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RESEARCH ARTICLE OPEN ACCESS

Genetyping of Carbapenem-resistant Organisms Isolated from Clinical Isolates Received from Tertiary Care Hospitals of Ahmedabad, Gujarat

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Abstract

The world is seeing a continuous rise in the levels of antibiotic resistance[1]. Organisms develop new resistance mechanisms, emerge, and spread the resistance worldwide, making it challenging to treat common infectious diseases. In the current study, clinical isolates received between the years 2017 to 2020 were cultured and the isolated organisms were screened for antibiotic resistance; isolates with multiple drug resistance were further subjected to confirmatory screening through Combined Disc Test (CDT) and Modified Hodge Test (M.H.T.), and molecular characterization to be finally tested for gene expression analysis. Molecular characterization involved screening of genes bla_{VIM-2}, bla_{KPC-3}, bla_{NDM-1}, and bla_{IMP-11} responsible for imparting carbapenem drug resistance[2]. From the laboratories of tertiary care hospitals, a total of 1452 clinical isolates were collected and identified. The organisms were subjected to antibiotic susceptibility screening and carbapenem resistance screening. The isolates found positive in the screenings were subjected to molecular characterization for genes, bla_{VIM-2}, bla_{KPC-3}, bla_{NDM-1}, and bla_{IMP-11}, responsible for imparting carbapenem drug resistance. Most of the isolates were resistant variably to aminoglycosides but were found to be resistant to fluoroquinolones and β-lactams group of antibiotics. Carbapenem activity was detected in twelve percent of total isolates and 27 percent among multidrug-resistant isolates. bla_{NDM-1} gene was found present in 77% isolates, and five organisms among the total number of organisms showed pan drug resistance.

Keywords: Carbapenem resistance, antibiotic resistance, bla_{VIM-2}, bla_{KPC-3}, bla_{NDM-1}, bla_{IMP-11}, Carbapenem-Resistant *Enterobacteriaceae,* Methicillin-Resistant *Staphylococcus aureus*

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INTRODUCTION

Patients' more prolonged stay at healthcare setups increases the chance of acquiring secondary infections. Antibiotic-resistant organisms aggravate the worsened situation. Each year, in the US, >2.8 million antibiotic resistant infections occur[1].According to the National Ambulatory Medical Care Survey 2016, conducted by the Center for Disease Control and Prevention (C.D.C.) in 2016, infectious and parasitic diseases accounted for 15.5 million doctor visits and 3.7 million emergency room visits in the U.S.A.[3]. As a result, more than 35,000 people die, making antibiotic-resistant infections a public health threat, according to the report published in the year 2019 by the C.D.C[4]. As per the annual report compiled by the Indian Council of Medical Research (ICMR) under the name of Antibiotic-Resistant Surveillance and Research Network in the year 2019, isolates from 107, 387 samples were studied taken/collected from various body parts[5].

As per the annual report compiled by the Indian Council of Medical Research (ICMR) under the name of Antibiotic-Resistant Surveillance and Research Network in the year 2019, a total of 32,672 significant clinical isolates belonging to various genera and species of family Enterobacteriaceae were screened for antibiotic resistance. 45% of the isolates were found to be resistant toimipenem, 35% were found to be resistant to meropenem, and 40% were found to be resistant to ertapenem antibiotic[4]. One in every thirty-one hospital patients has at least one hospital-associated infection on any given day in the U.S.A.[6]; similar situations are observed across the world, and attempts are being made by healthcare workers to prevent them. Extended-spectrum β-lactamases (ESβLs), Vancomycin-Resistant Enterococci (VRE), and Carbapenem-Resistant Enterobacteriaceae (C.R.E.), and Methicillin-Resistant *Staphylococcus aureus* (MRSA), are the most frequently isolated organisms responsible for Hospital Acquired Infections (HAIs)[6].

Antibiotic resistance is an ancient and dynamic mounting problem[7]. The resistance poses a global threat to the health of humans, animals and environment which is due to the emergence and existence of superbugs across animal, human and environmental due to excessive use of antibiotics in animals and humans. Antibiotic resistance has been declared as a global public health concern by many important organizations like Centers for Disease Control and Prevention (CDC), Infectious Disease society of America, World Economic Forum, and the World Health Organization[8,9]. Terrible complications may occur if effective global plans are not adopted soon. In this study we have tried to portray how resistance is spreading along with the intensity of the antibiotic resistance among different clinical specimen[10].

MATERIALS AND METHODS

The study does not involve samples of human origin, the strains used for processing were

Table 1. Isolate wise distribution of multidrug resistant strains from various clinical specimen

Specimen Type	Growth Observed in (n)	MDR (n)	Carbapenemase + MRSA (n)
Urine and Catheter Tip	490	262	42
Blood CS & Tip	29	15	5
Skin / Hair / Nail Scrapping	174	NA	NA
Semen	123	42	19
Swab	188	110	57
Aspirated Material / Drain / Tip	84	60	17
Sputum	117	67	6
Endotracheal Secretion, BAL and Tip	86	80	29
Stool	20	15	2
total	1311	651	177

MDR – Multidrug resistant, CS – Culture and sensitivity, BAL – Broncho Alveolar Lavage

submitted by associate laboratories of hospitals after isolation; hence, Helsinki declaration is not required. During the study, no humans were prescribed medicines or placebo; further, biological vitals such as height, sex, weight etc. were not measured. The resistance screening performed on the antibiotics against established formulas and at the concentrations prescribed.

Selection of Bacterial Strains

A total of 1452 non-repetitive clinical isolates, obtained from laboratories of tertiary care hospitals of Ahmedabad between the period of 2017 to 2020, were identified using Vitek-2 compact. In percentage, identified organisms were *E. coli* (45%), *Klebsiella* spp. (18%), *Enterobacter* spp. (11%) and *Proteus* spp. (3%), of *Enterobacteriaceae* group, *Acinetobacter* spp. (2%), *Burkholderia* spp. (4%), *Haemophilus* spp. (5%) and *Pseudomonas* spp. (13%), of the non-Enterobacteriaceae group, which were subjected to susceptibility testing.

Antibiotic Susceptibility Testing

The isolates were subjected to antibiotic susceptibility testing, performed using the protocol described by Kirby-Bauer disk diffusion susceptibility test protocol, and the results were interpreted according to the clinical breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) guideline[11,12]. According to the definition of C.D.C., in order not to miss any possible carbapenem producer, organisms showing resistance towards at least one carbapenem agent were considered for carbapenem confirmatory testing[11]. All consumable, used in the study, were procured from Hi-media Laboratories (Mumbai). Antibiotic discs taken are as following: Cefotaxime-clavulanic acid (30/10µg), aztreonam (30µg), cefepime (30µg), ceftriaxone (30µg), cefoxitin (30µg), cefotetan (30µg), meropenem (10µg), imipenem (10µg), ertapenem (10µg), cefotaxime (30µg), ceftazidime + clavulanic acid (30/10µg), ceftazidime (30µg)[11]. To differentiate among isolated cultures and to derive the antibiotic-resistance pattern of the organisms among extended-spectrum beta-lactamases (ESβLs), AmpC β-lactamases (AmpCβL) or Metallo-β-lactamases (MβL),the study used method suggested by Parul Sinha et al.[13]

Confirmation of Carbapenem Resistance

Modified Hodge Test

Using the ATCC *E. coli* 25922 strain a 5 ml Solution of 0.5 McF dilution was prepared.

Table 2. Gender wise distribution of MDR organisms

No.	Total (n)	Gender (n)		Type of Specimen (n)	Growth observed (n)		MDR strain isolated (n)		Carbapenem Strain Isolated (n)	
		M	F		M	F	M	F	M	F
1	3113	1676	1437	Urine and Catheter Tip 1270	410	860	81	162	24	21
				Blood CS & Tip 425	276	149	14	6	8	5
				Skin / Hair / Nail Scrapping 253	168	85	0	0	0	0
				Semen 317	317	0	69	0	17	0
				Swab 266	147	119	62	34	16	12
				Aspirated Material / Drain / Tip 270	153	117	20	24	24	22
				Sputum 184	118	66	29	26	2	5
				Endotracheal Secretion, BAL and Tip 104	75	29	59	13	20	7
				Stool 24	13	11	7	11	1	1

MDR – Multidrug resistant, CS – Culture and sensitivity, BAL – Broncho Alveolar Lavage

Prepared solution was diluted to 1:10 dilution factor for analysis. The prepared inoculum was lawn streaked on Muller Hinton agar plate and allowed to dry. At the center of the prepared plate meropenem (10µg) susceptibility disc was placed, and the test organism was streaked edge to edge on the plate. The results of the tests are considered positive when there is development of clover leaf-type indentation at the intersection of the test organism and ATCC strain within the zone of inhibition of the carbapenem susceptibility disc; results for the isolates developed otherwise were considered negative for carbapenem resistance[14].

Combined Disc Test (CDT) / Ethylene Diamine Tetraacetic Acid Test

Muller Hinton Agar plates, pre-inoculated with the sample of the test isolate (0.5 McF standard) were processed with two sets of antibiotic discs, each one containing imipenem (10µg), meropenem (10 µg) or ertapenem (10 µg). 10 µl 0.1M EDTA solution was added to one of the two antibiotic discs Immediately after the discs were placed onto the agar. Plates were incubated at 37°C for 16 to 20 hours; zone diameters were recorded at the end of the incubation period. Zone diameter difference of ≥5mm between the APBA-free and APBA-containing discs or between the EDTA-free and EDTA-containing discs were considered indicative of Class A and B carbapenemase production, respectively[15].

The organismspositive for at least one tests out of the two confirmatory tests, Combined Disc test (CDT) and Modified Hodge test, were

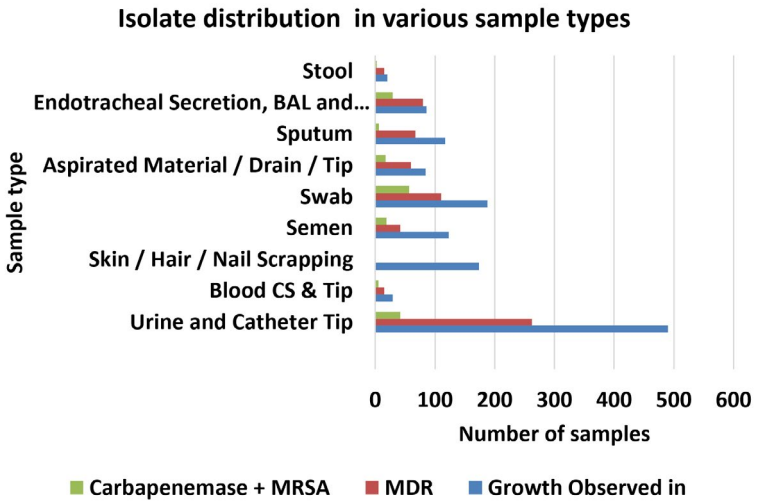

Fig. 1. Isolate wise distribution from various specimen types with MDR and carbapenem isolation pattern

Table 3. Results after phenotypic and automated isolation and deviation between both the techniques

Organisms identified as	Total isolates (n)	Identified as the same organism after phenotypic characterization (n)	Organism identified as after phenotypic characterization (n)	Isolates showing carbepenem resistance (n)
Acinetobacter spp.	3	2	01 (*E. coli*)	3
E. coli	17	14	03 (*Klebsiella* spp.)	17
Enterobacter spp.	7	7	0	7
Klebsiella spp.	33	30	03 (*E. coli*)	33
Proteus spp.	2	2	0	2
Pseudomonas spp.	19	19	0	19
Total	81	74	7	81

* Identification carried out using Vitek-2 compact.

selected; the remaining organisms were ruled out from the further analysis.

Molecular Characterization of Isolates

Using column extraction, genomic DNA of the isolates was extracted to perform Polymerase Chain Reaction (PCR) on a 7500 Real-Time RT-PCR machine using primers of Taqman chemistry for bla_{VIM-2}, bla_{KPC-3}, bla_{NDM-1}, and bla_{IMP-11} carbapenem genes while maintaining the conditions described by (Van der zee et al., 2014). 5.0µl sample DNA was mixed with 25 µl of master mix and 2.50µl of assay mix topped up by 17.50µl of H_2O ultimately making the final reaction volume to 50.0µl. RT-PCR reaction was set for single-plex PCR, despite primers have been showing positive reactions in multiplex conditions[16].

Table 4. Prevalence of carbapenem resistance among multidrug resistant isolates

No.	Organism	Total Isolates (n, %)
1	*Acinetobacter* spp.	4, 4.21%
2	*E. coli*	19, 20.00%
3	*Enterobacter* spp.	10, 10.53%
4	*Klebsiella* spp.	34, 40.00%
5	*Proteus* spp.	3, 3.16%
6	*Pseudomonas* spp.	21, 22.11%
Total		95, 100%

Data analysis

Data generated during the study were processed by conventional statistical tools and had a P value of <0.05 for critical testing; 7500-software provided by the manufacturer was used to analyze the data obtained from RT-PCR.

RESULTS AND DISCUSSION

The prevalence of multi-drug resistant and carbapenem-resistant organisms in healthcare

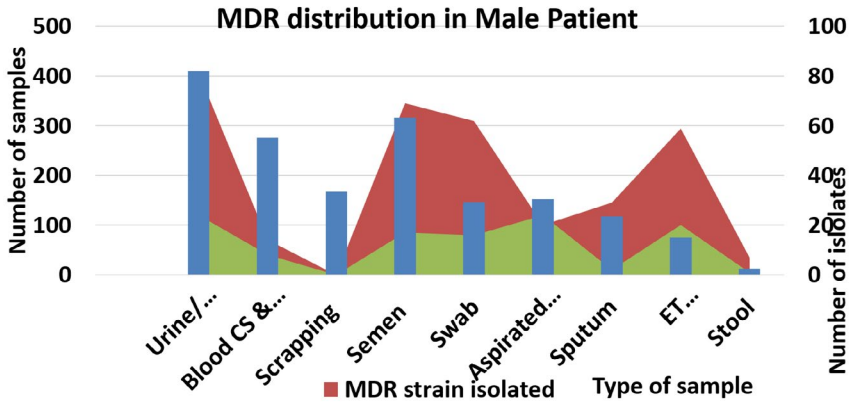

Fig. 2. MDR distribution among male patients

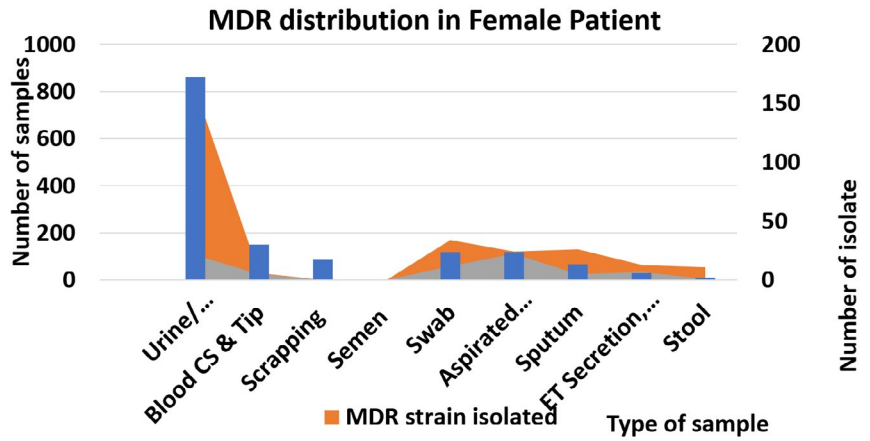

Fig. 3. MDR distribution among female patients

setup is a challenge for healthcare workers; in Egypt prevalence of carbapenem-resistant organisms among *Klebsiella pneumoniae* has been recorded as high as 44.3 %[17]. Similar trends were observed in this study. Out of a total number of isolates (n=1452) screened for antibiotic

Table 5. Molecular characterization and Carbapenem gene distribution in clinical isolates

Organism	Total No. of isolates (n)	blaNDM-1* (n)	blaIMP-11* (n)	blaVIM-2* (n)	blaKPC-3* (n)
Acinetobacter spp.	3	2	0	0	1
E. coli	17	4	3	2	8
Enterobacter spp.	7	2	0	0	5
Klebsiella spp.	33	25	16	18	30
Proteus spp.	2	0	0	1	1
Pseudomonas spp.	19	4	2	4	9

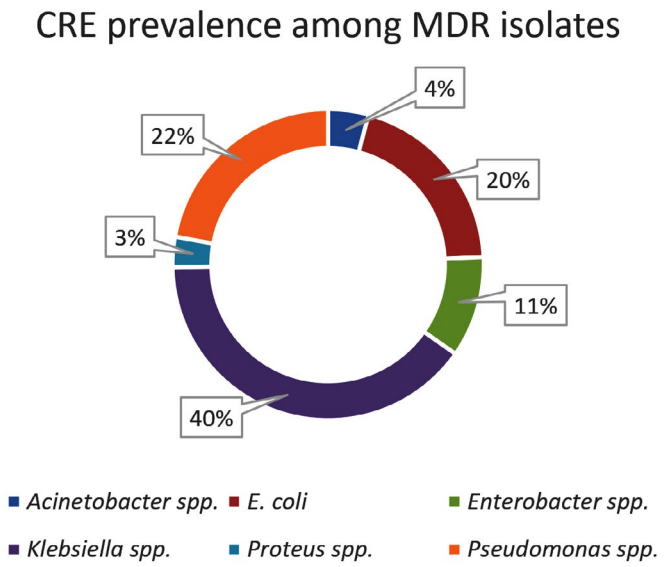

Fig. 4. CRE (Carbapenem Resistant Enterobacteriaceae) prevalence among MDR isolates

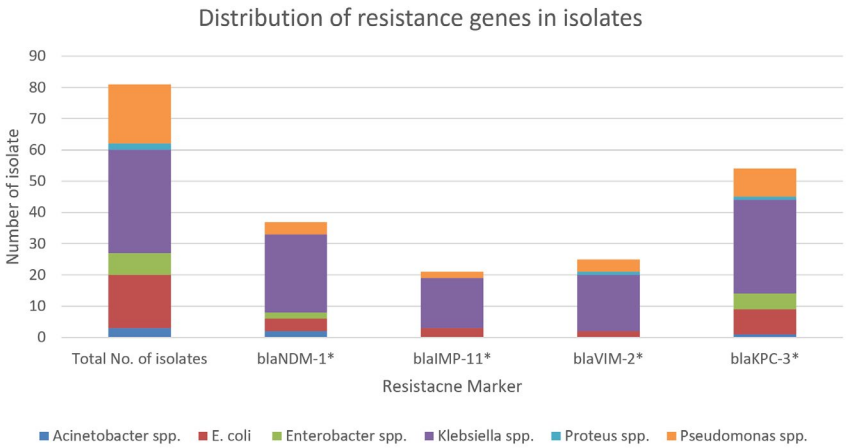

Fig. 5. Molecular characterization and Carbapenem gene distribution in clinical isolates.

resistance, n=651 isolates (45%) were found to be multi-drug resistant using current breakpoints recommended by CLSI (Table-1/Fig. 1)[18].

Most of the isolates collected during this study were resistant variably to aminoglycosides but were found to be resistant to fluoroquinolones and β-lactams group of antibiotics, which contradicts the finding of Endimiani et al[19]. Antibiotic susceptibility results of twelve isolates are worrisome, as the isolates showed pan-drug resistance, making it mandatory to develop new antimicrobials for the isolates along with a message for healthcare workers to maintain strict infection control measures to prevent cross-infection per se.

Carbapenem activity was detected in twelve percent of total isolates and 27 percent among multidrug-resistant isolates (n=177). CDT was positive for 25 percent of isolates (n=45), and M.H.T. was positive for 44 percent of isolates (n=77); whereas 31 percent of isolates (n=55) were found to be positive for both the tests (Table 2).

Gene bla_{NDM-1}, contradicting the findings of researchers from Turkey, where it emerged, consequently finding its reservoir to the Middle East and Africa, and expanding into India[21,22], agreeing to above research's prevalence of the gene was found as high as 77% among multidrug-resistant strains along with blaKPC-3 gene (Table 3) making Indian population vulnerable to carbapenem-resistant organism infections[21].

Furthermore, the presence of multiple bla_{KPC-3} and bla_{NDM-1} carbapenem resistance genes was observed, which explains why a total of five organisms showed pan-drug resistance.

Forty organisms that were positive during confirmatory screening, but showed negative results in RT-PCR, could be because of the combination of ESBLs and/or changes in an outer membrane protein[22,23].

CONCLUSION

It is evident, by looking at the findings of this study, that a high prevalence ratio of carbapenem-resistant strains is observed in clinical specimen. It is also clear that multiple genes are harbored by organisms, and such trends will continue until novel remedies are not developed for alternate therapeutic regimes. Hence, it seems to be the need of an hour to strictly implement antibiotic stewardship programs and stop over the counter sales of antibiotics; rigorous efforts are also to be made in finding drugs that has higher efficacy on the superbugs.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

1. Antibiotic resistance. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/antibiotic-resistance. 2020. Accessed May 27, 2021.
2. Netikul T, Kiratisin P. Genetic Characterization of Carbapenem-Resistant Enterobacteriaceae and the Spread of Carbapenem-Resistant Klebsiella pneumonia ST340 at a University Hospital in Thailand. *PLoS One.* 2015;10(9):e0139116. doi: 10.1371/journal.pone.0139116.
3. Hedegaard H, Minino AM, Warner M. Drug overdose deaths in the United States, 1999-2019. NCHS Data Brief, No. 394. Hyattsville, MD: National Center for Health Statistics. 2020.
4. Biggest threats and data - https://www.cdc.gov/drugresistance/biggest-threats.html, Accessed on-March 2, 2021.
5. Walia K, Madhumathi J, Veeraraghavan B, et al. Establishing Antimicrobial Resistance Surveillance & Research Network in India: Journey so far. *Indian J Med Res.* 2019;149(2):164-179. doi: 10.4103/ijmr.

IJMR_226_18

6. Olaisen RH, Rossen LM, Warner M, Anderson RN. Unintentional injury death rates in rural and urban areas: United States, 1999-2017 pdf icon. NCHS Data Brief, No. 343.
7. Singer AC, Shaw H, Rhodes V, Hart A. Review of Antimicrobial Resistance in the Environment and Its Relevance to Environmental Regulators. *Front Microbiol*. 2016;7:1728. doi: 10.3389/fmicb.2016.01728
8. Michael CA, Dominey-Howes D, Labbate M. The antimicrobial resistance crisis: causes, consequences, and management. *Front Public Health.* 2014;2:145. doi: 10.3389/fpubh.2014.00145
9. Spellberg B, Srinivasan A, Chambers HF. New societal approaches to empowering antibiotic stewardship. *Journal of the American Medical Association*. 2016;315(12):1229-1230. doi: 10.1001/jama.2016.1346
10. Laxminarayan R, Duse A, Wattal C, et al. Antibiotic resistance-the need for global solutions. *Lancet Infect Dis.* 2013;13(12):1057-1098. doi: 10.1016/S1473-3099(13)70318-9
11. Aslam B, Wang W, Arshad MI, et al. Antibiotic resistance: a rundown of a global crisis. *Infect Drug Resist.* 2018;11:1645-1658. doi:10.2147/IDR.S173867
12. Hudzicki J. Kirby-Bauer Disk Diffusion Susceptibility Test Protocol. [ebook] American Society for Microbiology. 2016. https://asm.org/getattachment/2594ce26-bd44-47f6-8287-0657aa9185ad/Kirby-Bauer-Disk-Diffusion-Susceptibility-Test-Protocol-pdf.pdf.
13. Parul S, Pallavi G, Rajni S, ArunaV, Maheshwari RK. Evaluation of a 12 Disc Test for Phenotypic Detection of β- lactamases Resistance in Gram-Negative Bacilli. *Int J Curr Microbiol App Sci.* 2016;5(6):105-114. doi: 10.20546/ijcmas.2016.506.013
14. Amjad A, Mirza IA, Abbasi SA, Farwa U, Malik N, Zia F. Modified Hodge test: A simple and effective test for detection of carbapenemase production. *Iran J Microbiol.* 2011;3(4):189-193.
15. Omair M, Usman J, Kaleem F, Hassan A, Khalid A, Fahim Q. Evaluation of combined disc method for the detection of metallo-β-lactamase producing Gram-negative bacilli. *Malays J Microbiol.* 2012;8(1):21-25.
16. Zee AVD, Roorda L, Bosman G, et al. Multicenter evaluation of Real time multiplex PCR for detection of Carbapenemase genes OXA-48, VIM, IMP, NDM and KPC. *BMC Infectious Diseases.* 2014;14:27. doi: 10.1186/1471-2334-14-27
17. Weiner-Well Y, Rudensky B, Yinnon A, et al. Carriage rate of carbapenem-resistant *Klebsiella pneumoniae* in hospitalized patients during a national outbreak. *J Hosp Infect.* 2010;74(4):344-349. doi: 10.1016/j.jhin.2009.07.022
18. Moemen D, Masallat DT. Prevalence and characterization of carbapenem-resistant *Klebsiella pneumoniae* isolated from intensive care units of Mansoura University hospitals. *Egyptian Journal of Basic and Applied Sciences.* 2017;4(1):37-41. doi: 10.1016/j.ejbas.2017.01.001
19. Endimiani A, Hujer AM, Perez F, et al. Characterization of blaKPC-containing *Klebsiella pneumoniae* isolates detected in different institutions in the Eastern U.S.A. *J Antimicrob Chemother.* 2009;63(3):427-437. doi: 10.1093/jac/dkn547
20. Poirel L, Bonnin RA, Nordmann P. Genetic features of the widespread plasmid coding for the carbapenemase OXA-48. *Antimicrob Agent Chemother.* 2012;56(1):559-562. doi: 10.1128/AAC.05289-11
21. ZA Memish, A Assiri, M Almasri, et al. Molecular characterization of carbapenemase production among gram-negative bacteria in Saudi Arabia. *Microbial Drug Resistance.* 2015;21(3):307-314. doi: 10.1089/mdr.2014.0121
22. Landman D, Salamera J, Singh M, Quale J. Accuracy of Carbapenem Nonsusceptibility for Identification of KPC-Possessing Enterobacteriaceae by Use of the Revised CLSI Break-points. *J Clin Microbiol*. 2011;49(11):3931-3933. doi: 10.1128/JCM.01176-11
23. Ganesh KS, Adithan C, Harish BN, Sujatha GS, Roy G, Malini A. Antimicrobial resistance in India: A review. *J Nat Sci Biol Med.* 2013;4(2):286-291. doi: 10.4103/0976-9668.116970