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RESEARCH ARTICLE OPEN ACCESS

Prevalence and Characterization of Multi Drug Resistant Gram Negative Bacilli Isolates from a Tertiary Care Centre of Western U.P., India

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Abstract

Infections with MDR GNB are associated with mortality rates 21% higher than those of non resistant GNB and results in longer in patient stays and higher treatment costs. Several Indian studies have reported prevalence of carbapenemase producing Enterobacteriaceae, Pseudomonas and Acinetobacter species in a range of 11% to 81%, because of ample variation reported in prevalence and incidence of carbapenemases reported from different geographical region from time to time, we aimed to determine prevalence of carbapenemase producing organism and carbapenemase encoding genes among clinical MDR-GNB isolates from our area and also to assess the performance of the phenotypic tests. This was a cross sectional study. A total of 510 multi drug resistant isolates included were subjected to MHT and MBL E strip Test to detect carbapenamase production. In addition these isolates were subjected to PCR assay to confirm presence of carbapenamase genes encoding for these enzymes. The study found carbapenemase prevalence of 58.6% by phenotypic tests. bla_{NDM} was the most common gene (24.7%) found by PCR assay followed by bla_{KPC} (14.9%), bla_{VIM} (9.6%) and bla_{OXA-48} (8.6%). Awareness of the prevalence and incidence of the carbapenem resistance and carbapenemase enzymes is crucial in the prevention of their spread and selection of appropriate treatment options. Study shows high prevalence rate of carbapenam resistant gram negative bacilli in this area, which indicates danger of limited treatment options and requirement of continuous detection of these cases to limit spread of resistant cases.

Keywords: Carbapenemase producing GNB, carbapenem resistant GNB, carbapenemase encoding genes.

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INTRODUCTION

Bacterial resistance to anti-microbial treatment is emerging as one of the major public health problem. Carbapenamases may be defined as beta lactamases that significantly hydrolyze at least imipenem or meropenem. Resistant to carbapenams is mostly due to the production of carbapenemases, which are β- lactamase enzymes with a capacity to hydrolyze not only the carbapenams but also all the other beta lactam agents[1,2]. The most common carbapenemases include verona integron metallo-beta–lactamases types (VIM), imipenemase (IMP) types, *Klebsiella pneumoniea* carbapenemase (KPC), oxacillinase-48 (OXA-48), and New Delhi metallo-beta-lactamase-1 (NDM-1), encoded by carbapenem resistance determining gene bla_{VIM}, bla_{IMP}, bla_{KPC}, bla_{OXA-48} and bla_{NDM} respectively[1]. Phenotypic assay are used to identify carbapenemase activity while molecular assay have been developed to identify carbapenemase encoding genes[2]. Recently, increasing resistance to carbapenams in health care associated infections has been reported worldwide[3-5]. Thus, resistance to carbapenams becomes a real threat to the survival of patients with infections caused by MDR-GNB as mortality in such infections has been reported up to 50% who acquire blood stream infections and overall mortality rates are 21% higher than those of non resistant GNB and results in longer in patient stays and higher treatment costs[1,6]. Several Indian studies have reported prevalence of carbapenemase producing Enterobacteriaceae, Pseudomonas and Acinetobacter species in a range of 11% to 81%[7-11]. This study set out to determine the burden of carbapenam resistance, prevalence of carbapenemase producing organism and carbapenemase encoding gene among clinical MDR-GNB isolates obtained from patients. We also aimed to determine performance of Modified Hodge test (MHT) and Metallo β-lactamase (MBL) E Test by comparing them with results of Polymerase chain reaction (PCR) assay.

MATERIALS AND METHODS

Study design and setting

This was a cross sectional laboratory based prospective study which was carried out in Microbiology department of Teerthankar Mahaveer Medical College and Research Centre, Moradabad, Uttar Pradesh, India, during the period of April 2016 to December 2018.

Sample collection

A total of 2562 non-duplicate samples from patients suspected of infection caused by Gram-negative bacteria like Urine, Pus, Blood, Body fluids, Tracheal secretion, Sputum, HVS, Foley's Tip etc were collected as per standard sample collection technique reported earlier[12].

Collected samples were subjected to conventional methods i.e. Gram staining, Culture, Biochemical tests. Out of 2562 samples 1507 were gram negative and out of 1507 gram negative bacterial isolates 510 were Multi Drug Resistant. Drug susceptibility test was done by Kirby Bauer disk diffusion method with following antibiotic disks Imipenem (IPM) 10µg, Meropenem (MRP) 10µg, Ertapenem (ETP) 30µg, Cefixime (CFM) 5mg, Cefepime (CPM) 30mg, Ceftazidime(CAZ) 30µg, Ceftazidime/Clavulanic Acid (CAC) 30µg, Ceftriaxone (CTX) 30µg, Cefoperzone/Sulbactum (CFS) 30µg, Ciprofloxacin (CIP) 5µg, Piperacillin/Tazobactam (PIT) 10µg, Amikacin (AK) 30µg, Tigecycline (TGC) 15µg and interpreted according to CLSI guidelines[13]. This was in order to find out MDR Strain and also to find relation between resistance to these drugs and carriage of carbapenemase gene. Multi Drug Resistant (MDR) strains were differentiated according to criteria given by Mattner *et al*.[6] In brief, isolates that were resistant to three different classes of antibacterials but sensitive to carbapenems were included and isolates that were resistant to any one carbapenem but sensitive to other anti-bacterials were also included.

Detection of carbapenemase production

Phenotypic detection of carbapenemase production was done by MHT and MBL E Test. MHT was performed and interpreted according to guideline provided by CDC[14]. MBL E test strip were obtained by HiMedia Pvt. Ltd and test was performed and interpreted according to kit insert provided with kit[15].

PCR amplification for carbapenemase genes

The entire molecular / PCR test (DNA extraction, amplification and gel electro-phoresis) were done in molecular laboratory of Subharti Medical College, Meerut.

DNA extraction was done using Qiagen DNeasy blood and tissue kit following

manufacturer's instructions[16]. for reaction mixture preparation, commercially available Genei® Master Mix kit was used. Manufacturer's instruction manual was followed for using the kit. Primers of PrimeX targeting bla_{VIM}, bla_{KPC}, bla_{OXA-48} and bla_{NDM} were obtained from Valine Life Sciences, India, as described in study by Asthana S *et al*.[17] For reaction mix preparations following contents were added Molecular grade water 15µl, Master Mix. in kit 25µl, Primers 0.2µl and Template DNA 10µl. The amplification was done using Applied Biosystem Veriti 96 well thermal cycler. For bla_{KPC}, bla_{OXA-48} and bla_{NDM} the programme was initial denaturation at 94°C for 5 minutes followed by 30 cycles of 30 seconds denaturation at 94°C, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. An additional final extension step was performed at 72°C for 7 minute. For bla_{VIM} the same programme was used except that annealing temperature was adjusted to 45°C for 60 seconds and had a final extension step of 72°C for 10 minutes.

5µl of PCR product were analyzed by electrophoresis in 1.0% agarose stained with ethidium bromide to detect the specific amplified product by comparing with 100 base-pair standard DNA ladder and visualized under gel doc. system. For quality control, of MHT well characterized strains were used. *E. coli* ATCC 25922 was used as a susceptible strain, *K. pneumoniae* ATCC BAA-1705 as a positive control while *K. pneumoniae* ATCC BAA-1706 was used as a negative control and for PCR tests, the following control strains were used; *K. pneumoniae* ATCC strain BAA-1705 for bla_{KPC}, *K. pneumoniae* ATCC strain BAA-2146 for bla_{NDM}, and *E. coli* ATCC BAA-2523 for $bla_{OXA-48.}$

Ethical Approval

The study protocol was carefully reviewed and approved by the Institutional Ethics Committee of the Teerthankar Mahaveer Medical College and Research Centre.

Data Analysis

Data analysis was done using SPSS ver. 16.0. All categorical variables were represented by percentages and Comparison of categorical variables was done by Chi-square test. A p value of <0.05 was considered as evidence of significant statistical difference.

RESULTS

Distribution and Characteristics of Isolates Included in study

A total of 2562 samples were processed during study period of April 2016 to December 2018 in which 1507 were gram negative bacilli and out of 1507 gram negative bacterial isolates 510 were Multi Drug Resistant. MDR strains were differentiated according to criteria given by Mattner *et al*.[6] most of which were resistant to three different classes of anti-bacterial. Overall 25.1% isolates were resistant to one or more carbapenem tested. Individually, 11%, 8.1%, 6% resistant rate was observed by Imipenem, Ertapenem and Meropenem respectively. Out of imipenem, ertapenem and meropenem resistant isolates genes were present in 59% 64%, and 68.8% isolates respectively.132 isolates were resistant to three different classes of anti-bacterial but sensitive to carbapenems tested. Distribution of antibiotic resistant isolates is shown in Table 1. Out of 510 MDR strains 267 were from male and 243 from female patients. The age of patients ranged from 1day to 82 years with a median of 35 years. Most common species isolated among MDR-GNB was *E. coli* (34.7%, 177/510) followed by *K. pneumoniae* (18.2%, 93/510) and *P. aeruginosa* (9.4%, 48/510). It was also observed that highest frequency of *E. coli* was from Gyne ward and that of *K. pneumoniae* was from Medicine ICU. Distribution of isolates from various sources is shown in Table 2. Highest numbers of MDR organisms were from MICU followed by SICU. Most common sample was Urine (140/510) followed

Table 1. Cross Tabulation showing total drug resistant isolates and number of isolates with gene detected

Drug Resistant	Gene Detected	No gene Detected	Total Isolates
Imipenem	92	74	166
Ertapenem	75	47	122
Meropenem	57	33	90
Carbapenem resistant MDR-GNB	224	154	378
Carbapenem sensitive MDR-GNB	17	115	132
Total	241	269	510

by Pus (113/510) and Blood (85/510). Majority of Urine samples was received from FMW, Gyne and MMW. Majority of Pus samples were from MMW and Blood samples were from Medicine ICU and Surgical ICU as shown in Table 3.

Prevalence of Carbapenemase producing organism based on MHT and MBL E-test

Carbapenemase activity was detected in 15.6% (80/510) isolates by MHT method, 23.5% (120/510) by MBL E test method. 19.4% (99/510) isolates were positive for both tests. Therefore, total number of isolates positive by MHT was 179/510 (35.0%) and by E test was 219/510 (42.9%) correlation of phenotypic results with

Table 2. Shows Ward wise Distribution of Species isolated

Wards	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*A. lwoffi*	*C. freundi*	*E. aerogenes*	Other Organism*	Total
ICCU	02	01	—	01	—	—	—	04
MICU	14	14	04	09	09	—	13	63
NEU. ICU	03	02	03	06	02	01	09	26
NICU	05	05	—	01	—	02	05	18
PICU	01	—	01	—	02	01	02	06
SICU	14	07	07	06	05	03	07	49
ENT	—	—	01	—	—	—	02	03
FMW	18	08	01	—	01	01	05	34
FOW	01	—	—	—	—	—	01	02
FSW	06	04	—	—	01	01	05	17
GYNE	22	02	—	01	01	01	04	31
L/R	06	03	—	01	—	01	01	12
OBS	14	03	01	03	05	03	05	34
MMW	14	07	03	—	—	01	02	27
MOW	07	08	09	05	02	03	04	38
MSW	13	09	06	—	03	03	06	40
URO	01	—	—	—	—	—	—	01
OPD	09	05	01	01	—	04	09	29
PEDIA	04	02	—	01	—	—	01	08
PVT.	05	03	01	—	—	01	01	11
TBC	05	03	05	04	01	01	08	27
E/W	15	05	05	02	—	01	01	29
TOTAL	177	93	48	41	32	28	91	510

*Others Organism Include K. oxytoca (n=21), E. cloacae (n=17), C. koseri (n=13), P. mirabilis (n=4), P. vulgaris (n=3), Pseudomonas Species (n=29), A. baumanni Complex (n=4)

ICCU: Intensive Critical care Unit, MICU: Medicine Intensive care Unit, Neu ICU: Neuro Intensive care Unit, NICU: Neonatal Intensive care Unit, PICU: Paediatric Intensive care Unit, SICU: Surgical Intensive care Unit, FMW: Female Medicine Ward, FOW: Female Orthopaedic Ward, FSW: Female Surgery Ward, L/R Labour Room, MMW: Male Medicine Ward, MSW: Male Surgery ward, URO: Urology Ward, PVT.: Private ward, OPD: Out Patient Department, E/W: Emergency ward

that of number of gene detected is shown in Table 4. Among 510 MDR isolates total 299 were carbapenemase producers by phenotypic methods and 211 were non carbapenemase producers. Table 5.

Prevalence of genes encoding for Carbapenemase enzymes

Based on PCR assays 47.2% (241/510) of the isolates were positive for one or more gene. *E.coli* was species with highest number of gene detected, whereas percentage wise highest rate of gene among MDR isolates were detected in *E. cloacae* (64.7%) followed by *C. freundi* (62.5%). 82 out of 177 MDR *E. coli* strains were positive for one or more gene followed by *K. pneumoniae* (40/93, 43%), *A. lwoffi* (25/41, 60.9%), *P. aeruginosa* (20/48, 37.9%) and *C. freundi* (20/32, 62.5%). 82 (16.07%) isolates had presence of one or

Table 3. Distribution of type of samples obtained from different wards

Wards	Urine	Pus	Blood	Sputum	ET. Secr.	Other Samples[#]	Total
ICCU	02	—	—	01	—	01	04
MICU	08	01	20	07	20	07	63
NEU. ICU	02	—	02	01	18	03	26
NICU	—	—	10	—	—	08	18
PICU	—	—	04	—	—	03	07
SICU	07	09	16	02	12	03	49
ENT	—	03	—	—	—	—	03
FMW	21	02	05	02	—	04	34
FOW	—	01	—	01	—	—	02
FSW	04	07	02	—	02	02	17
GYNE	22	05	—	—	—	04	31
L/R	03	—	—	—	—	09	12
OBS	16	05	—	—	—	13	34
MMW	21	—	02	02	—	02	27
MOW	—	35	02	01	—	—	38
MSW	05	18	07	03	03	04	40
URO	01	—	—	—	—	—	01
OPD	13	15	01	—	—	—	29
PEDIA	02	03	03	—	—	—	08
PVT.	03	03	02	01	01	01	11
TBC	01	03	—	13	—	10	27
E/W	09	03	09	08	—	—	29
TOTAL	140	113	85	42	56	74	510

[#]Other Samples Include: - BAL (n=12), Bronchial Biopsy specimen (n=2), Catheter Tip (n=1), CSF (n=2), D&E Specimen(n=1), Drain Fluid (n=3), Peritoneal Fluid (n=7), Rectal Swab (n=7), Vaginal Swab (n=3), Pleural Fluid (n=1), Foley's Tip (n=18), HVS (n=17)

more genes. Distribution of gene in various MDR isolates is shown in Table 6. All target genes were unevenly distributed among the isolated species with overall highest prevalence of bla_{NDM} (24.7%) followed by bla_{KPC} (14.9%), bla_{VIM} (9.6%) and bla_{OXA} (8.6%). Species wise distribution of genes detected is shown in Table 7.

Correlation of phenotypic and genotypic tests

Out of 80 isolates positive by MHT 51 isolates showed presence of one or more gene and in 29 isolates no gene was detected. Out of 120 isolates positive by MBL E test genes were detected in 104 samples and in rest of 16 isolates which were phenotypically positive but no gene

Table 4. Cross tabulation of results of phenotypic tests with gene detection

MHT Result	MBL E-test Result	Total no. of Samples	Number of samples in which Gene found	Number of Samples in which gene not found
Positive	positive	99	86	13
Positive	negative	80	51	29
Negative	positive	120	104	16
Negative	negative	211	nil	211
Total number of sample Processed		510	241	269

Table 5. Distribution of Number of Carbapenemase Producing and Non Carbapenemase producing isolates from different wards

ICU'S	CPP	CPN	Total Isolates
MICU	44	19	63
SICU	34	15	49
PICU	3	4	7
NICU	6	12	18
NEURO ICU	20	6	26
ICCU+ CCU	3	1	4
	110	57	167
WARDS	CPP	CPN	Total
MSW	26	14	40
MOW	19	19	38
MMW	12	15	27
FMW	20	14	34
FSW	8	9	17
FOW	1	1	2
GYNE	15	16	31
L/R	8	4	12
OBS	18	16	34
E/W	19	10	29
PEDIA	4	4	8
ENT	3	0	3
URO	1	0	1
TBC	14	13	27
PVT.	7	4	11
	175	139	314

Table 6. Species wise distribution of Gene detected in number of MDR isolates

Organism Isolated	Total Number of MDR organisms	Genes Detected In
E. cloacae	17	11(64.7%)
C. freundi	32	20(62.5%)
A. lwoffi	41	25(60.9%)
K. oxytoca	21	12(57.1%)
P. mirabilis	4	2(50%)
E. coli	177	82(46.3%)
C. koseri	13	6(46.1%)
K. pneumoniae	93	40(43.0%)
P. aeruginosa	48	20(41.6%)
E. aerogenes	28	11(39.2%)
Pseudo. Spp	29	11(37.9%)
Acb complex	4	1(25%)
P. vulgaris	3	0(0%)
Total	510	241

was detected in them. 99 isolates which were positive for both test MHT and MBL E test 86 were positive for gene detection and in 13 isolates no gene was detected. Table 4

Sensitivity and specificity of Modified Hodge test and MBL E test was calculated considering PCR as gold standard. MHT gave better performance for detection of Class A and Class D genes, sensitivity and specificity for bla_{KPC} was 93.4% and 75.1% and sensitivity and specificity for bla_{OXA} calculated was 84.7% and 69.8% whereas MBL E test is better for MBL detection, sensitivity and specificity for bla_{NDM} was 99.2% and 78.3%. Overall sensitivity and specificity of MHT found was 56.8% and 78.8% and sensitivity and specificity of E-test was 82.4% and 86.7% respectively. Statistically it was also evident that MBL E Test had strong association with detection of bla_{NDM} and bla_{VIM} ($p<0.05$) and MHT showed a good association with detection of bla_{KPC} and bla_{OXA-48} genes. ($p<0.05$).

DISCUSSION

Antibiotic resistance to reserve antibiotic class is on a continuous rise among gram negative bacteria especially in the family Enterobacteriaceae and among species of Acinetobacter and Pseudomonas (EPA Species)[1,2]. Recently, a newspaper article reported 13% of mortality rate in India is due to antibiotic resistant cases, which is more than double when compared to developed nations where mortality rate due to drug resistant cases is 2-7%[18]. Worldwide several studies had reported increased prevalence of carbapenemase producing organisms[5,19,20]. Our findings show out of 510 MDR strains 52.3% were from males and 47.6% were from females. Ratio of male to female patient in this study was 1:1.09 this shows almost equal distribution of Antibiotic resistant strains among both sexes. We found most common MDR organism was *E.coli* followed by *K. pneumoniae* and *P. aeruginosa* and the hotspot zone of these organism were medical and surgical ICU'S. Similar findings were reported by Mathias *et al*. from Ludhiana, Diwakar J. *et al*. from Etawah and Manohar *et al*. from Tamil Nadu region[8,9,21]. These findings may have vital role in making of hospital infection control policy. This study shows a prevalence rate of carbapenemase enzyme of 58.6% by phenotypic tests among EPA species

Table 7. Distribution of different Carbapenemase genes detected in various Enterobacteriaceae, *Pseudomonas* and *Acinetobacter* species.

Group	Organism	NDM	KPC	VIM	OXA	NDM, KPC	NDM, VIM	NDM, OXA	KPC, VIM	KPC, OXA	VIM, OXA	NDM KPC VIM	NDM KPC OXA	NDM VIM OXA	KPC OXA VIM	NO. of isol.
Entero-bacteria-ceae	*E.coli*	28	14	10	11	06	—	04	02	05	—	—	01	01	—	82
	K.pneumoniae	15	07	08	01	04	01	01	02	—	—	—	—	—	01	40
	K.oxytoca	06	01	—	01	02	01	01	—	—	—	—	—	—	—	12
	E.aerogenes	06	01	—	01	—	02	—	—	—	—	01	—	—	—	11
	E.cloacae	04	00	—	01	02	01	02	01	—	—	—	—	—	—	11
	C.freundi	10	01	01	01	02	—	02	01	—	—	01	—	01	—	20
	C.koseri	02	01	—	—	—	01	—	—	—	—	01	—	—	01	06
	P.mirabilis	01	01	—	—	—	—	—	—	—	—	—	—	—	—	02
	P.vulgaris	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	Total	72	26	19	16	16	06	10	06	05	—	03	01	02	02	184
Pseudomonas	*P.aeruginosa*	08	05	04	01	01	—	—	—	—	01	—	—	—	—	20
	Pseudo. spp	05	02	03	—	—	—	—	—	—	01	—	—	—	—	11
	Total	13	07	07	01	01	—	—	—	—	02	—	—	—	—	31
Acineto-bacter	A.lwoffi	11	04	02	02	01	—	01	—	02	—	—	02	—	—	25
	Acb Complex	01	—	—	—	—	—	—	—	—	—	—	—	—	—	01
	Total	12	04	02	02	01	—	01	—	02	—	—	02	—	—	26
Grand Total		97	37	28	19	18	06	11	06	07	02	03	03	02	02	241

resistant to three different classes of antibacterials or resistant to any one of the carbapenem tested. Our phenotypic prevalence is lower than then that reported by Diwakar *et al.* from Etawah, Mate *et al.* from Imphal and Saini *et al.* from Jaipur who reported phenotypic prevalence of 81.8%, 60% and 83% respectively[9,10,22], whereas, higher as compared to that reported by Mathias *et al.* from Ludhiana, Gupta *et al.* from Varanasi and Singh *et al* from Navi Mumbai[8,23,24]. They reported phenotypic prevalence of 57%, 50% and 43.7% respectively. The difference in these findings could be because of variation in geographical regions which occur from time to time and also because of different inclusion criteria and test done. Our prevalence is also much higher than that reported in studies from western countries like United States, Canada, and Latin America[19,25,26]. These differences may be due to restricted use of antibiotics in these countries compared to India where many drugs are available over the counter without prescription of a clinician. In parallel, we found prevalence of genes encoding for carbapenemase was 47.2%. Variable rate of genotypic prevalence has been reported by various Indian studies ranging from 18% to 100%[7-11,23]. The difference might be due to different target genes as in some studies only single class of gene was targeted whereas in our study common genes of all classes of carbapenemase were included. The most prevalent gene among 510 MDR GNB isolates was bla_{NDM}, (27.4%) This was in accordance to studies elsewhere in India viz., Delhi, Guwahati, Mumbai, Vellore and Puducherry reporting bla_{NDM} as the commonest gene detected[7,27-29]. Although in western world most common gene encoding carbapenemase found is bla_{KPC} [19,30]. Whereas a study from Africa reported highest prevalent gene were bla_{IMP} types while another one reported bla_{VIM} as the most common gene encoding for carbapenemase enzyme[1,2]. These findings are suggestive of inter-regional spread of the specific mechanism of carbapenem resistance. In our study we found 58(11.3%) samples were phenotypically positive but no gene was detected in them by PCR. This might be due to limited number of genes targeted in our study as well as to other mechanisms of resistance such as porin loss or mutations.

When we compared the performance of phenotypic tests to results obtained by PCR, it was found that Modified Hodge test was more sensitive and specific for Class A enzyme i.e. bla_{KPC} (93% sensitive and 75.11% specific) and Class D enzyme i.e. bla_{OXA} whereas MBL E test performed better for detection of Class B enzymes i.e. bla_{NDM} and bla_{VIM}. Similar results were reported by Girlich *et al*.[31]

CONCLUSION

Carbapenemases are globally distributed and their prevalence and incidence vary considerably across each continent, nation, region and even centre to centre, so awareness of the prevalence and incidence of the carbapenem resistance and carbapenemase enzymes is crucial in the prevention of their spread and selection of appropriate treatment options. Our study shows high prevalence rate of carbapenamase producing gram negative bacilli in this area, which indicates danger of limited treatment options and requirement of continuous detection of these cases to limit spread of resistant cases. We also found that combination of two phenotypic tests MHT and E strip Test can be done together to rule out false negative results whereas E Test should be done on regular basis to detect MBL as MBL encoding genes were more prevalent in our region as it is not feasible to do PCR on regular basis on every sample.

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CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

AUTHOR'S CONTRIBUTION

SM performed the tests, collected data, did data analysis and wrote the manuscript. UF guided the study and reviewed the manuscript. SM and UF approved it for publication.

FUNDING

None.

DATA AVAILABILITY

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

ETHICS STATEMENT

The study protocol was carefully reviewed and approved by the Institutional Ethics Committee of the Teerthankar Mahaveer Medical College and Research Centre, Moradabad U.P. India.

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