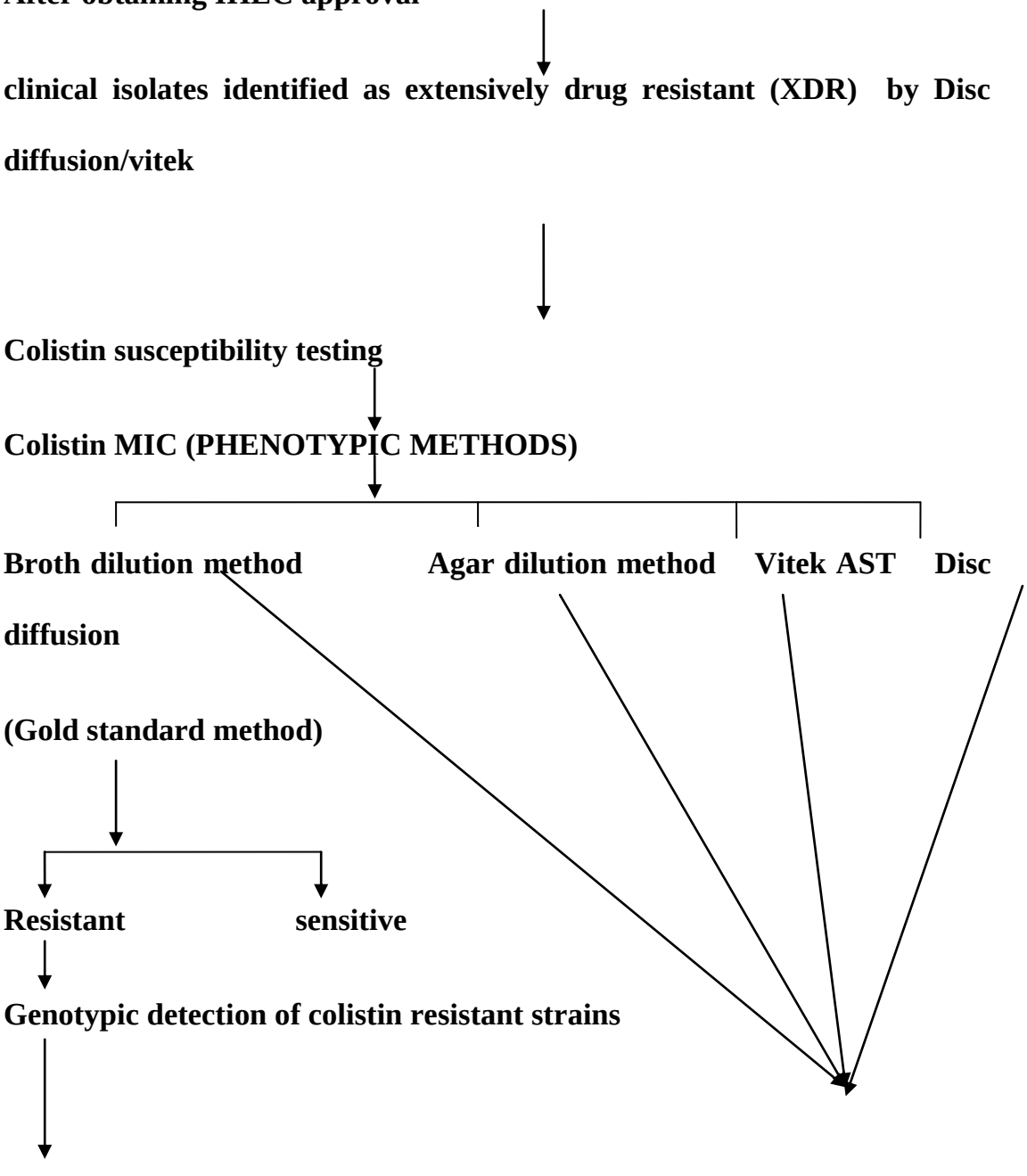
Dynamic screening:

Rapid diagnostic methods such as automated methods and molecular methods should be available for screening the XDR strains. Proper surveillance of screening in transplant patients , nasopharyngeal secretions,rectal swab screening to be done to rule out XDR infections[99]..Adoption of proper track to identify the route of XDR infection by epidemiological measures.[100]

MATERIALS AND METHODS

METHODOLOGY:

(Flow chart)



Period of study : 18 months

Study Design : This is a prospective experimental study had been conducted at the diagnostic microbiology department of PSG hospital.

Study Population: NA

Geographic Area:PSG Hospitals, Coimbatore

Sample Size determination

Formula used: n= t2 Xp(1-p)/m^2

Where;

n=required sample size

t=confidence level at 95%(standard value of 1.96)

p=estimated prevalence of colistin sensitivity strains

m=margin of error at 5%(standard value of 0.05)

Estimated prevalence from hospital statistics (p)=0.02

n=1.96X1.96X 0.2(1-0.2)/0.05X0.05

n= 245.8 **(250)**

Sample size – **250** clinical isolates of XDR gram negative bacilli

Sampling – Consecutive sampling .

Inclusion criteria : All Gram negative clinical Isolates identified as extensively drug resistant . That is , resistant to all antibiotic groups except one /two .

Exclusion criteria :

1. All Microorganisms sensitive to most of the antibiotics tested.
2. *Proteus sp , Morganella, H pylori, Serratia marscescens, Chromobacterium sp,* (Intrinsically resistant to Colistin)

METHODOLOGY:

A total of 219 Gram negative clinical isolates identified as extensively drug resistant or pan drug resistant by antibiotic susceptibility testing method such as discdiffusion/vitek out of the total 8040 no of Gram negative isolates.were included in this study . They were subjected to the following

I **PHENOTYPIC DETECTION**

- Microbroth dilution (gold standard reference method)
- Agar dilution method
- Vitek-2 automated system
- Disc diffusion

II GENOTYPIC DETECTION

- MCR-1 GENE (plasmid mediated resistant gene)

Colistin susceptibility testing:

Though disc diffusion method is not recommended by CLSI for colistin susceptibility testing .In routine ,for antibiotic susceptibility testing most laboratories uses disc diffusion method..

Disc diffusion method:

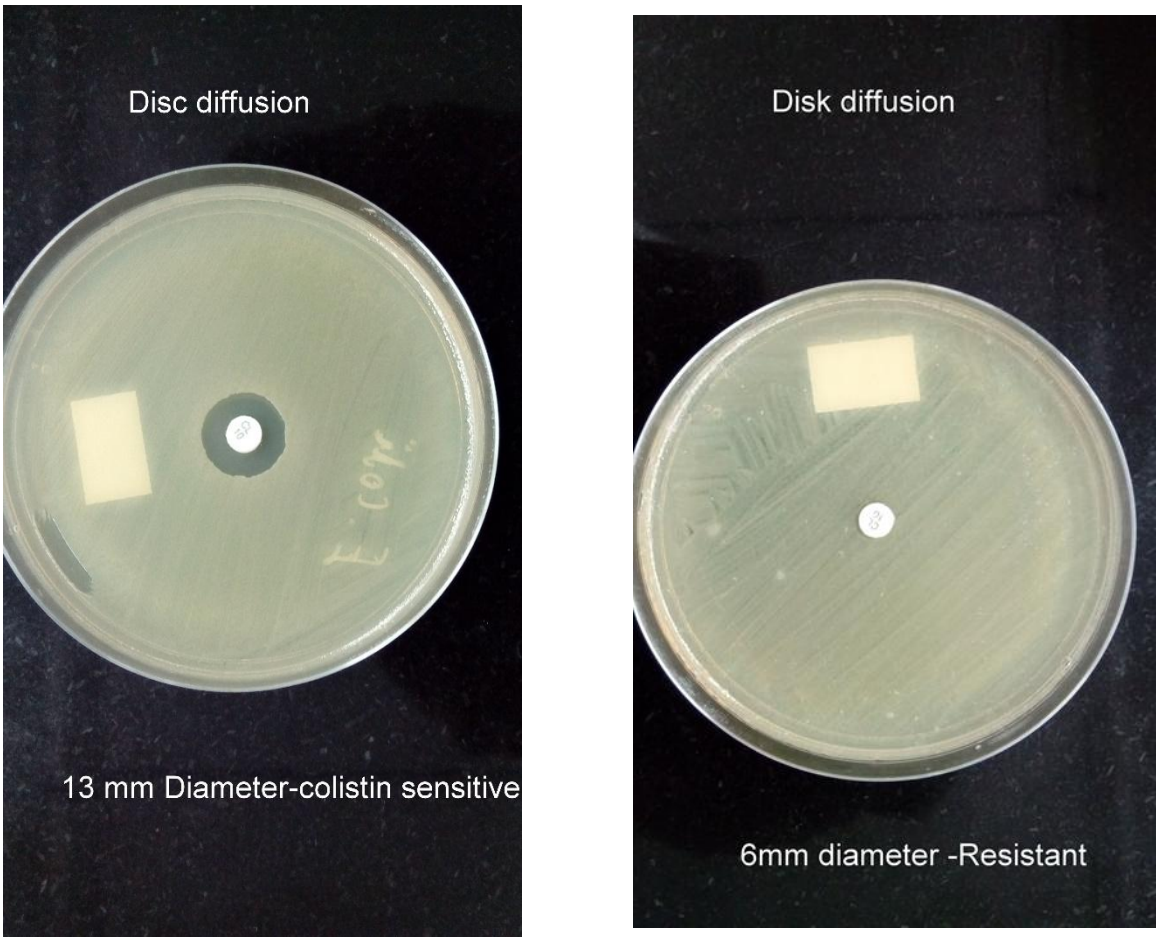
About 219 clinical isolates were taken they were inoculated in peptone water and matched with 0.5 Mc Farland,then sterile Mueller Hinton agar plate was taken,a lawn culture was made for all the 219 isolates.Then colistin antibiotic disc was placed for each isolate.The disc used was HIMEDIA Laboratories Colistinsulphate 10µg.The plates were incubated overnight @ 37℃ over 18 hours. After incubation the zone size was interpretated. According to CLSI(Clinical Laboratory Standard Institute 2015) there is no zone interpretation for *Enterobactericeae* for colistin testing. only for *pseudomonas aeruginosa* the zone diameters are given.The zone diameter as per CLSI follows.

ORGANISM	DISK CONTENT	ZONE DIAMETER SENSITIVE(mm)	RESISTANT(mm)
Pseudomonas aeruginosa	10µg	≥11mm	≤10mm

The zone diameters showingthe sensitive and resistant diameter are shown in fig 5

FIG5: COLISTIN SUSCEPTIBILITY BY DISC DIFFUSION METHOD

(a) **(b)**



(a)Disc diffusion method showing sensitive as 13mm zone diameter,(b)Disc diffusion method showing 6mm zone diameter as resistant

VITEK -2 COMPACT:

Vitek 2 compact is an automated machine. It is rapid and accurate for identification of organisms and for reporting the sensitivity and resistance pattern. It eliminates unnecessary biochemical test. The advance expert system(AES) in vitek2 compact gives the minimum inhibitory concentration value(MIC) for the antibiotics. Procedure was performed according to manufacturer's instructions.

EQUIPMENTS:

Vitek 2 compact machine

Gram negative cassettes

Vortex mixer

Materials Required :

Pure isolates of 24hour duration

Sterile saline

Mc farland

Disposable tips

Disposable gloves

Procedure :

Work was done under sterile precautions.all the 219 clinical isolates were were checked for purity by plating on a nutrient agar. Colonies were isolated individually.

Preparation of inoculums:

Colonies were picked and mixed with saline, it was vortexes until the colonies get mixed up properly. It was matched with 0.5Mc Far land. Then from the matched Mcfarland inoculums 148µl was transferred to corresponding another sterile Vitek tube.

Working method:

Once the inoculums are transferred, Vitek gram negative cassettes are brought to the ambient room air. Then the cassettes are inserted into the corresponding Vitek tube containing the inoculums. Then it is loaded in the Vitek loader ,samples are entered in the system. vitek starts working automatically. Reports are generated in a computer .sensitivity and resistance pattern with their corresponding MIC values are ready in a printed form within 6 hours .

MIC BY AGAR DILUTION METHOD:

In Agar dilution method different concentrations of the antimicrobial substance are incorporated into a agar medium , followed by the application of a standardized number of cells to the surface of the agar plate. After 16-20

hours of incubation Growth is assessed and the MIC value is read. Minimum inhibitory concentration was determined by using Agar dilution method using isosensitest agar as per the British society of antimicrobial chemotherapy(BSAC). IsoSensitest agar(ISA,Oxoid,Basingstoke,UK) was used. It is a semi defined medium that has been designed for antimicrobial susceptibility testing. The advantage of this media when compared to Mueller Hinton agar is ,it has better penetration of the drug and also used to detect heteroresistance.

MATERIALS REQUIRED:

- Isosensitest agar 3.1 grams for 100 milli litre of media
- Sterile petridish plates
- Freshly prepared antibiotic stock solution
- Freshly prepared inoculum standardised with 0.5 mc farland
- Multipipettes of size 100µl(microlitre),50µl,1-10µl
- Disposable tips
- Discarding jar with 1% sodium hypochlorite
- Disposable gloves
- *ATCC E.COLI,ATCC PSEUDOMONAS* for control strains
- Incubator at 37˚c

PREPARATION OF ANTIBIOTIC STOCK SOLUTION:

Colistin sulphate is used for antibiotic susceptibility testing, because it has better penetration in the agar,as per CLSI guidelines.

Antibiotic stock solution were prepared according to the formula:

$$\frac{1000}{P} \times V \times C = W$$

Where P= potency of the drug given by manufacturer(µg/mg), required(millilitre mL),C= Final concentration of solution(multiples of 1000)mg/L, and W= weight of the antibiotic in milligram(mg) to be dissolved in volumeV(ml).

For preparation of further stock solutions, from the initial 10,000 mg/L solution, prepare the following:

1 mL of 10,000 mg/L solution + 9 mL diluent= 1000 mg/L

100 µl of 10,000 mg/L solution + 9.9 mL diluent = 100 mg/L

PREPARATION OF AGAR DILUTION PLATES:

Sterile test tubes were labelled .Dilution was done ranging from 16µg to 0.25µg,that is from higher concentration to lower concentration. From the freshly prepared stock solution 128µg of drug was taken and added to first tube which was labelled as 16µg,then further doubling dilution was done.Now 1ml of desired diluted drug was added to 19 ml of IsoSensitest agar.Then the agar

containing particular concentration of drug was poured in to the sterile petri plates which were labelled as 16,8,4,2,1,0.5,0.25 µg/ml.Growth control plate was also labelled.

PREPARATION OF STANDARD INOCULUM:

- Sterile tubes of 7.5_x3 cm tubes were taken, 3ml of sterile Saline was added and labelled as 1,2,3 according to the particular clinical isolate.
- From a overnight pure culture the colonies are picked and the organism are diluted in 3ml of saline to obtain a suspension of approximately of 10^7 colony forming unit/millilitre(CFU/ML).It was matched with Mc Farland.

INOCULATION:

Now the petri plates containing particular concentration of drug were taken and allowed for drying.once the plates were dried , 2µl of inoculum that is 10^4cfu /ml was added from the standard inoculum to the particular drug containing plate.

INCUBATION:

Inoculated plates were incubated at 37℃ for 16-20 hours in an ambient air incubator.

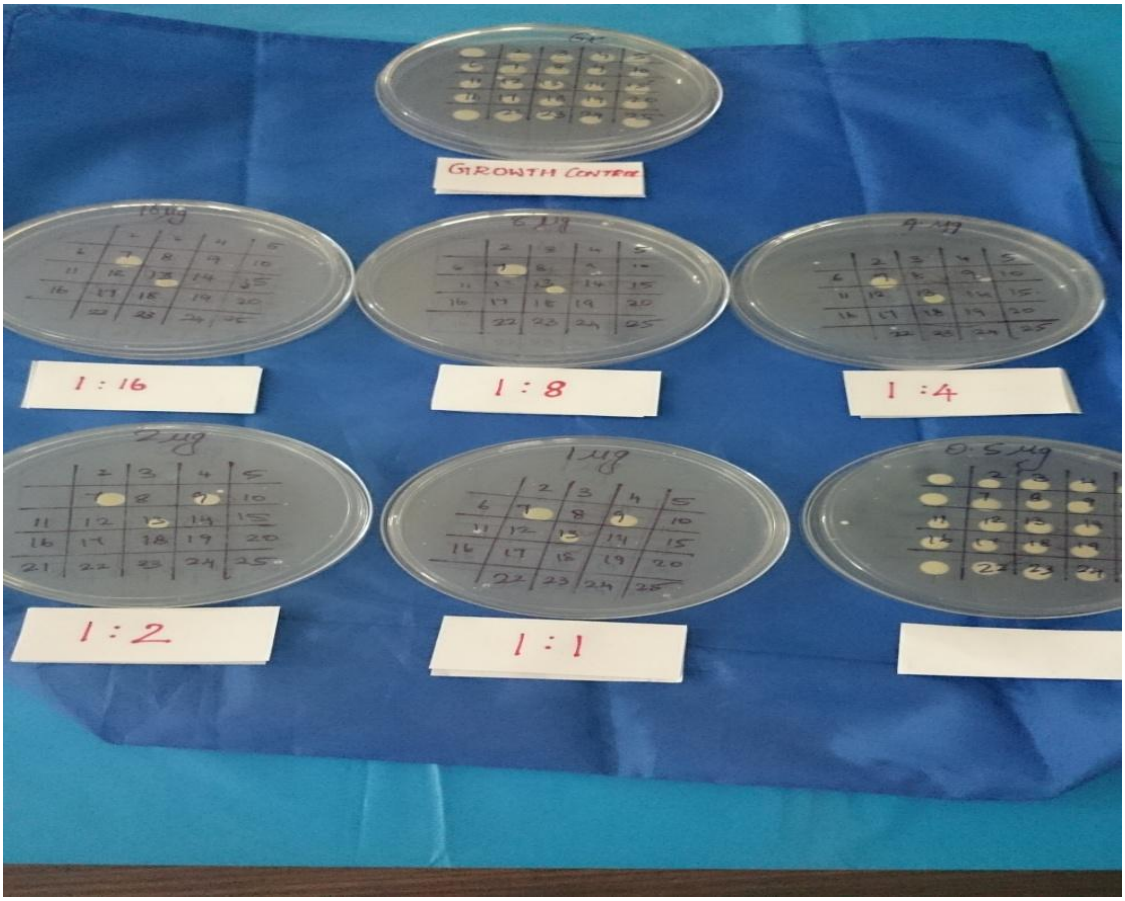
INTERPRETATION OF MINIMUM INHIBITORY CONCENTRATION :

- Growth Control Petriplate : Growth should be visible in growth control plate.

  Highest concentration where there is no visible growth of the organism or where there is inhibition of growth is considered as the MIC of the organismas shown in **figure 6** .

  .MIC values have been interpreted based on the CLSI guidelines.

Fig 6:Showing Minimum inhibitory concentration by agardilution method for XDR-GNB isolates included in the study



MIC VALUES AS PER CLSI GUIDELINES 2016 :

MIC breakpoints for *Pseudomonas aeruginosa*

SENSITIVE	INTERMEDIATE	RESISTANT
≤2µg/ml	4µg/ml	≥8µg/ml

MIC breakpoints for *Acinetobacter spp*

SENSITIVE	RESISTANT
≤2µg/ml	≥4µg/ml

There are no MIC breakpoints for *Enterobactericeae* as per CLSI guidelines .only EUKAST(European committee of antimicrobial susceptibility testing) guidelines and BSAC guidelines gives the MIC breakpoints for *Enterobactericeae.*

MIC BREAK POINTS AS PER EUKAST GUIDELINES

MIC breakpoints for *Enterobacteriaceae*

SENSITIVE	RESISTANT
≤2µg/ml	≥2µg/ml

MIC breakpoints for *Acinetobacter spp*

SENSITIVE	RESISTANT
≤2µg/ml	≥2µg/ml

MIC breakpoints for *Pseudomonas aeruginosa*

SENSITIVE	RESISTANT
≤4µg/ml	≥4µg/ml

MIC VALUES AS PER BSAC GUIDELINES 2015

MIC breakpoints for *Enterobacteriaceae*

SENSITIVE	RESISTANT
≤2µg/ml	≥2µg/ml

MIC breakpoints for *Acinetobacter spp*

SENSITIVE	RESISTANT
≤2µg/ml	≥2µg/ml

MIC breakpoints for *Pseudomonas aeruginosa*

SENSITIVE	RESISTANT
≤4µg/ml	≥4µg/ml

MICRO BROTH DILUTION METHOD:

Micro broth dilution method is the gold standard reference method for antibiotic susceptibility testing of colistin according to CLSI guidelines 2016.Reproducibility of this method is good. Procedure was done according to the CLSI guidelines.

Materials required:

Sterilised Micro titre plates

Cat ion adjusted Mueller Hinton broth

Freshly prepared antibiotic stock solution

Freshly prepared inoculums

Mc far land black/white lines

Micropipettes100µl,1000µl.

Disposable tips

Discarding jar with0.1%sodiumhypochlorite

MIC mirror reader

Disposable gloves

ATCC E.COLI,ATCC PSEUDOMONAS

Procedure:

Preparation of inoculums:

219 clinical isolates were taken and plated in a Mac Conkey agar, checked for the purity, colonies were picked up and diluted in sterile saline 10^7 CFU/ml matched with 0.5 Mc Far land.

Preparation of antibiotic stock solution:

Antibiotic stock solution were freshly prepared according to the formula as mentioned by the CLSI guidelines and it was diluted from higher concentration to lower concentration and the dilution were labelled as 64µg,32µg,16µg,8µg,4µg,2µg,1,0.5µg respectively.

Antibiotic dilution range:

Once the antibiotics were diluted it was added to 19mlcation adjusted Mueller Hinton broth and it was mixed, drug was added in order as labelled from64µg,32µg,16µg,8µg,4µg,2µg,1,0.5 µg.

Procedure of Micro Broth dilution method:

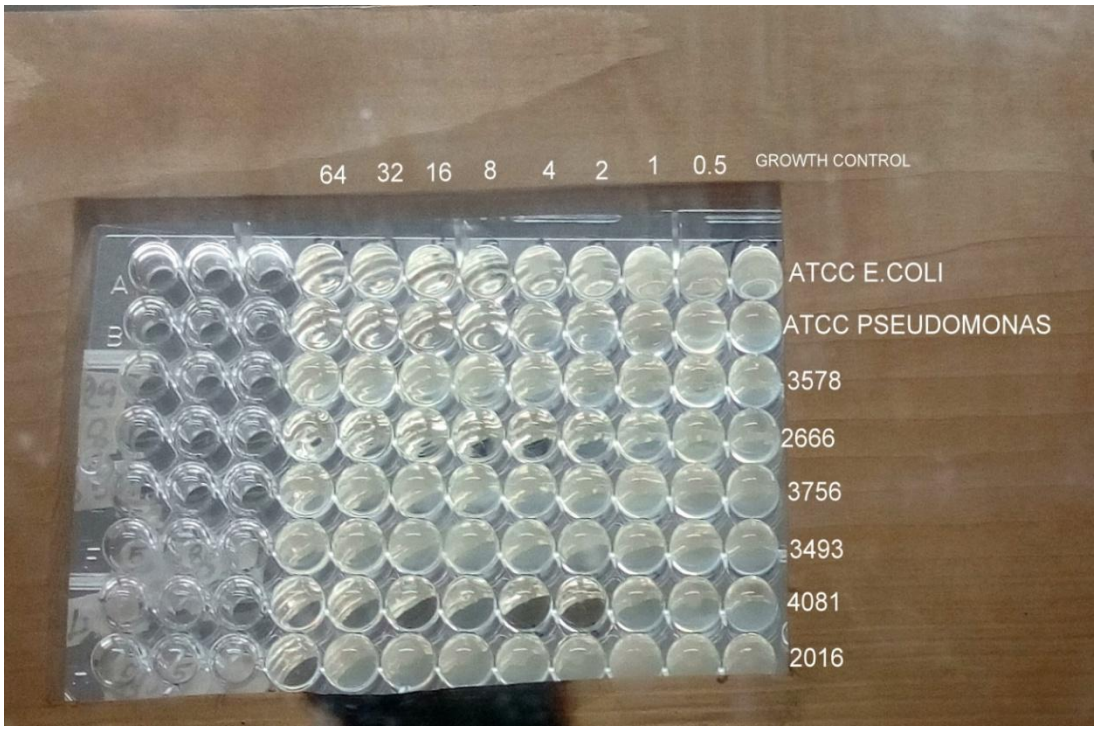
Antibiotic stock solution were kept ready and it was diluted in different concentration .Inoculum were freshly prepared and matched with 0.5 Mc Farland.Micro titre plates were taken and the plates were labelled. To the growth control well 100µl of inoculums without antibiotics were added .*ATCC* control strains were added. Then 100µl of desired drug concentration was

added to each well.Last 150µl of inoculums was added to each well. Inoculated plates were incubated at 37℃ for 16-20 hours in an ambient air incubator. .

INTERPRETATION OF MINIMUM INHIBITORY CONCENTRATION :

- GROWTH CONTROL IN MICROTITRE WELL : Visible Growth or turbidity should be visible in growth control well.
- Turbidity or ≥2mm button was taken as growth .
- Highest concentration where, there is no visible growth of the organism or where there is inhibition of growth is considered as the MIC of the organismas shown in figure 7.

FIGURE 7: DETERMINATION OF COLISTIN MIC BY THE GOLD STANDARD BROTH DILUTION METHOD



MOLECULAR DETECTION :Detection of MCR-1 gene

In this study Clinical isolates of 11 in number out of 219 extensively gram negative bacilli were subjected to molecular detection which were found to be phenotypically resistant. Extraction was done by both boiling method and QIAamp DNA MINI extraction kit(QIAGEN).

DNA EXTRACTION BY BOILING METHOD

Individual colonies were inoculated into luria bertani broth incubated at 37℃ for 48 hours .Allowed for centrifugation, then incubating under water bath, then the supernatant contains DNA which is used for PCR amplification.

Materials required for boiling method:

Sterile Eppendroff tubes 1ml in size

Vortex mixer

Water bath at 76℃

Centrifuge machine at 5000rpm

Distilled water

Procedure:

Colonies inoculated into luria bertani broth were taken in eppendroff tubes,it was centrifuged at 5000rpm for 5minutes,pellets are formed,then the

supernatant were discarded,then the pellets were washed with l00µl of sterile distilled water and centrifuged again at 5000rpm for 5min.Then the washed pellets were incubated in water bath at 76℃ for 1hour.Then it is cooled for 5minutes and centrifuged again,then the supernatant was used for PCR.Extracted DNA was stored at -20℃.

DNA EXTRACTION BY KIT METHOD:

Extraction was done using QIAamp DNA MINI extraction kit(QIAGEN).Procedure for extraction was done according to the manufacturer instruction.

Instruments and materials:

PCR Thermo cycler

Eppendrof tubes

Vortex mixer

Refrigerated microcentrifuge-Legend Micro21R

Dry bath-Thermocon DB900

Micropipettes1000µl,200 µl,100 µl

Barrier tips 1000 µl and 200 µl

DNA MINI kit components:

Lysis buffer(AL)

Proteinase K

Wash buffer 1& II

Elution buffer

Spin column tube

Collection tubes of 2ml size

Storage of kit:Room temperature (15-25℃)

Extraction procedure:

Pre extraction steps

25ml of absolute alcohol was added to wash buffer 1(AW1)

30 ml of absolute ethanol was added to wash buffer2(AW2)

Dry bath was set at 56℃

Extraction steps

- Eppendroff tubes containing the inoculums were taken to this 100µl of ATL and 20µl of proteinase K was added.
- Vortex was done and incubated in dry bath at 56℃ for 1hour with intermittent vortex until the pellet were completely lysed
- From the dry bath the tube was removed and AL of 200µl was added.

- Then the tube removed from the dry bath.To this tube 200µl of absolute ethanol was added and it was vortexed.
- Without wetting the rim the lysate was transferred to the upper reservoir of a labelled spin column tube.In cold centrifuge the tube was centrifuged at 8000 rpm for 1minute.
- Contents in the collection tube were discarded.spin column was transferred to a new 2ml collection tube.Then 500µl of was added to the collection tube and then centrifuged at 8000 rpm for 1 minute
- Then 500µl of AW2 was added to the solute and centrifuged at 14000 rpm for 3 minutes
- Spin column tube was placed in a new sterile 1.5ml eppendrof tube for elution of DNA
- Elution buffer of 200µl was added to the lysate and it was held at room temperature for 5minutes.centrifugation was done for 1minute at 8000rpm and the eluted material was stored at 4℃ for PCR

  Cells get lysed during the incubation period with proteinase K due to the presence of salts and detergents present in the lysis buffers. On centrifugation it gets digested ,the DNA present in it binds to the silica in the spin column tubes.Impurities like proteins and polysaccharides are removed on subsequent washing.

PCR FOR DETECTION OF MCR-1 GENE FOR ENTEROBACTERICEAE:

Clinical isolates that were identified resistant phenotypically were exposed to molecular detection .Then the DNA Extraction was done by using quiagen kit.The primers used were obtained from Sigma Aldrich,Mumbai.

Genes	primer name and sequence(5'-3')	Amplicon size(bp)	Reference
MCR-1	CLRF-CGGTCAGTCCGTTTGTTC CLR R-CTTGGTCGGTCTGTAGGG	309	Yi-liu et al 2016

Reaction mixture:

Each single reaction mixture is 25µl ,which contains 12.5µl of Master Mix(10mm dNTPs,1U TAQ DNA polymerase,25mM $Mgcl_2$ and 2.5µl of 10X Taq buffer),5µl of DNA suspension and 1µm of forward primer,1µm of reverse primer(Sigma-Aldrich,Mumbai).The remaining volume was adjusted with PCR grade water.

Polymerase Chain Reaction PCR:

PCR was performed under following conditions95℃ for 15minutes,30 cycles of 95℃ for 30 seconds,55℃ for 30 seconds,72℃ for 1minute and 72℃ for 5minutes.

PCR FOR DETECTION OF mgrb GENE FOR *KLEBSIELLA PNEUMONIAE*:

PCR was performed for 11 clinical isolates which were identified as *Klebsiella pneumonia* and found to be resistant for colistin and MIC's of ≥8 µg in vitek ,agardilution method,microbroth dilution were subjected to PCR.The primers used were obtained from Sigma Aldrich,Mumbai.Primers used were based on the reference article from a previous study.The primers used were as follows.

Gene	primer name and sequence(5'-3')	Amplicon size(bp)	Reference
mgrb	mgrbF-TCTAGATCTTATGATGCACACCTGTCGGGCACC mgrb R-CTCGAGGACATTTTTCTGCCGACTGATTTTCTGCGC	350	Cannetelli et al 2014

Reaction mixture:

Each single reaction mixture is 25µl ,which contains 12.5µl of Master Mix(10mm dNTPs,1U TAQ DNA polymerase,25mM $Mgcl_2$ and 2.5µl of 10X Taq buffer),5µl of DNA suspension and 1µm of forward primer,1µm of reverse primer(Sigma-Aldrich,Mumbai).The remaining volume was adjusted with PCR grade water.

Polymerase chain reaction:

The PCR was performed by conventional method under following temperatures

Initial denaturation step was 180seconds at 95℃,35 cycles , denaturation67℃ for 30seconds,72℃for 30 seconds and final extension step of 300 seconds at 72℃.After amplification, the products were stored at -20℃

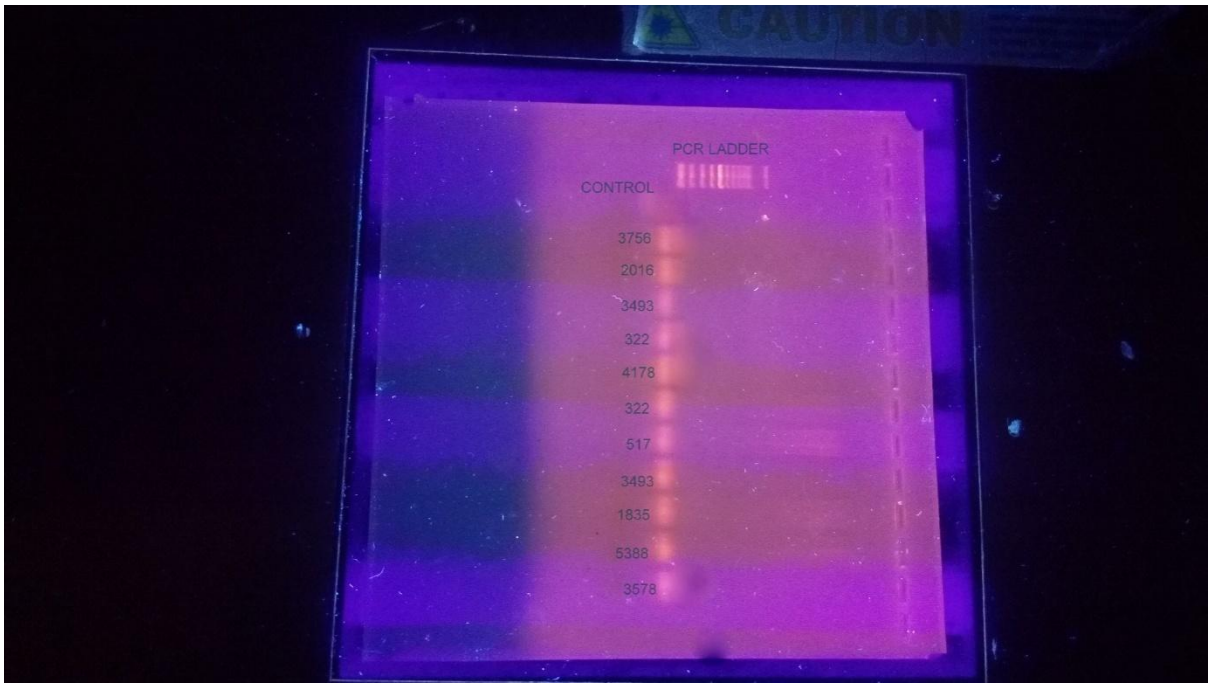
AGAROSE GEL ELECTROPHORESIS:

Agarose gel electrophoresis was done to visualise the amplified product.Agarose gel was prepared by adding 0.8% of agarose gel powder with Tris borate EDTA buffer(TBE).The mixture was heated in microwave for 2minutes until it becomes a clear solution.Ethidium bromide was added to visualize the amplified DNA product under UV light.The mixture was allowed to set in an electrophoresis tank with the comb in place.A 100 base pair molecular ladder was added as a molecular marker to the first well.and rest of the wells the amplified product of 6µl was added.After adding it was placed in a electrophoretic tank containing tris borate EDTA(TBE) at 5 volts for 10minutes followed by 96 volts for 45 minutes.

INTERPRETATION:

Ethidium bromide was used to stain the amplified DNA .It was visualised under UV illuminator. Then the images were captured by gel doc.In this study 11 clinical isolates that were subjected to PCR, was found to be negative for MCR-1 gene. Only the primer dimer of 11 isolates were seen in the gel..Further study would be done to detect the resistant pattern of colistin strains.

Figure8: Gel electrophoresis showing negative for mCR -1 gene



RESULTS AND ANALYSIS

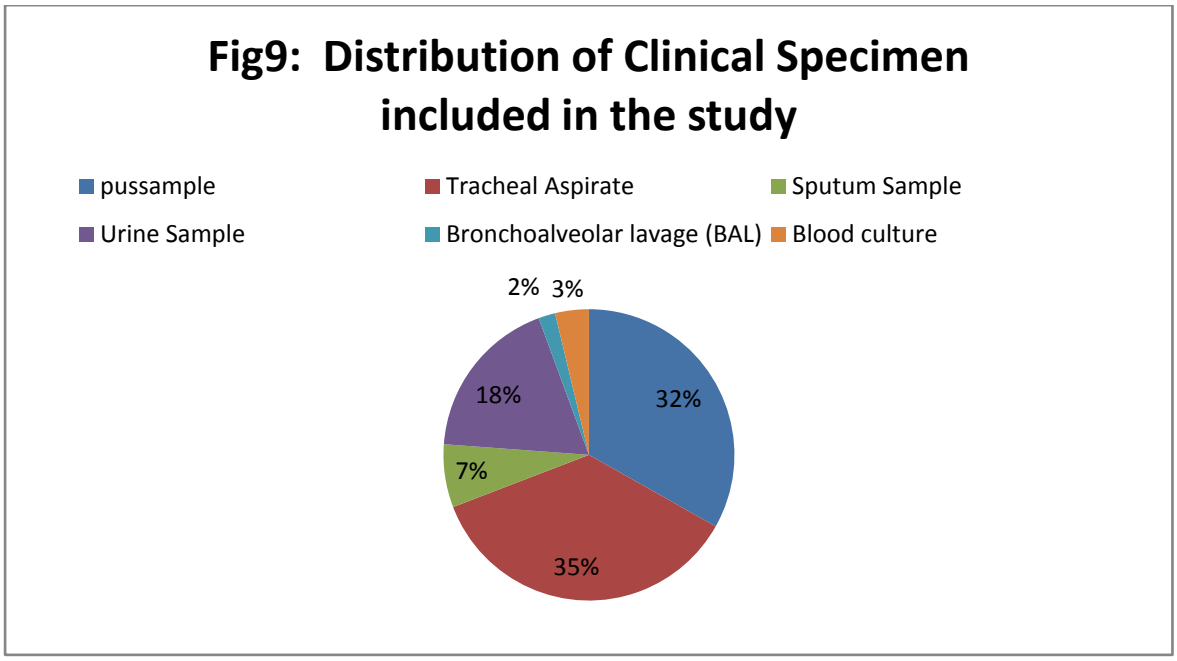
RESULTS & ANALYSIS:

Of the 219 clinical isolates included in the study most of them were from tracheal aspirate or pus sample followed by urine and others as shown in table-2

Table 2: showing distribution of clinical specimen included in the study

Sample Name	**Number(%)**
Pussample	71
Tracheal Aspirate	77
Sputum Sample	15
Urine Sample	39
Bronchoalveolar lavage (BAL)	4
Blood culture	8
Total	**219**

Distribution of clinical specimen that are included in the study is shown in fig 9

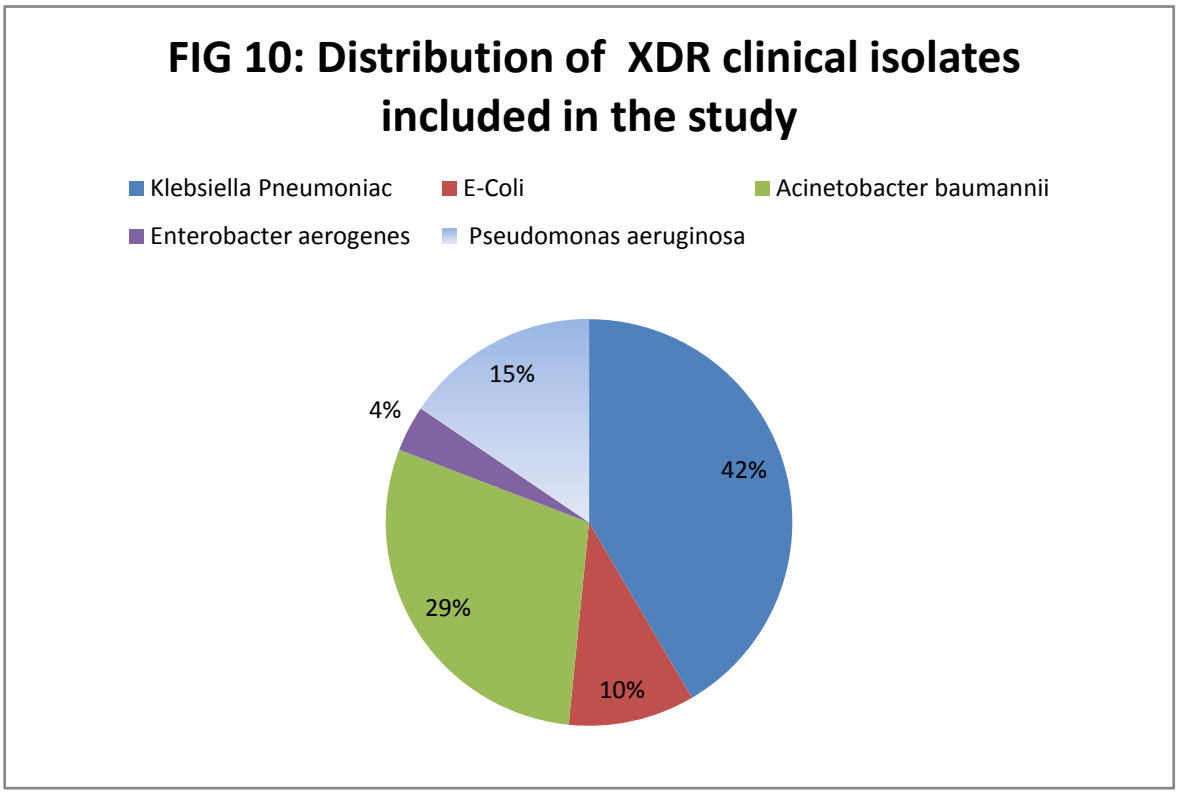


of the 219 clinical isolates *Klebsiella pneumonia* was found to be 41%,followed by *Acinetobacter baumannii*(29%),*Pseudomonas aeruginosa*(15%) respectively as shown in table 3.

Table3:SHOWING DISTRIBUTION OF CLINICAL ISOLATE INCLUDED IN THE STUDY

ISOLATES	Number(%)
Klebsiella pneumoniae	91(41.55)
E coli	22(10.05)
Acinetobacter baumannii	64(29.22)
Enterobacter aerogenes	8(3.65)
Pseudomonas aeruginosa	34(15.53)
TOTAL	**219(100)**

Out of 219 gram negative isolates *klebsiella pneumoniae* was higher, which was around 42%,*Acinetobacter baumannii* was 29%,followed by *Pseudomonas aeruginosa* 15%,*E.coli* was 10% and *Enterobacter aerogenes*was 4%.Distribution of these extensively drug resistant isolates are shown in fig10.



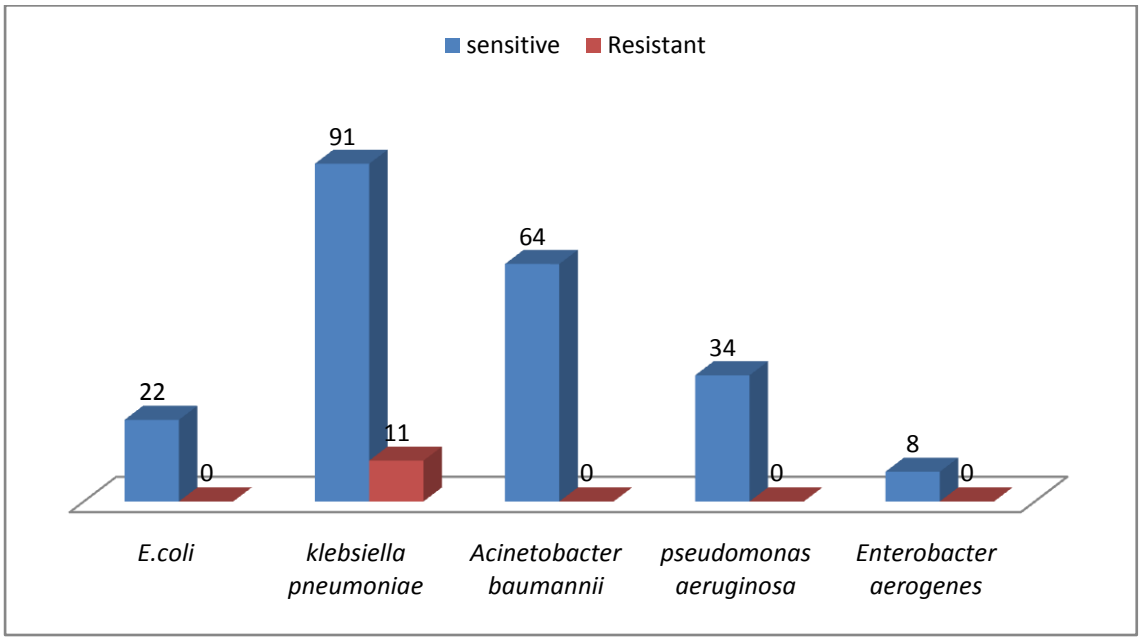
Out of the 219 extensively drug resistant clinical isolates of gram negative bacilli that were subjected to Colistin susceptibility testing by various phenotypic methods , the agar dilution method was comparable with the Gold standard broth dilution method . Discdiffusion method was found to be the least useful with only 196 (94%) isolates correctly identified as sensitive. In Vitek – 2 system identified 205 out of 208 sensitive isolates. As shown in table4.

TABLE: 4 SHOWING THE RESULTS OF PHENOTYPIC METHODS OF COLISTIN SUSCEPTIBILITY

S.NO	PHENOTYPIC METHODS	SENSITIVE
1	DISC DIFFUSION	196(94%)
2	VITEK 2COMPACT	205(98.5%)
3	AGAR DILUTION	208(100%)
4	BROTH DILUTION	208(100%)

Of the 219 XDR isolates 91 isolates were *klebsiellapneumoniae* ;out of 91isolates 11 were found to be colistin resistant,no reistant strain for other organism,64 isolates were *acinetobacter* , 34 isolates were *pseudomonas aeruginosa* followed by others as shown in fig11.

FIG11: **SHOWING DISTRIBUTION OF SUSCEPTIBILITY OF XDR CLINICAL ISOLATES INCLUDED IN THE STUDY**



In our study Colistin susceptibility testing was compared with the standard reference method there were discordant numbers seen in the disc diffusion method and Vitek .Agar dilution showed the same susceptibility pattern with the reference method their values are shown in table5.

TABLE:5 COMPARISION OF COLISTIN SUSCEPTIBILITY BY VARIOUS PHENOTYPIC METHODS AGAINST THE REFERENCE METHOD

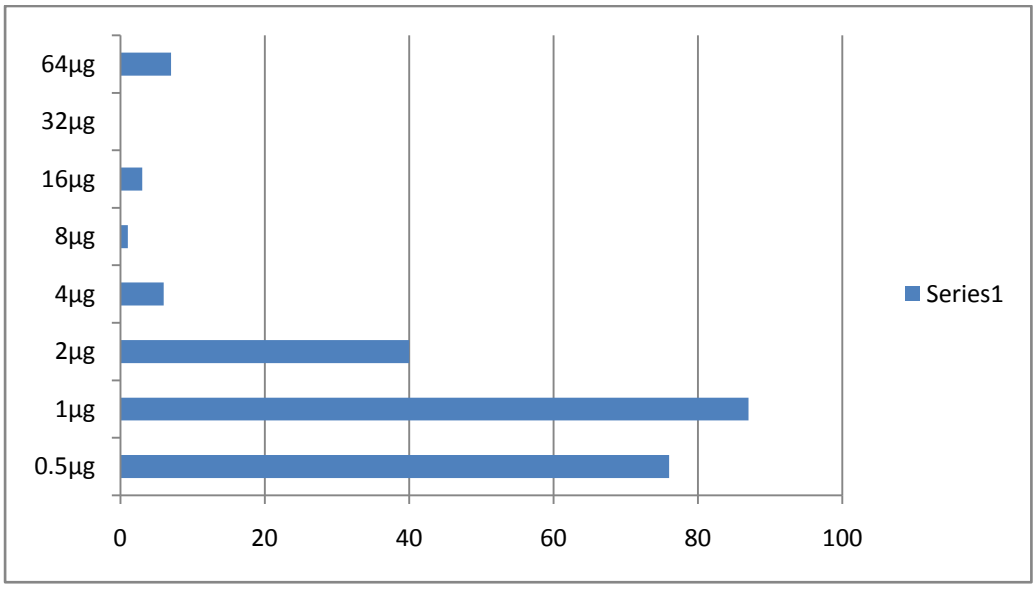
S.NO	METHODS	SENSITIVE	RESISTANT	DISCORDANT NO
1	DISC DIFFUSION	192	11	16
2	VITEK 2COMPACT	203	11	5
3	AGAR DILUTION	208	11	-

Minimum inhibitory concentration values of XDR isolates from higher concentration to lower concentration that is from 64µg to 0.5µg.out of 219 XDR isolates 7 isolates had an MIC of 64µg,3isolates had an MIC of 16µg,1 isolate was 8µg,further lower concentration of MIC values are shown in table6 and represented in Fig.12.

TABLE6: SHOWING THE MIC OF COLISTIN AMONG THE XDR ISOLATES TESTED BY MICRO BROTH DILUTION METHOD

MIC VALUES	NO OF ISOLATES
0.5µg	76
1µg	87
2µg	40
4µg	6
8µg	1
16µg	3
32µg	-
64µg	7
Total	219

FIG12: SHOWING THE MIC OF COLISTIN AMONG THE XDR ISOLATES TESTED BY MICRO BROTH DILUTION METHOD



In our study out of 219 clinical isolates there were 11 isolates that were resistant to colistin ,resistant strain were seen during the period of March 2015 to December 2015. 5 strains were resistant during the period of march,4 strains were found to be resistant during the period of October and 2 strains were found resistant during the period of December 2015.After that there were no resistant strains found.They are represented in the Fig 12.

FIG-12: ISOLATION OF COLISTIN RESISTANT ISOLATES DURING THE PERIOD OF STUDY

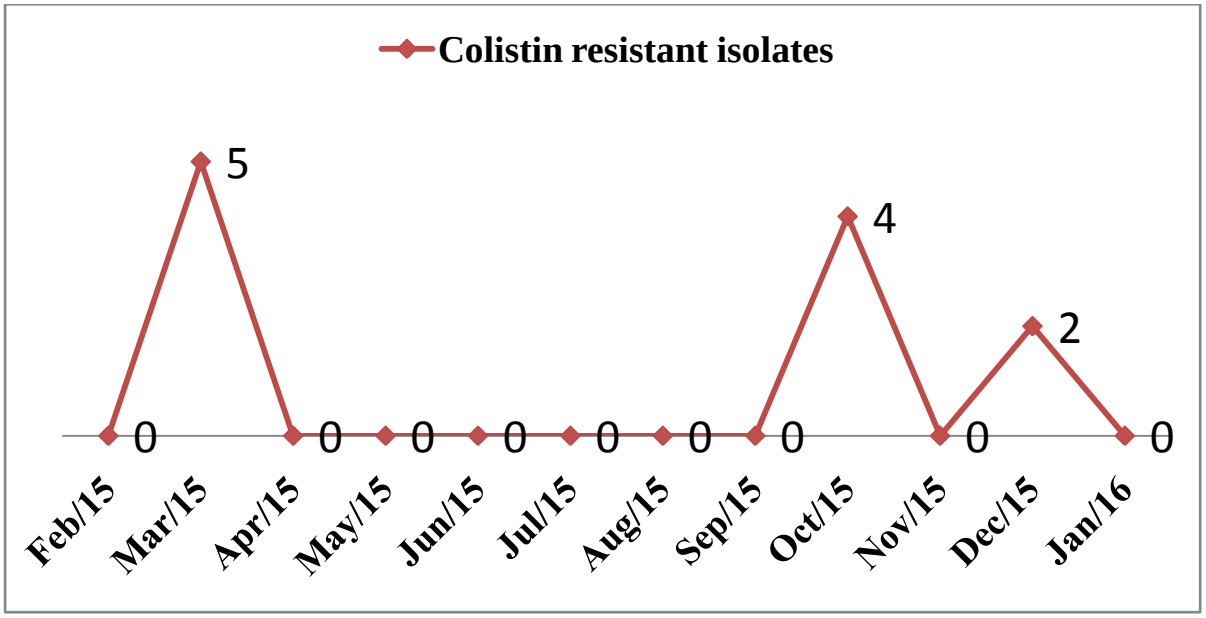


TABLE7: CLINICO MICROBIOLOGICALPROFILE OF PATIENTS WITH COLISTIN RESISTANT ISOLATES

S. No	LAB NO	PATIENT AGE/SEX	PATIENT DIAGNOSIS	℞	CLINICAL SAMPLE	ISOLATED ORGANISM	OUTCOME
1	5388	86/F, PULMO	Intracranial hemorrhage	Inj.mr, inj.col	Wound swab	*k.pneumoniae*	AMA
2	237	72/M	Diabetic ulcer, Amputation done	Inj.mr,	Wound swab	*k.pneumoniae*	Discharged
3	4178	66/F, SURGERY	CA rectum	Inj.mr	trachealaspirate	*k.pneumoniae*	Death
4	322	45/M, HPBLT	DCLD case	Injmr,	BAL	*k.pneumoniae*	Death
5	517	54/M, HPBLT	Dcld case	Inj.mr	urine	*k.pneumoniae*	Discharged
6	2016	42/M, HPBLT	Dcld case	Inj.col, Inj.dr	Pigtail drain	*k.pneumoniae*	AMA
7	3451	79/M, MICU	Post jejunostomy	Inj.col, inj.van	Tracheal aspirate	*k.pneumoniae*	Death
8	3756	39/F, MICU	Postlscswound ,peritonitis	Injmr, inj.col	urine	*k.pneumoniae*	Death

9	3369	27/M, NEURO	RTAcase, craniotomy	Inj.mr ,inj.col	Tracheal	*k.pneumoniae*	Discharged
10	4071	58/M, HPBLT	cholangitis	Inj.mr,	Pig tail	*k.pneumoniae*	Death
11	3493	80/F, MICU	Bullous ph	Inj.col, inj.van	Blood culture	*k.pneumoniae*	Death

S. No	LAB NO	CLINICAL SAMPLE	ISOLATED ORGANISM	MICROBROTH µg/ml	AGAR DILUTION µg/ml	VITEK	DISCDIFFUSION
1	5388	Wound swab	*k.pneumoniae*	8	8	≥8	Resistant
2	4071	Wound swab	*k.pneumoniae*	≥64	8	8	Resistant
3	4178	Trachealaspirate	*k.pneumoniae*	≥64	16	≥16	Resistant
4	322	BAL	*k.pneumoniae*	≥64	8	≥8	Resistant
5	517	Urine	*k.pneumoniae*	16	8	8	Resistant
6	2016	Pigtail drain	*k.pneumoniae*	16	8	8	Resistant
7	3451	Tracheal aspirate	*k.pneumoniae*	16	8	8	Resistant
8	1835	Tracheal aspirate	*k.pneumoniae*	≥64	16	≥8	Resistant
9	3578	Tracheal	*k.pneumoniae*	≥64	8	≥16	Resistant
10	3756	Pig tail	*k.pneumoniae*	≥64	16	≥8	Resistant
11	3493	Blood culture	*k.pneumoniae*	≥64	16	≥8	Resistant

DISCUSSION

DISCUSSION:

Colistin is used as a last resort of drug to treat severe infections caused by XDR organisms. Over the yeas the usage of colistin is increased by about 10 fold. Due to increased usage of colistin there is rise in resistance. The prevalence of resistance to colistin are less recognized and documented in literature

Although resistance to polymyxins is generally less than 10%, it is higher in the Mediterranean and South-East Asia (Korea and Singapore), and in these areas colistin resistance rates are continually increasing.Organisms that are more prone for colistin resistance are carbapenem producing *K.pneumoniae*[101]*,Acinetobacter spp,Pseudomonas spp.*In our study out of 219 XDR isolates tested 11 isolates were found to be phenotypically resistant, to colistin and all the 11 isolates were *Klebsiella pneumoniae*.

Increasing MIC of colistin has been reported world wide. [13].Many studies indicate that colistin MIC Value of≥4µg/ml are known to produce increasing mic value which leads to resistance.In our study out of 219 samples,6samples were found to be ≥4µg/ml out of 6 isolates 4 were *Pseudomonas aeruginosa* and 2 were *Klebsiella pneumoniae* which represents that they are moving towards resistance.

SENTRY antimicrobial surveillance reported from2006 to 2009 have also shown that isolates with polymyxins with an MIC value of≥ 4µg /ml showed resistance in *Acinetobacter* isolates which was detected in the regions

of USA(1.1%), 0.9% in latin America followed by APAC region and Europe which was around 0.7% and 0.4% respectively.heyner et al

Micro broth dilution method is considered as a gold standard reference method. Various studies on broth dilution method recognised that heteroresistance to colistin can be detected by this method . This helps to prevent antibiotic adjusting and to prevent inappropriate usage of antibiotics.Heteroresistance or the subpopulation was noted in *Acinetobacter baumannii.*[102].In our study there were no subpopulation or heteroresistance detected

Other than microbroth reference method, automated colistin susceptibility testing methods play a role in detecting the resistance and sensitivity based on the MIC values.Automated method such as vitek compact2 identifies the resistance/sensitive based on the MIC values.Many studies previously have stated that vitek invitrosusceptibility testing have shown concordant values and holds good in susceptibility testing for colistin on comparing with the golds standard[55].But studies recently document that vitek especially for enterobactericeae was found to be unreliable compared with the reference method[56].In our study out of 219 isolates 5 isolates showed discordant results, out of 5 isolates 3were *Pseudomonas aeruginosa* and 2 were *Klebsiellapneumoniae* in vitek, that is in reference method they were found to be sensitive were as in vitek they were found to be resistant.

Disc diffusion method is not a reliable method for susceptibility testing of colistin. Disc diffusion performing on the Mueller hinton agar shows poor penetration of the drug.Isosensitest agar can be used to detect heteroresistance[55].In our study out of 219 isolates 16 isolates have shown discordant results,that is in disc diffusion method it is resistant where as in reference method it is sensitive. Isosensitest agar can be preferred as per BSAC guidelines.

MCR-1 gene coding for colistin resistance are on the plasmid , which transfers rapidly from one cell to other .it has been identified by research workers from China . Though many researchers are trying to various genotypic methods to detect MCR -1 gene , till date no reports of MCR-1 in India have been reported.[76]In our study out of 219 isolates,phenotypically 11 isolates were found to be resistant,those isolates were subjected to molecular detection of MCR-1 gene ,but they were found to be negative.Those 11 isolates negative for MCR-1, may be due to other resistant gene such as MCR-2 which is also a plasmid mediated gene isolated in Belgium during June 2016,or due to chromosomal mediated resistant gene ,alteration in the two component system. Strict infection prevention and control measures should be initiated at the earliest as these mobilecan be easily transferred between organisms.

Clinical outcome of the patient is better in combination therapy rather than monotherapy[96].out of 11 patients who developed colistin resistant ,seven patients were in combination therapy 5 patient's were treated in combination of carbapenem with colistin.Two patient was normally discharged,other 5 patients

expired in combination therapy.In a study by Garnacho-Montero, clinical cure of 57% was observed in combination therapy. Out of 11 patients two patient's were previously treated with colistin.It is known that patient on colistin therapy they develop resistance.

Antibiotic stewardship should be enforced to prevent and control extensively drug resistant gram negative organisms. In our study , colistin resistance was found to be the highest during the period of March 2015 and least during Dec2015 and not isolated so far since January 2016 .Strengthened antimicrobial stewardship program by the infection control team of the institute maybe one of the resaons . Pre-authorization of high end antibiotics , restricting the usage of High end antibiotics including colistin and providing feedbacks to the treating physicians was implemented since October 2015 .There was a marked drop in the usage of colistin followed by reduced XDR isolates .

In critical wards and in pre transplant patients routine screening such as rectal swabs,nasopharyngeal secretions for isolation for XDR gram negative organisms should be done.

SUMMARY

SUMMARY:

- Rising colistin resistance among clinical isolates from seriously ill patients are alarming, as there are no or limited therapeutic options
- . To preserve this last resort drug effective measures should be taken to identify their resistance as early as possible ..
- Around 219 clinical isolates of XDR strains were collected which was routinely identified by the disc diffusion method and automated method vitek 2 compact.
- Minimum inhibitory concentration (MIC) of the isolates were determined by three methods - Microbroth dilution (standard reference method).Automated vitek 2 compact,Agar dilution method.
- Agar dilution method when compared with microbroth dilution method ,the susceptibility pattern was similar there were no discordant numbers.
- In vitek 2 compact 5 isolates were found to be resistant, but it was sensitive in reference method.
- In disc diffusion method 16 isolates did not match the reference method.
- Disc diffusion should not be used to determine colistin susceptibility
- Eleven Clinical isolates found to be resistant by the gold standard broth dilution method was subjected to PCR for detection of MCR-1 plasmid mediated gene

- All the 11 isolates o *Klebsiella pneumoniae* were found to be negative for MCR-1 gene.Resistance maybe attributed to MCR-2 gene or other mechanisms.
- Combination therapy showed a good outcome among patients rather than monotherapy
- Further studies are required to identify the gene/s responsible for resistance and simple screening method to detect colistin resistance.

CONCLUSION

CONCLUSION:

Microbroth dilution the gold standard method should be used for colistin susceptibility testing..As this method is cumbersome and cannot be performed routinely everyday , alternative methods such as agar dilution, automated method vitek 2 compact can be used. There are no new antibiotics against Gram negative organisms in the pipeline therefore Colistin the last resort of drug should be preserved by Implementing antibiotic stewardship and appropriate infection control measures.

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APPENDIX

APPENDIX

- **ISOSENSITEST AGAR: composition**

Typical Formula*	**gm/litre**
peptone	3gm
Hydrolysed casein	11.0
Glucose	2.0
Sodium chloride	3.0
Disodium hydrogen phosphate	2.0
Sodium acetate	1.0
Magnesium glycerophosphate	0.2
Calcium gluconate	0.1
Cobaltous sulphate	0.001
Cupric sulphate	0.001
Zinc sulphate	0.001
Ferrous sulphate	0.001
Manganous chloride	0.002
Menadione	0.001
Cyanocobalamin	0.001
L-Cysteine	0.02

hydrochloride	
L-Tryptophan	0.02
Pyridoxine	0.003
Pantothenate	0.003
Nicotinamide	0.003
Biotin	0.0003
Thiamine	0.00004
Adenine	0.01
Guanine	0.01
Xanthine	0.01
Uracil	0.01
Agar	8.0

pH 7.4 ± 0.2 @ 25°C

31.4 grams of isosensitest agar was suspended in 1litre of distilled water. After dissolving ,the media was sterilised by autoclaving at 121°C for 15 minutes.

- **Cation adjusted Mueller Hinton broth**

Ingredients Gms / Litre

Beef, infusion from 300.000

Casein acid hydrolysate 17.500

Starch 1.500

Final pH (at 25°C) 7.3±0.1

**Formula adjusted, standardized to suit performance parameters

ANNEXURES

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

To
Dr T Kalaiselvi
Postgraduate
Department of Microbiology
PSG IMS & R
Coimbatore

Ref: Project No. 14/394

Date: December 15, 2014

Dear Dr Kalaiselvi,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 05.12.2014 to conduct the research study entitled "*Study of colistin sensitivity among clinical isolates of extensively drug resistant gram negative bacilli*" during the IHEC meeting held on 12.12.2014.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol
3. Confidentiality statement
4. Application for waiver of consent
5. Proforma
6. Current CVs of Principal investigator, Co-investigators
7. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 12.12.2014 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Dr. P. Sathyan (Chairperson, IHEC)	DO, DNB	Clinician (Ophthalmology)	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr. S.Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr. D. Vijaya	M Sc, Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

Dr S Bhuvaneshwari
Member-Secretary
Institutional Human Ethics Committee

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INSTITUTIONAL HUMAN ETHICS COMMITTEE

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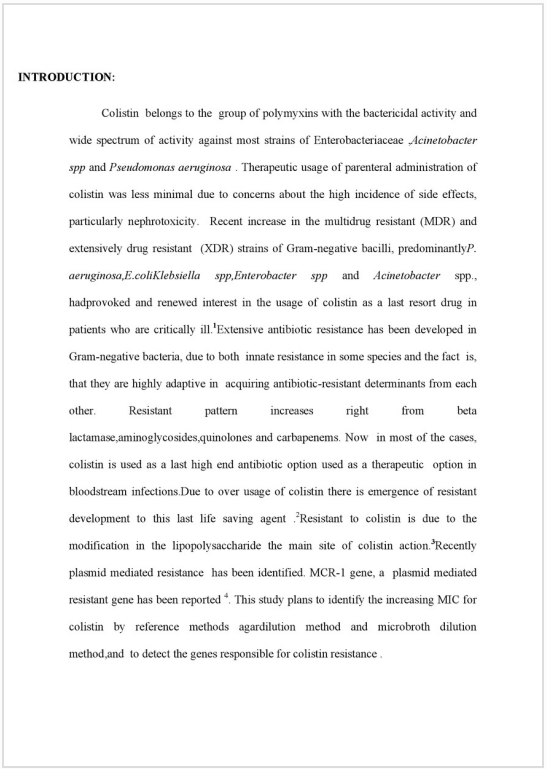
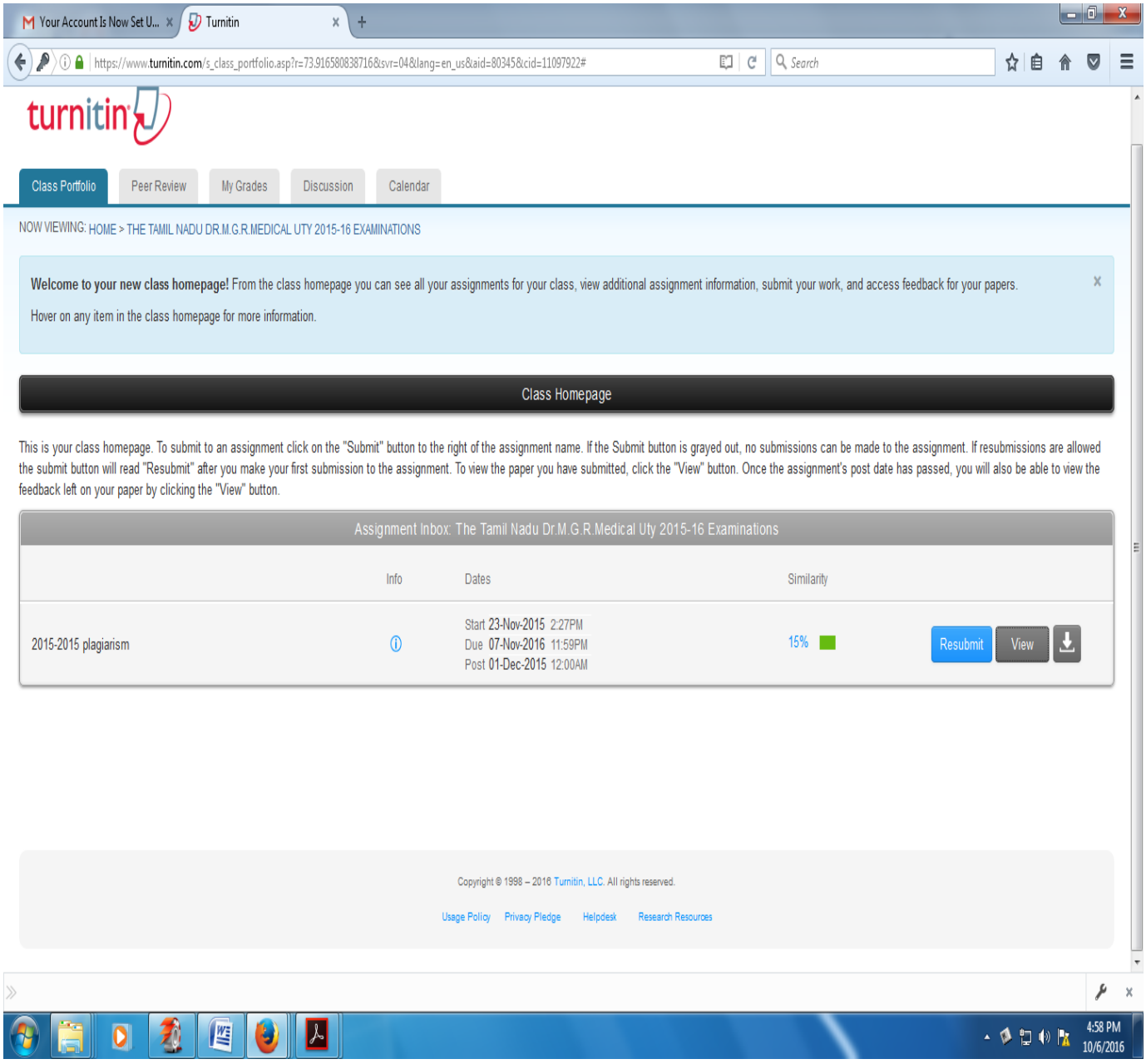


INTRODUCTION:

Colistin belongs to the group of polymyxins with the bactericidal activity and wide spectrum of activity against most strains of Enterobacteriaceae ,*Acinetobacter spp* and *Pseudomonas aeruginosa* . Therapeutic usage of parenteral administration of colistin was less minimal due to concerns about the high incidence of side effects, particularly nephrotoxicity. Recent increase in the multidrug resistant (MDR) and extensively drug resistant (XDR) strains of Gram-negative bacilli, predominantly*P. aeruginosa,E.coliKlebsiella spp,Enterobacter spp* and *Acinetobacter* spp., hadprovoked and renewed interest in the usage of colistin as a last resort drug in patients who are critically ill.[1]Extensive antibiotic resistance has been developed in Gram-negative bacteria, due to both innate resistance in some species and the fact is, that they are highly adaptive in acquiring antibiotic-resistant determinants from each other. Resistant pattern increases right from beta lactamase,aminoglycosides,quinolones and carbapenems. Now in most of the cases, colistin is used as a last high end antibiotic option used as a therapeutic option in bloodstream infections.Due to over usage of colistin there is emergence of resistant development to this last life saving agent .[2]Resistant to colistin is due to the modification in the lipopolysaccharide the main site of colistin action.[3]Recently plasmid mediated resistance has been identified. MCR-1 gene, a plasmid mediated resistant gene has been reported [4]. This study plans to identify the increasing MIC for colistin by reference methods agardilution method and microbroth dilution method,and to detect the genes responsible for colistin resistance .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