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ORIGINAL ARTICLE

Isolation and Characterization of Multidrug Resistant Bacterial Strains from Hospital Settings

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ABSTRACT
Multidrug resistance (MDR) is one of the severe global health threats confronting mankind linked with greater mortality, morbidity and economic expenses. The clinically isolated bacteria responsible for difficult-to-treat nosocomial infections are significantly important. This present investigation aimed to isolate, characterize and identify the multidrug resistance bacteria from the clinical samples collected from SUM hospital, Bhubaneswar, Odisha, India. The predominant bacterial strains were isolated and their phenotypic, biochemical, and antibiotic susceptibility tests were performed. Identifications of bacterial strains were performed using VITEK-II system and 16S rRNA sequencing. A total of ten predominant bacterial strains (80% gram-negative and 20% gram-positive) were characterized and antibiotic resistance patterns were determined. Bacterial isolates BT_MDR2 identified as Klebsiella pneumonia was resistant against all the tested antibiotics except colistin with MIC value (≥ 128 µg/ml) for Ticarcillin/Clavulanate and Piperacillin/Tazobactam. Similarly, the bacterial isolate BT_MDR9 was identified as Enterococcus faecalis, which showed resistance against various antibiotics with MIC value (≥ 64 µg/ml) for benzyl penicillin. Clinical sample revealed the presence of diverse MDR bacterial strains resistant against commercially available antimicrobials agents. This investigation emphasized the urgency of early identification of MDR bacterial pathogens to develop novel antimicrobial agents to reduce disease burden.
Keywords: Antibiotic susceptibility, Biochemical characterization, Multidrug resistance.

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INTRODUCTION

Multidrug resistant (MDR) bacteria pose greater threat to the public healthcare and economic sector worldwide [1,2]. MDR is referred to acquired ability of microbes to resist at least three or more antimicrobial categories [3]. Increasing cases of infections with MDR bacterial strains have risen alarmingly and responsible for seven lakh deaths per year globally and estimated ten million deaths by 2050 [4]. MDR causes stumbling blockage in disease control and significantly reduces the drug effectiveness associated with higher rate of mortality and morbidity [5]. MDR pathogens includes both gram-positive and gram-negative bacteria including S. aureus, A. baumannii, K. pneumoniae, E. faecium, E. faecalis, P. aeruginosa, E. coli and Enterobacter sp. spreading rapidly worldwide [6]. Nosocomial infections led by MDR pathogens are evading the bactericidal activities of antimicrobial drugs and creating new paradigms in pathogenesis, resistance mechanism and transmission [5,8,9]. The MDR bacterial strains are not only limited to the hospital settings and clinical regions, but also prevalent in all environmental samples [4,9]. Sources of MDR bacteria include wastewater, soil, sewage, raw meat, livestock and dairy products, gastrointestinal tract, respiratory system, skin of humans and animals [10,11]. Although, the antimicrobial resistance in microbes is a natural phenomenon that develops over time, but excessive and improper of antibiotics in human, animals and aquaculture, inadequate hygienic conditions, accumulation of antibiotics in environment, extreme intake of different wide-spectrum antimicrobial agents, lack of aseptic diagnostic devices in healthcare and lack of antimicrobial stewardship are considered to be the primary factors responsible for accelerating the persistence and

spreading of MDR in the environment [12-16]. The present study demonstrated that the polluted habitats possess relatively higher resistant bacteria compared to natural habitats, which indirectly imply that human have significantly contributed to increased proportion of MDR in the environment [17]. Besides, MDR strains have dramatically increased healthcare expenditure and limited therapeutic options to treat infections [18-20]. Multidrug resistance exhibited by the bacterial pathogens is a complex process that involves molecular and cellular based bacterial machineries to avert, expel, negate, destroy or withstand antimicrobial agents [21]. Bacterial resistance against antimicrobial agents is classified as intrinsic, acquired and/or adaptive [22,23]. The term intrinsic/inherent resistance refers to the inbuilt structural and functional characteristics of microorganisms (presence of restricted outer membrane or constitutive efflux pumps) to limit the efficacy of antibiotics [10,24]. Secondly, acquired resistance involves incorporating additional genetic material (plasmids, transposons, integrons or bare DNA) through the horizontal gene transfer (conjugation, transduction or transformation) or by mutations in targeted genes caused by selective pressure and natural selection [23, 25, 26]. However, the adaptive resistance has transient nature, which provides relatively greater ability of bacteria to withstand the antimicrobial action through alternations in gene expression induced by the environmental triggers but often reverts to original form after removal of inducing factor [23]. Adaptive resistance triggers the evolution of more effective and persistent resistance mechanisms. Mechanisms of resistance exhibited by MDR pathogens to resist antibiotic toxicity are classified into four categories such as (a) limiting drug uptake (especially in gram-negative bacteria) that limits uptake of antimicrobial agents by reducing outer membrane permeability); (b) modifications and alterations in drug target (includes point mutation in gene encoding target site, change in target site of enzyme and bypassing original site) (c) drug inactivation or modification either by enzymatic degradation of drug molecule or by addition or removal of specific moiety to drug molecules by enzymes synthesized; and (d) activation of drug efflux pumps (to evacuate drug molecules out of the cell and confers target resistant) [5,6]. Due to the structural differences, the mechanism of resistance conferred by gram-positive and gram-negative bacteria varies from each other. Moreover, presence of lipopolysaccharide layer in gram-negative pathogenic bacteria renders them more resilient to antimicrobial agents in contrast to gram positive bacteria [16]. Furthermore, gram negative strains are the culprits for majority of the nosocomial infections and considered as critically priority MDR bacteria. Nosocomial infections by MDR pathogenic bacteria are life-threatening that constitute global challenges and thereby new innovative strategies to reduce the prevalence of MDR pathogens is prerequisite. Several investigations have suggested the hospital setting as the hotspot for antibiotic resistance inputs from the bacterial strains and metabolized drugs from the patient excrement potentially contains MDR pathogens that impose negative impacts on public health [5, 17,27-30]. Several researchers reported that quick spread of genes encoding resistance in MDR pathogens is due to uncontrolled use of antibiotics by human, selection pressure on resistant bacteria to adapt the adverse conditions and release of hospital effluents [16, 31]. Clinically isolated bacterial pathogens serve as an antibiotic resistance reservoir and possible source of genes encoding resistance. In the present-day context, increasing antibiotic resistance by MDR pathogens is a major concern for clinical therapeutics. Taking into account, the study was designed with an aim to isolate MDR bacteria pathogens followed by their microbiological and biochemical characterization and susceptibility test using Kirby-Bauer method. Subsequently, the MDR bacterial pathogens were identified and confirmed through VITEK-II analysis and 16S rRNA ribotyping. The proposed study was designed with an objective to isolate MDR bacterial pathogens from hospital settings not only for the early detection but also the rapid screening of novel antibacterial agents that inhibit their transmission and pathogenesis.

MATERIAL AND METHODS

Study area and sampling

Various clinical samples (urine, stool, pus, blood, body fluids and swabs) were taken from hospitalized patients (IMS and Sum Hospital, Bhubaneswar, Odisha) for the isolation of bacteria. The study was approved by institutional ethical committee of IMS and Sum Hospital (IEC Code No.: IMS SH/IEC/2018/37).

Sample processing and bacteria isolation

The clinical samples collected were headed to laboratory carefully in cool and sterile circumstance. Clinical samples were inoculated into different enrichment media such as nutrient agar, blood agar and MacConkey agar and incubated for 24 hours at optimal temperature 37°C in a BOD incubator for proper bacterial growth. After incubation, the colonies so formed on different media plates were picked and continuously sub-cultured through quadrant streaking to obtain pure culture. Pure bacterial cultures were stored in glycerol stock at 4°C for further analysis. Moreover, MTCC 109 and MTCC 439 were used

as reference strains, which were procured from Institute of Microbial Technology (IMTECH), Chandigarh, India.

Phenotypic and Biochemical characterization of bacterial isolates

Colonies from purified bacterial culture were subjected for preliminary identification by phenotypic characterization. Various biochemical characterizations such as catalase, coagulase, oxidase, methyl red, indole, Voges-Proskauer, oxidation fermentation, citrate, urease, triple-sugar-iron, nitrate reduction, carbohydrate fermentation, phenylalanine deaminase and motility test were performed following standard microbiological methods [32]. MTCC strains were used as reference in each biochemical analysis.

Antibiotic susceptibility test by Kirby-Bauer's method

All the bacterial isolates from clinical samples and two MTCC reference strains used in the study were screened for antibiotic sensitivity tests by Kirby-Bauer disc-diffusion assay using the standard Mueller-Hinton agar [32]. For the purpose, an aliquot of 10 µl of 0.5 McFarland equivalent overnight grown bacterial culture was spread on already solidified MHA agar followed by introducing high potency different antibiotic discs separately over MHA agar plates and incubated at 37°C for 24 hours in a BOD incubator. The diameter of zone of inhibition (in mm) were recorded using antibiotic zone scale following the standard antibiotic susceptibility test chart of Clinical Laboratory Standard Institute (CLSI) guidelines. In the study, twelve standard antibiotic discs such as amoxiclav, ampicillin, methicillin, gentamicin, amikacin, ciprofloxacin, nalidixic acid, ofloxacin, levofloxacin, colistin, cefepime and tigecycline were used against gram-negative bacteria, whereas fifteen commercial antibiotic discs such as penicillin, ampicillin, methicillin, amikacin, gentamicin, cefotaxime, cefepime, ciprofloxacin, levofloxacin, azithromycin, linezolid, erythromycin, vancomycin, clindamycin and tetracycline were used for antibiotic susceptibility test against the gram-positive bacteria.

Identification of bacteria using VITEK-II system

The ID-GPC test cards are substituted with 46 fluorometric tests including change in pH tests and various derivatives for aminopeptidases and -osidases detection. Various substrates for aminopeptidases tests are associated with 7-amino-methylcoumarin (7AMC), whereas substrates for -osidases detection are associated with 4-methylumbelliferone (4MU). In the present study, 21 different test substrates were used including ten diverse 4MU derivatives and eleven diverse 7AMC derivatives. Additionally, ID-GPC card are substituted with 16 fermentation tests using D-maltose, D-galactose, D-raffinose, D-amygdaline, D-sorbitol, D-xylose, D-glucose, L-arabinose, D-Trehalose, D-melibiose, D-mannitol, N-acetylglucosamine, lactose, salicin, glycerol, arbutine as substrate, decarboxylase test and six miscellaneous tests. The GN test card differentiate fermenting and non-fermenting Gram-negative bacilli. Similarly, VITEK-II GN card used for 47 biochemical tests to measure carbon utilization, inhibition/resistance and enzymatic activities. The card was loaded and sealed in vacuum condition and then inserted into VITEK-II reader-incubator module at 35.5°C. Kinetic fluorescence was measured in every 15 min. The outcomes were analyzed using the ID-GPC and ID-GNB database.

Bacteria identification by 16S rRNA sequencing

Genomic DNA of bacteria were extracted following the protocol prescribed by Quick-DNA™ Fungal/Bacterial Miniprep Kit (Catalogue No.: D6005). Extracted DNA concentration was measured by Nanodrop and stored at -80°C for future analysis. Ratio of absorbance at 260 nm and 280 nm was measured for DNA purity assessment. Ratio between (~1.8 to 2.0) reveals purity of isolated DNA. 16S rRNA gene amplification was done by PCR with the following reaction mixture (Forward and reverse primers: 10 pmol each, $MgCl_2$: 2.5 mM, 200 µM each four dNTPs, Taq DNA polymerase: 0.5 U, 1X PCR buffer and 50-100 ng of isolated genomic DNA) using specific primers 27F (5′AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′TACGGTTACCTTGTTACGACTT3′). Amplification by PCR involves template denaturation by heating at 95°C for 5 min, followed by 39 cycles of denaturation for 30 sec at 95°C, annealing for 45 sec at 72°C and 1 min elongation at 72°C and 7 min final extension at 72°C. Amplicons were resolved in 0.8% agarose gel electrophoresis using 1X TAE buffer at 50V for 30-45 min until DNA fragments were well migrated and bands in agarose gel was documented using gel documentation system. The obtained PCR products were purified and sequenced (ABI 3730XL, Thermo Fisher Scientific). The derived sequences were compared for homology using NCBI BLAST database [34]. Multiple sequence alignment of 16S rRNA gene sequences were generated and the phylogenetic tree was constructed using MEGA 11.0 software [35].

RESULTS

In the present study, total ten clinical bacterial isolates and two reference strains (MTCC 439 and MTCC 109) were analyzed. Morphological characterization of the clinically isolated bacterial strains revealed

that 80% bacterial isolates (BT_MDR1 to BT_MDR8) were found to be rod shaped and gram-negative bacteria (n=8) and 20% of the bacterial isolates (BT_MDR9 and BT_MDR10) were cocci and gram-positive bacteria (n=2). Reference strains MTCC 109 was rod shaped, gram-negative bacteria whereas MTCC 439 found as coccus and gram-positive bacteria (Table 1). Biochemical characterization of clinical bacterial strains (BT_MDR1 to BT_MDR10) revealed wide variation (Table 1). All the clinical isolates except BT_MDR9 showed positive result for catalase test. Bacterial isolate (BT_MDR10) was found to be coagulase positive. None of the bacterial isolates were able to produce oxidase enzyme and revealed negative result for oxidase production except BT_MDR4, which was found to be oxidase positive. Indole test revealed that two bacterial isolates (BT_MDR1 and BT_MDR9) out of ten were determined to be indole positive. It is evident from the methyl red (MR) test that only three bacterial isolates (BT_MDR2, BT_MDR4 and BT_MDR9) showed positive result whereas the rest seven bacterial isolates revealed negative result for methyl red test. Three bacterial isolates (BT_MDR2, BT_MDR9 and BT_MDR10) out of all clinically isolated bacterial strains were observed to be positive for Voges Proskauer (VP) test whereas rest seven isolates showed negative result. Oxidation fermentation test revealed wide variations among the clinically isolated bacterial strains with one isolate (BT_MDR4) as oxidative, one isolate (BT_MDR3) as facultative and the rest were found to be fermentative in nature. Bacterial isolates (BT_MDR2, BT_MDR3 and BT_MDR4) were observed to utilize citrate as the energy source and showed positive result for citrate test. Among all the clinically isolated bacterial strains, three isolates (BT_MDR2, BT_MDR3 and BT_MDR10) were observed to produce urease enzyme and revealed positive result for urease test, whereas the rest seven isolates showed negative results. Besides, all the bacterial isolates showed positive result for nitrate reduction (NR) test. Moreover, the triple sugar iron test and carbohydrate fermentation test revealed wide variations among the isolates. Further, the mortality test revealed that six bacterial isolates (BT_MDR1, BT_MDR3, BT_MDR4, BT_MDR5, BT_MDR6 and BT_MDR7) were motile and four bacterial isolates (BT_MDR2, BT_MDR8, BT_MDR9, BT_MDR10) were found to be non-motile. However, none of the bacterial isolates except BT_MDR3 showed positive result for phenylalanine deaminase test. Based on biochemical characterization of clinically isolated bacterial strains following Bergey's manual of bacteriology, the isolates BT_MDR1, BT_MDR2, BT_MDR3, BT_MDR4, BT_MDR5, BT_MDR6, BT_MDR7, BT_MDR8, BT_MDR9 and BT_MDR10 were identified as Escherichia coli, Klebsiella pneumoniae, Proteus sp., Pseudomonas aeruginosa, Salmonella typhi, Salmonella paratyphi A, Salmonella paratyphi B, Shigella sp., Enterococcus faecalis and *Staphylococcus aureus* respectively (Table 1). Additionally, the biochemical characterization of two reference strains (MTCC 109 and MTCC 439) was depicted (Table 1). Reference strain (MTCC 109) was non-motile and showed positive results for catalase, VP, citrate, urease and nitrate reduction test, and negative for oxidase, indole, methyl red and phenylalanine deaminase test similar to BT_MDR2 and identified as *K. pneumoniae* whereases MTCC 439 was observed as non-motile, positive results for indole, VP and nitrate reduction test and negative for catalase, oxidase, methyl red, citrate, urease and phenylalanine deaminase test similar BT_MDR9 and identified as *E. faecalis*.

The antibiotic resistance pattern of bacterial strains against different standard antibiotics belonging to five different classes with their zone of inhibition (in mm) was presented through heat map (Table 2). Formation of clear zone around antibiotic disc represents their sensitivity to antibiotic, whereas absence of zone of inhibition around antibiotic disc represents their resistance against antibiotic. Accordingly, bacterial strains were classified as resistant, sensitive and intermediate based on their zone on inhibition following CLSI guidelines. Antibiotic resistance pattern exhibited by eight gram-negative and two-gram positive bacterial strain has been presented in form of radar plot for comparative study (Figure 1). The antibiotic sensitivity analysis of *E. coli* (Met^r Cl^r Ak^i Tgc^i) revealed highly sensitive to ciprofloxacin (33.9 mm) and levofloxacin (31.8 mm) (Figure 1a). Resistance pattern by *K. pneumoniae* (Amc^r Amp^r Met^r Ak^i Na^i Of^i Cl^i Cpm^i Tgc^i) revealed resistance against amoxyclav, ampicillin and methicillin with no zone of inhibition and sensitive to gentamicin (20.5 mm) and ciprofloxacin (21.9 mm). Resistance pattern by *Proteus sp*. (Met^r Cl^r Tgc^r) showed relatively higher sensitive against ampicillin (28.1 mm), ciprofloxacin (36.8 mm) and levofloxacin (29.4 mm). Study indicated that *P. aeruginosa* (Amc^r Amp^r Met^r Na^i Tgc^r) higher sensitivity towards ciprofloxacin (38.6 mm). Subsequently, the three gram-negative bacteria such as *S. typhi* (Met^r Cl^i Tgc^i), *S. paratyphi A* (Met^r Cl^i) and *S. paratyphi B* (Met^r Cl^i Tgc^i) revealed similar antibiotic resistance patterns. Further, the *Shigella sp.* was found to be highly sensitive to ciprofloxacin (34.5 mm) and levofloxacin (32.8 mm) (Figure 1a). Antibiotic resistance patterns exhibited by the gram-positive *E. faecalis* (Met^r) was highly sensitive to clindamycin (34.5 mm), linezolid (24.8 mm) and erythromycin (22.8 mm) whereas *S. aureus* (Met^r Ak^i Cip^i Le^i Va^i) was observed to be highly sensitive to clindamycin (31.2 mm), azithromycin (23.2 mm) and gentamicin (25.2 mm) (Figure 1b). Antibiotic resistance patterns exhibited by the clinical bacterial isolate BT_MDR2 using VITEK-II system was found

to be *Klebsiella pneumonia*, which showed resistant against all antibiotics with lower MIC value (8 μg/ml) for three antibiotics (levofloxacin, tigecycline, doripenem) except colistin with intermediate resistance (Table 3). Similarly, the clinical bacterial isolate BT_MDR9 was identified as *Enterococcus faecalis* that showed resistant against antibiotics such as penicillin, gentamicin, ciprofloxacin, levofloxacin, erythromycin, linezolid where as sensitive to antibiotics such as teicoplanin, vancomycin, tetracycline, tigecycline, nitrofurantoin revealed by VITEK-II system (Table 4).

Table 1: Biochemical characterization of the bacterial strains isolated from clinical samples (BT_MDR1 to BT_MDR10) as well as the reference strains *i.e., Klebsiella pneumoniae* (MTCC 109) and *Enterococcus faecalis* (MTCC 439).

Bacterial strains	Shape/ size	Gram staining	Cat test	Coa test	Oxi test	Indole test	MR test	VP test	OF test	Cit test	Ure test	TSI test	NR test	CF test				Mort test	PD test	Species identified
														G	F	L	S			
BT_MDR1	Rod	-ve	+		-	+	+	-	Fer	-	-		+	+		+	+	+		*Escherichia coli*
BT_MDR2	Rod	-ve	+		-	-	-	+	Fer	+	+	+	+	+		+	+	-		*Klebsiella pneumoniae*
BT_MDR3	Rod	-ve	+		-	-	+	-	Facul	+	+		+	+		-	-	+	+	*Proteus* sp.
BT_MDR4	Rod	-ve	+	-	+	-	-	-	Oxd	+	-		+	-		-	-	+		*Pseudomonas aeruginosa*
BT_MDR5	Rod	-ve	+		-	-	+	-	-	-	-	+	+	+		-	-	+		*Salmonella typhi*
BT_MDR6	Rod	-ve	+		-	-	+	-	-	-	-	+	+	+		-	-	+		*Salmonella paratyphi A*
BT_MDR7	Rod	-ve	+		-	-	+	-	-	-	-	+	+	+		-	-	+		*Salmonella paratyphi B*
BT_MDR8	Rod	-ve	+		-	-	+	-	-		-		+			-	-	-		*Shigella* sp.
BT_MDR9	Cocci	+ve	-		-	+	-	+	Fer	-	-		+	+		+	+	-		*Enterococcus faecalis*
BT_MDR10	Cocci	+ve	+	+	-	-	+	+	Fer		+		+	+	+	+	+	-		*Staphylococcus aureus*
MTCC 109 (Reference)	Rod	-ve	+		-	-	-	+	Fer	+	+	+	+	+		+	+	-		*Klebsiella pneumoniae*
MTCC 439 (Reference)	Cocci	+ve	-		-	+	-	+	Fer	-	-		+	+		+	+	-		*Enterococcus faecalis*

NB: Biochemical tests performed in the study: Catalase test (Cat test); Coagulase test (Coa test), Oxidase test (Oxi test); Methyl Red test (MR test); Voges-Proskauer test (VP test); Oxidation fermentation test (OF test: Fer- Fermentative, Facul- Facultative, Oxd- Oxidative); Citrate test (Cit test); Urease test (Ure test); Triple-Sugar-Iron test (TSI test); Nitrate reduction test (NR test); Carbohydrate fermentation test (CF test); Carbohydrates: (G: Glucose, F: Fructose, L: Lactose, S: Sucrose); Mortality test (Mort test); Phenylalanine deaminase test (PD test).

Molecular identification of the selected clinically isolated bacterial strains (BT_MDR2 and BT_MDR9) was conducted by 16S rRNA sequencing and the phylogenetic analysis was performed. Maximum Likelihood approach was employed to figure out the evolutionary history based on Tamura-Nei model [35]. Besides, Neighbor-Joining and BioNJ algorithms were utilized to construct initial tree(s) for heuristic search by Tamura-Nei model approach, and then topology with superior log likelihood value were selected. The trees with the highest log likelihood for BT_MDR2 (-3214.4006) and BT_MDR9 (-22048.24) were presented. The analysis involved 10 (BT_MDR2) and 9 (BT_MDR9) nucleotide sequences respectively. All positions with gaps and missing data were removed. A total 3948 and 3789 positions are present in the final dataset for BT_MDR2 and BT_MDR9 respectively. Phylogenetic trees were constructed in MEGA 11.0 software [35]. Based on high sequence similarity and molecular phylogenetic analysis, the clinical bacteria BT_MDR2 and BT_MDR9 were identified as *Klebsiella pneumoniae* (Figure 2a) and *Enterococcus faecalis* (Figure 2b) respectively.

Table 2: Heat map representing the antibiotic susceptibility pattern exhibited by gram negative and gram-positive bacteria isolated from the clinical samples with zone of inhibition (in mm). Column represents bacterial isolates whereas row represents antibiotics. Red blocks indicate resistance, green blocks indicate sensitivity and yellow blocks represent intermediate action of antibiotics. Gradient in colour (green → red) showed gradual increase in resistance exhibited by bacteria isolated from the clinical sample.

Bacterial isolates (n=10)	**Antibiotics**											
Gram Negative Bacteria (n=8)	**AMC**	**AMP**	**MET**	**GEN**	**AK**	**CIP**	**NA**	**OF**	**LE**	**CL**	**CPM**	**TGC**
Escherichia coli	17.4	16.3	0	23.6	14.8	33.9	18.5	28.6	31.8	0	21.6	11.8
Klebsiella pneumoniae	0	0	0	20.5	13.5	21.9	12.8	13.6	15.4	12.5	11.5	13.6
Proteus sp.	20.2	28.1	0	17.4	16.7	36.8	16.6	25.4	29.4	0	24.6	0
Pseudomonas aeruginosa	0	0	0	26.4	24.3	38.6	8.6	20.5	25.8	15.5	22.7	0
Salmonella typhi	19.5	19.5	0	26.8	20.9	37.6	22.2	35.6	35.9	12.5	26.9	11.9
Salmonella paratyphi A	22.4	24.8	0	26.5	21.6	40.2	19.3	31.8	35.3	13.8	25.6	16.8
Salmonella paratyphi B	18.3	20.6	0	25.8	21.8	39.1	18.6	21.6	27.6	13.1	31.3	10.6
Shigella sp.	16.5	17.3	0	21.8	12.7	34.5	19.8	19.6	32.8	0	22.8	9.5
Resistance percentage (%)	25%	25 %	100%	0%	0%	0%	0%	0%	0%	37.5%	0%	25 %

Gram Positive bacteria (n=2)	**PE**	**AMP**	**MET**	**AK**	**GEN**	**CTX**	**CPM**	**CIP**	**LE**	**AZM**	**ER**	**LZ**	**VA**	**CD**	**TE**
Enterococcus faecalis	18.5	23.5	0	16.8	28.6	19.5	23.7	18.9	16.9	30.5	22.8	24.8	16.5	34.5	20.2
Staphylococcus aureus	17.5	19.2	0	14.9	25.2	17.3	24.2	13.6	14.7	23.2	27.4	28.1	13.8	31.2	23.6
Resistance percentage (%)	0%	0%	100 %	0%	0 %	0%	0%	0%	0%	0 %	0 %	0%	0%	0%	0 %

NB: Antibiotics (µg/disc): AMC: Amoxyclav (30 µg/disc); PE: Penicillin (5 µg/disc); AMP: Ampicillin (10 µg/disc); MET: Methicillin (10 µg/disc); AK: Amikacin (30 µg/disc); GEN: Gentamicin (30 µg/disc); CTX: Cefotaxime (10 µg/disc); CPM: Cefepime (30 µg/disc); CIP: Ciprofloxacin (5 µg/disc); LE: Levofloxacin (10 µg/disc); AZM: Azithromycin (30 µg/disc); ER: Erythromycin (10 µg/disc); LZ: Linezolid (10 µg/disc); VA: Vancomycin (10 µg/disc); CD: Clindamycin (10 µg/disc); TE: Tetracycline (30 µg/disc); NA: Nallidixic acid (10 µg/disc); OF: Ofloxacin (5 µg/disc); CL: Colistin (10 µg/disc); TGC: Tigecycline (10 µg/disc).

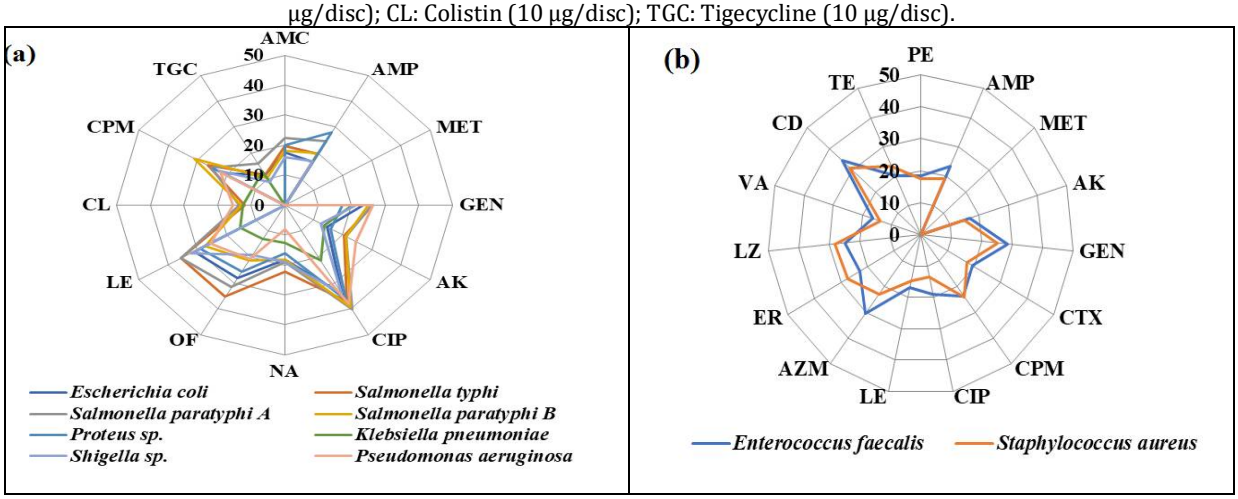

Figure 1: Radar graph of zone of inhibition exhibited by the clinically isolated gram-negative bacteria and gram-negative bacteria against different antibiotics.

Table 3: Antibiogram/susceptibility of *Klebsiella pneumoniae* (BT_MDR2) against different antibiotics using VITEK-II system.

Antimicrobial agents	MIC (µg/ml)	Interpretation	Antimicrobial agents	MIC (µg/ml)	Interpretation
Ticarcillin/ Clavulanic Acid	≥ 128	R	Cefoperazone/ Sulbactam	≥ 64	R
Imipenem	≥16	R	Gentamicin	≥ 16	R
Ceftazidime	≥ 64	R	Ciprofloxacin	≥ 4	R
Amikacin	≥ 64	R	Levofloxacin	≥ 8	R
Cefepime	≥ 64	R	Minocycline	≥ 16	R
Aztreonam	≥ 64	R	Tigecycline	≥ 8	R
Doripenem	≥ 8	R	Colistin	≤ 0.5	I
Piperacillin/ Tazobactam	≥ 128	R	Trimethoprim/ Sulfamethoxzole	≥ 320	R
Meropenem	≥ 16	R			

NB: R: Resistant; S: Sensitive; I: Intermediate

Table 4: Antibiogram/susceptibility of *Enterococcus faecalis* (BT_MDR9) against different antibiotics using VITEK-II system.

Antimicrobial agents	MIC (µg/ml)	Interpretation	Antimicrobial agents	MIC (µg/ml)	Interpretation
Benzyl penicillin	≥ 64	R	Daptomycin	-	-
Oxacillin	-	-	Teicoplanin	≤ 0.5	S
Gentamicin (Synergy)	SYN-R	R	Vancomycin	≤ 0.5	S
Ciprofloxacin	≥ 8	R	Tetracycline	≤ 1	S
Levofloxacin	≥ 8	R	Tigecycline	≤ 0.12	S
Inducible Clindamycin	-	-	Nitrofurantoin	32	S
Erythromycin	≥ 8	R	Rifampicin	-	-
Linezolid	≥ 8	R			

NB: R: Resistant; S: Sensitive; I: Intermediate

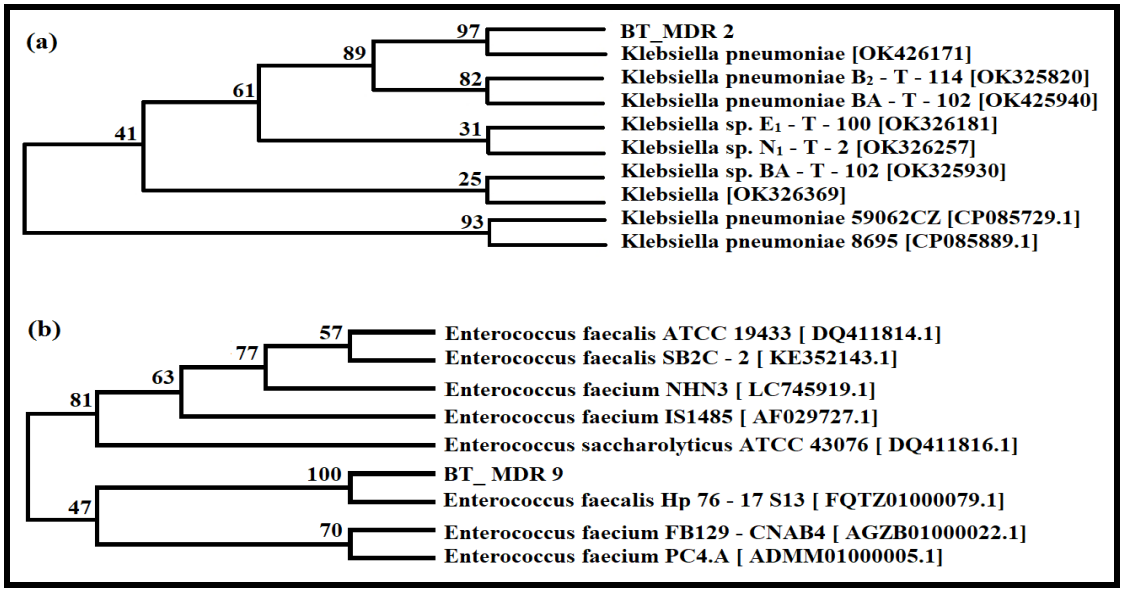

Figure 2: Phylogenetic analysis of the bacterial isolates (BT_MDR2 and BT_MDR9), which were identified to be (a) *Klebsiella pneumoniae* and (b) *Enterococcus faecalis* using Maximum Likelihood method based on the Tamura-Nei model.

DISCUSSION

In recent years, the global prevalence of antibiotic resistance has skyrocketed. Use of antibiotics for disease treatment in hospitals is highly significant as clinical samples were associated with pathogenic multidrug bacteria leading to deadly infections, cross transmission and longer therapy and hospitalization [36]. Understanding the spread of MDR bacteria in hospital settings is therefore critical for infection control and the sensible use of antibiotics. Bacterial isolates form clinical samples showed

resistance against commercially available drugs, which might be due to the excessive and misuse of antibiotics and accumulation of antibiotics in hospital setting. The study also revealed the dominance of gram-negative bacterial strains in clinical samples. The findings have been substantiated by several workers [5,17,37]. However, the patterns of antibiotic resistance differ greatly in hospital and non-hospital samples due to varying environmental conditions and antibiotics exposure [17]. Antibiotic resistance patterns exhibited by the gram-negative bacteria revealed the highest resistance against methicillin (100%) followed by colistin (37.5%) and then amoxyclav, ampicillin and tigecycline (25%). In contrast, antimicrobial agents such as ciprofloxacin and levofloxacin were found as highly efficient antibiotics to gram-negative bacterial isolates. Similarly, antibiotic resistance pattern of gram-positive bacteria revealed that all the clinical bacterial isolates were highly sensitive to clindamycin followed by azithromycin, erythromycin, linezolid, and gentamicin. Nevertheless, all the gram-positive and gram-negative bacterial isolates were found to be resistance against methicillin. Further, the VITEK II analysis revealed the variation in the antibiotics resistant patterns exhibited by gram-positive as well as gram-negative isolates such as *Klebsiella pneumonia* and *Enterococcus faecalis* against different antibiotics. Based on the findings, the present study clearly suggested that the gram-negative bacteria exhibited relatively higher resistance to antibiotics compared to the gram-positive bacteria due to presence of outer membrane barrier [10,38].

CONCLUSION

Multidrug resistance bacteria against commonly used antibiotics are prevalent in the clinical settings. MDR gram-positive as well as gram-negative bacterial isolates in hospital settings are responsible for various nosocomial infections in hospitalized patients resulting in health and economic burdens. Thereby, the proper identification of MDR bacteria and their antibiotic resistance pattern is prerequisite not only for the potential use of commercial antibiotics but also provide avenues for the development of novel and effective antimicrobial agents for controlling disease transmission and pathogenesis.

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AUTHOR CONTRIBUTIONS

All the authors have made significant contribution for literature survey, sample collection, designing of experiments, data analysis and interpretation. Final draft including compilation of the results was done by corresponding authors. All authors have read and agreed to the publication version of the manuscript.

CONFLICT OF INTEREST

All authors declare no conflicts of interest.

REFERENCES

1. Catalano A, Iacopetta D, Ceramella J, Scumaci D, Giuzio F, Saturnino C, Aquaro S, Rosano C, Sinicropi MS. (2022). Multidrug resistance (MDR): A widespread phenomenon in pharmacological therapies. Molecules. 2;27(3):616. https://doi.org/10.3390/molecules27030616
2. Mai W, Liu Y, Meng Q, Xu J, Wu J. (2023). Bacterial Epidemiology and Antimicrobial Resistance Profiles of Respiratory Specimens of Children with Pneumonia in Hainan, China. Infection and Drug Resistance. 31:249-61. 10.2147/IDR.S397513
3. De Oliveira DM, Forde BM, Kidd TJ, Harris PN, Schembri MA, Beatson SA, Paterson DL, Walker MJ. (2020). Antimicrobial resistance in ESKAPE pathogens. Clinical microbiology reviews.17;33(3):e00181-19. https://doi.org/10.1128/cmr.00181-19
4. Yang X, Ye W, Qi Y, Ying Y, Xia Z. (2021). Overcoming multidrug resistance in bacteria through antibiotics delivery in surface-engineered nano-cargos: recent developments for future nano-antibiotics. Frontiers in Bioengineering and Biotechnology. 8;9:696514. https://doi.org/10.3389/fbioe.2021.696514
5. Benkő R, Gajdács M, Matuz M, Bodó G, Lázár A, Hajdú E, Papfalvi E, Hannauer P, Erdélyi P, Pető Z.(2020). Prevalence and antibiotic resistance of ESKAPE pathogens isolated in the emergency department of a tertiary care teaching hospital in Hungary: a 5-year retrospective survey. Antibiotics.19;9(9):624. https:// doi.org/10.3390/antibiotics9090624

6. Mancuso G, Midiri A, Gerace E, Biondo C. (2021). Bacterial antibiotic resistance: The most critical pathogens. Pathogens. 2;10(10):1310. https://doi.org/10.3390/pathogens10101310
7. Pendleton JN, Gorman SP, Gilmore BF. (2013). Clinical relevance of the ESKAPE pathogens. Expert review of anti-infective therapy. 1;11(3):297-308. https://doi.org/10.1586/eri.13.12
8. Lerminiaux NA, Cameron AD. (2019). Horizontal transfer of antibiotic resistance genes in clinical environments. Canadian journal of microbiology. ;65(1):34-44. https://doi.org/10.1139/cjm-2018-0275
9. Cerceo E, Deitelzweig SB, Sherman BM, Amin AN. (2016). Multidrug-resistant gram-negative bacterial infections in the hospital setting: overview, implications for clinical practice, and emerging treatment options. Microbial Drug Resistance.1;22(5):412-31. https://doi.org/10.1089/mdr.2015.0220
10. Exner M, Bhattacharya S, Christiansen B, Gebel J, Goroncy-Bermes P, Hartemann P, Heeg P, Ilschner C, Kramer A, Larson E, Merkens W. (2017). Antibiotic resistance: What is so special about multidrug-resistant Gram-negative bacteria?. GMS hygiene and infection control. 12. 10.3205/dgkh000290
11. Serwecińska L. (2020). Antimicrobials and antibiotic-resistant bacteria: a risk to the environment and to public health. Water. 25;12(12):3313. https://doi.org/10.3390/w12123313
12. Bebell LM, Muiru AN. (2014). Antibiotic use and emerging resistance: how can resource-limited countries turn the tide?. Global heart. 1;9(3):347-58. https://doi.org/10.1016/j.gheart.2014.08.009
13. Tanwar J, Das S, Fatima Z, Hameed S. (2014). Multidrug resistance: an emerging crisis. Interdisciplinary perspectives on infectious diseases. 234-240. https://doi.org/10.1155/2014/541340
14. Vikesland P, Garner E, Gupta S, Kang S, Maile-Moskowitz A, Zhu N. (2019). Differential drivers of antimicrobial resistance across the world. Accounts of chemical research. 8;52(4):916-24. https://doi.org/ 10.1021/acs. accounts.8b00643
15. Vivas R, Barbosa AA, Dolabela SS, Jain S. (2019). Multidrug-resistant bacteria and alternative methods to control them: an overview. Microbial Drug Resistance. 1;25(6):890-908. https://doi.org/10.1089/mdr.2018.0319
16. Bharadwaj A, Rastogi A, Pandey S, Gupta S, Sohal JS. Multidrug-Resistant Bacteria: Their mechanism of action and prophylaxis. BioMed Research International. 2022 Sep 5;2022. https://doi.org/10.1155/2022/5419874
17. Moges F, Endris M, Belyhun Y, Worku W. Isolation and characterization of multiple drug resistance bacterial pathogens from waste water in hospital and non-hospital environments, Northwest Ethiopia. BMC research notes. 2014 Dec;7:1-6. https://doi.org/10.1186/1756-0500-7-215
18. Van Duin D, Paterson DL. Multidrug-resistant bacteria in the community: trends and lessons learned. Infectious disease clinics. 2016 Jun 1;30(2):377-90. https://doi.org/10.1016/j.idc.2016.02.004
19. Reygaert WC. An overview of the antimicrobial resistance mechanisms of bacteria. AIMS microbiology. 2018;4(3):482. 10.3934/microbiol.2018.3.482
20. Dhingra S, Rahman NA, Peile E, Rahman M, Sartelli M, Hassali MA, Islam T, Islam S, Haque M. Microbial resistance movements: an overview of global public health threats posed by antimicrobial resistance, and how best to counter. Frontiers in Public Health. 2020 Nov 4;8:535668. https://doi.org/10.3389/fpubh.2020.535668
21. Varela MF, Stephen J, Lekshmi M, Ojha M, Wenzel N, Sanford LM, Hernandez AJ, Parvathi A, Kumar SH. Bacterial resistance to antimicrobial agents. Antibiotics. 2021 May 17;10(5):593. https://doi.org/10.3390 /antibiotics10050593
22. Fernández-García L, Fernandez-Cuenca F, Blasco L, López-Rojas R, Ambroa A, Lopez M, Pascual Á, Bou G, Tomás M. (2018). Relationship between tolerance and persistence mechanisms in Acinetobacter baumannii strains with AbkAB toxin-antitoxin system. Antimicrobial agents and Chemotherapy. 62(5):e00250-18. https://doi.org /10.1128/aac.00250-18
23. Christaki E, Marcou M, Tofarides A. (2020). Antimicrobial resistance in bacteria: mechanisms, evolution, and persistence. Journal of molecular evolution. 88:26-40. https://doi.org/10.1007/s00239-019-09914-3
24. Martinez JL. General principles of antibiotic resistance in bacteria. Drug Discovery Today: Technologies. 2014 Mar 1;11:33-9. https://doi.org/10.1016/j.ddtec.2014.02.001
25. Friedrich AW. (2019). Control of hospital acquired infections and antimicrobial resistance in Europe: the way to go. Wiener Medizinische Wochenschrift. 169:25-30. https://doi.org/10.1007/s10354-018-0676-5
26. McInnes RS, McCallum GE, Lamberte LE, van Schaik W. (2020). Horizontal transfer of antibiotic resistance genes in the human gut microbiome. Current opinion in microbiology. ;53:35-43. https://doi.org /10.1016/j.mib.2020.02.002
27. Hauhnar L, Pachuau L, Lalhruaitluanga H. (2018). Isolation and characterization of multi-drug resistant bacteria from hospital wastewater sites around the city of Aizawl, Mizoram. Advances in Bioscience and Biotechnology. 12;9(07):311. 10.4236/abb.2018.97020
28. Caselli E, Arnoldo L, Rognoni C, D'Accolti M, Soffritti I, Lanzoni L, Bisi M, Volta A, Tarricone R, Brusaferro S, Mazzacane S. Impact of a probiotic-based hospital sanitation on antimicrobial resistance and HAI-associated antimicrobial consumption and costs: a multicenter study. Infection and Drug Resistance. 2019 Feb 27:501-10. 10.2147/IDR.S194670
29. Chaoui L, Mhand R, Mellouki F, Rhallabi N. (2019). Contamination of the surfaces of a health care environment by multidrug-resistant (MDR) bacteria. International journal of Microbiology.29;2019. https://doi.org /10.1155/2019/3236526
30. Wang M, Wei H, Zhao Y, Shang L, Di L, Lyu C, Liu J. (2019). Analysis of multidrug-resistant bacteria in 3223 patients with hospital-acquired infections (HAI) from a tertiary general hospital in China. Bosnian journal of basic medical sciences. 19(1):86. https://doi.org/10.17305%2Fbjbms.2018.3826

31. Gil-Gil T, Ochoa-Sánchez LE, Baquero F, Martínez JL. (2021). Antibiotic resistance: time of synthesis in a post-genomic age. Computational and Structural Biotechnology Journal.;19:3110-24. https://doi.org/10.1016/j.csbj.2021.05.034
32. Pelczar MJ, Bard, RC, Burnett, GW.(1957). *Manual of microbiological methods*. McGraw-Hill Book Company.
33. Hsueh PR, Ko WC, Wu JJ, Lu JJ, Wang FD, Wu HY, Wu TL, Teng LJ. (2010). Consensus statement on the adherence to Clinical and Laboratory Standards Institute (CLSI) Antimicrobial Susceptibility Testing Guidelines (CLSI-2010 and CLSI-2010-update) for Enterobacteriaceae in clinical microbiology laboratories in Taiwan. Journal of Microbiology, Immunology and Infection.1;43(5):452-5.
34. Martínez-Absalón S, Rojas-Solís D, Hernández-León R, Prieto-Barajas C, Orozco-Mosqueda MD, Peña-Cabriales JJ, Sakuda S, Valencia-Cantero E, Santoyo G. (2014). Potential use and mode of action of the new strain Bacillus thuringiensis UM96 for the biological control of the grey mould phytopathogen Botrytis cinerea. Biocontrol Science and Technology. 2;24(12):1349-62. https://doi.org/10.1080/09583157.2014.940846
35. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution.1;28(10):2731-9. https://doi.org/10.1093/molbev/msr121
36. Toubes E, Singh K, Yin D, Lyu R, Glick N, Russell L, Mohapatra S, Saghal N, Weinstein RA, Trenholme G. (2003). Risk factors for antibiotic-resistant infection and treatment outcomes among hospitalized patients transferred from long-term care facilities: does antimicrobial choice make a difference? Clinical infectious diseases. 15;36(6):724-30. https://doi.org/10.1086/368081
37. Kot B, Grużewska A, Szweda P, Wicha J, Parulska U.(2020). Antibiotic resistance of uropathogens isolated from patients hospitalized in district hospital in central Poland in 2020. Antibiotics. 16;10(4):447. https://doi.org/10.3390/antibiotics10040447
38. Miller SI. (2016). Antibiotic resistance and regulation of the gram-negative bacterial outer membrane barrier by host innate immune molecules. MBio. 2;7(5):e01541-16. 10.1128/mBio.01541-16.