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Original Research Paper

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PHENOTYPIC DETECTION, BIOFILM PRODUCTION AND MOLECULAR CHARACTERIZATION OF *K. PNEUMONIAE* ISOLATES RECOVERED FROM BLOOD STREAM INFECTIONS IN A TERTIARY CARE HOSPITAL

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ABSTRACT

Objective: To examine Carbapenem resistance, & biofilm production in K.pneumoniae isolates recovered from BSIs.

Methods: A prospective, Cross-sectional study was conducted in a 650 bedded tertiary care centre in Jhajjar, Haryana India from July 2019 to April 2020. A total of 76 isolates were recovered from Blood stream infections (BSIs). Carbapenem resistance, biofilm production was checked phenotypically and the presence of -lactamase genes by PCR. Isolate relatedness was determined by REP PCR, and ERIC PCR.

Results: From July 2019 to April 2020, in this study a total of 76 K.pneumoniae isolates recovered from BSIs, 58 isolates were ESBL producers positive for TEM, SHV and CTX-M genes,42 were carbapenemase producers and 29 were metallo beta lactamase producers positive for DDST, CDST and MBL (IP/IPI) E-test. Out of these 29 MBL producers18 had blaNDM-1 gene, and 11 had blaVIM-2 gene respectively.

Conclusions: The significant presence of both metallo beta lactamase genes and biofilm co-existence in the same isolates is worrisome.

KEYWORDS : BSIs, Biofilm, *K.pneumoniae*, ESBL, *blaTEM-1, blaSHV, blaCTXM, blaNDM-1*, and *blaVIM-2*

INTRODUCTION-

Bloodstream infections are the leading cause of morbidity and mortality in people of all ages, particularly in debilitated and immunocompromised patients. These infections are very frequent and present as a life-threatening condition in hospital environment. Worldwide, BSIs affects about 30 million people leading to 6 million deaths, In the determination of causative agents of bloodstream infection, blood cultures are the best method of choice because they are highly sensitive and easier to perform. BSIs are characterized by the presence of viable microbe in the bloodstream that elicit inflammatory response and often accompanied by alteration of clinical, laboratory, and haemodynamic parameters. [1] [2] [3] [4] [5] β-lactam antibiotics are the most frequently used antimicrobials for empirical therapy. ESBL-producing strains of *K.pneumoniae* are a serious threat as these plasmid-mediated bacterial enzymes confers significant resistance to oxyimino cephalosporin and monobactam antimicrobials. Carbapenems are often used to treat infections caused by ESBL producing *K.pneumoniae*. However, resistance to carbapenems most frequently mediated by the enzymatic hydrolysis of the drugs by *K.pneumoniae*. [1] [2] [3] [4] [5] Carbapenemases enzymes recognize almost all hydrolysable β-lactams, and most are resistant to inhibition by all commercially viable β-lactamase inhibitors. Ambler class A (including KPC and GES), A, and Ambler class D (CHDLs or OXA-48) beta-lactamases had serine at their active site, while Ambler class B (including IMP, VIM, SIM, and NDM) are all metalloenzymes with zinc in active-site. Biofilms are the microbial communities that are irreversibly associated with a surface and are enclosed in a self-produced extracellular polymeric matrix. Metalloenzymes producing *K.pneumoniae* within the biofilm are more resistant to antimicrobial agents leave patients with very few or no antimicrobial options. [1] [2] [3] [4] [5] This study provides an insight into the acquisition and spread of the MBL genes along with Biofilm production.

MATERIALS AND METHODS

The Bacterial Isolates -A prospective, cross-sectional study was conducted in a 650 bedded tertiary care centre in Jhajjar, Haryana, India from July 2019 to April 2020. A total of 76 clinically significant, non-duplicate *K.pneumoniae* isolates were recovered from BSIs of hospitalized patients admitted to the Medical and Surgical intensive care units. Bacterial identification was performed by routine conventional microbial culture and biochemical tests using standard recommended techniques [3] [6] All the strains were preserved in 15% glycerol-supplemented Luria-Bertani medium at − 80°C for molecular analysis.

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility test was performed by the Kirby Bauer's disc diffusion technique on Mueller–Hinton agar, as per Clinical Laboratory Standard Institute (CLSI) guidelines.[7]

The antibiotics tested were as follows (potency in μg/disc): Ampicillin (10), Cefuroxime (30), Cefpodoxime(30), Cefixime(5), Ceftazidime (30), Cefazolin(30), Cefoxitin(30), Cefepime (30), Cefotaxime (30), Piperacillin (100), Piperacillin-Tazobactam (100/10), Aztreonam (30), Imipenem (10), Meropenem (10), Ertapenem (10), Doripenem(10), Colistin(10), Gentamicin (10), Tobramycin (10), Amikacin (30), Netilmicin (30), Ciprofloxacin (5), and Levofloxacin (5) (Hi Media Laboratories Pvt. Ltd., Mumbai, India). *Paeruginosa* ATCC 27853, *E.coli* ATCC 25922, *E.coli* ATCC 35218 and *K.pneumoniae* ATCC 700603 were used as quality control strains. The degree of susceptibility of the test isolate to each antibiotic was interpreted as sensitive (S), intermediate resistant (I) or resistant (R) by measuring the zone diameter of inhibition. [7]

MIC Determination

Minimum inhibitory concentrations (MIC) of antibiotics were determined by Microbroth dilution Assays and further confirmed by the E-test (bioMérieux, Marcy l'Etoile), France for individual antibiotic. [3] [6] [7]

Phenotypic Screening For ESBL Detection

Isolates with reduced susceptibility to **Ceftazidime (17 mm), Aztreonam (17 mm)**, **Cefotaxime (22 mm)** and **Ceftriaxone (19mm)** as recommended by CLSI guidelines, were selected for confirmation of ESBL production. Isolates were tested for ESBL production by standard CLSI double-disc diffusion method and double disc synergy test and using E test (bioMérieux, Marcy l'Etoile, France) for detecting the MIC. These tests were checked for quality using standard control ESBL negative strain of E. coli ATCC 25922. [3] [6] [7]

Phenotypic Screening for Carbapenemase Production

Isolates with reduced susceptibility to ertapenem, meropenem and imipenem (diameter of zones of inhibition ≤18mm) by disc diffusion method were screened for the production of carbapenemase. [3] [6] [7]

Phenotypic Screening For Metallo Beta Lactamase Production

MHT, DDST, CDST and MBL (IP/IPI) E-test was performed to detect Carbapenemase as well as Metallo-beta-lactamase production as described previously.[3][4][5][6][7]

DNA Extraction And Molecular Detection

DNA was extracted from the bacterial isolates using the spin column method (QIAGEN; GmbH, Hilden, Germany) as per manufacturer's instructions. PCR-based detection of beta lactamase (ESBL) genes (bla_{TEM}, bla_{SHV}, bla_{CTXM} and bla_{OXA}), Ambler class B MBLs ($bla_{IMP,}$ $bla_{VIM,}$ bla_{GIM}, $bla_{SIM,}$ $bla_{SPM,}$ *and* bla_{NDM}), Ambler class D ($bla_{OXA-23,}$ $bla_{OXA-24,}$ *and* bla_{OXA-48}) and serine carbapenemases ($bla_{KPC,}$ $bla_{GES,}$ *and* bla_{NMC}) were carried out on the isolates by using Gene Amp 9700 PCR System (Applied Biosystems, Singapore). [4] [5] PCR products were run on 1.5% agarose gel, stained with ethidium bromide visualized under UV light and photographed. The amplicons were purified using QIAquick PCR purification kit (QIAGEN; GmbH, Hilden, Germany) DNA sequencing and sequence analysis Automated sequencing was performed on an ABI 3730XL DNA analyzer using the Big Dye system (Applied Biosystems Foster City, CA, USA). Sequences were compared with known sequences using the BLAST facility (http://blast.ncbi.nlm.nih.gov).[3][4][5][6]

Detection Of Biofilm Production:

K.pneumoniae isolates producing biofilm were detected using Congo Red Agar method (qualitative) and Microtiter plate quantitative assay. The biofilm formation on microtiter plate were detected using 0.1% crystal violet and safranin separately and then the optical density (O.D) of each well were measured at 490 nm and 570 nm wavelength using automated (Lisa *Scan* EM Microplate Reader). The biofilm producers were differentiated as high, moderate, weak and non-adherent based upon the comparison with control.[4][5]

Molecular Typing Of The Strains

Repetitive element-based PCR (REP-PCR) and Enterobacterial Repetitive Intergenic Consensus (ERIC – PCR) assays were performed, to rapidly characterize metalloenzymes producing *K.pneumoniae* strains which were recovered from the patients. Similarity clustering analysis was performed using unweighted pair group method with arithmetic mean and Dice coefficient. Clinical isolates with a similarity coefficient >85% were considered clonal.[4][5]

RESULT AND DISCUSSION

Out of 76 isolates, 58 (76%) were found to be ESBL producers resistant to **Ceftazidime, Aztreonam**, **Cefotaxime** and **Ceftriaxone (MIC ≥ 16** μg/mL**).** Out of these 42 (55%) exhibited reduced susceptibility to **ertapenem**, **meropenem** and **imipenem (MIC ≥ 8** μg/mL**).** Among these 42 isolates, 29 had shown metallo beta lactamase enzyme production (38%). (Table -1) These *29 K.pneumoniae* isolates were detected producing biofilm using Congo Red Agar method (qualitative) and Microtiter plate quantitative assay as compared with controls. Table -2 showing antibiograms of Metallo beta lactamase producing *K.pneumoniae*. Intensive care units provide an ideal environment for the dissemination of resistant determinant genes within the organisms. There are multiple risk factors associated with both environment and the patient that allow the development and spread of such pathogens. ICU patients often have frequent hospital admissions with their respective underlying medical conditions and there has been increase in the risk of colonization by multi-drug-resistant pathogens. Carbapenem resistant isolates are generally resistant to most other classes of antibiotics, while usually retaining susceptibility to tigecycline, Polymyxin B and colistin. PCR amplification for Ambler class D ($bla_{OXA-23,}$ $bla_{OXA-24,}$ *and* bla_{OXA-48}) and serine carbapenemases ($bla_{KPC,}$ $bla_{GES,}$ *and* bla_{NMC}) was found to be negative for all isolates. Out of 29 MBL producing *K.pneumoniae, 18(62%) had* bla_{NDM-1}, while 11 (38%) had $bla_{VIM-2.}$

bla_{TEM-1}, $bla_{SHV-12,}$ $bla_{CTXM-15}$ and bla_{NDM-1} gene were found in 11, seven had copresence bla_{TEM-1},$bla_{SHV-28,}$ $bla_{CTXM-14}$ and $bla_{NDM-1.}$ bla_{TEM-1}, $bla_{SHV-12,}$ $bla_{CTXM-15}$ and bla_{VIM-2} gene were present in 8 isolates, while bla_{VIM-2} in association with bla_{TEM-1}, $bla_{SHV-28,}$ and $bla_{CTXM-14}$ was present in three isolates. The results of Microtiter plate assay for detection of quantitative biofilm formation were compared by staining with both 0.1% crystal violet and 0.1% safranin. Cut off value for Crystal violet had shown Strong= ≥ 0.72 O.D. while Safranin had ≥ 0.83 O.D. for strong biofilm. Strain molecular typing of 29 strains of *K.pneumoniae* by REP PCR generated 5 cluster with an average of 6 to 10 fragments while ERIC PCR produced 6 clonal clusters with an average of 8 to 16 fragments. This showed that ERIC -PCR was much better as compared to REP PCR.

CONCLUSION

This study identify the metalloenzymes producing *K.pneumoniae* and their association with biofilm production. The study revealed that carbapenem resistant isolates are strong biofilm producer hence can be useful for clinical infection control purposes.

Declarations-

Financial Support And Sponsorship- Nil.

Conflict Of Interest-None to declare

Table -1 Showing Phenotypic Characterization Of *K.pneumoniae* Isolates Recovered From BSIs

Number of isolates	Carbapenem resistance by disc diffusion	ESBL	CDST	DDST	MBL -test	Biofilm
76	42	58	29	29	29	29

Table -2 Antibiograms Of Metallo Beta Lactamase Producing *K.pneumoniae* Along With Interpretive Criteria For Each Drug.

Antibiotic	Zone size in mm	MIC (μg/mL)
Ampicillin	≤13	≥32
Ampicillin sulbactam	≤11	≥32/16
Amoxicillin-Clavulanic Acid	≤13	≥32/16
Amikacin	≤16	≥64
Aztreonam	≤17	≥16
Cefazolin	≤19	≥8
Cefepime	≤18	≥16
Cefotaxime	≤22	≥4
Cefoxitin	≤14	≥32
Ceftazidime	≤17	≥16
Ceftriaxone	≤19	≥4
Cefuroxime	≤14	≥32
Cefixime	≤15	≥4
Ciprofloxacin	≤21	≥1
Colistin		≤2
Ertapenem	≤18	≥2
Gentamicin	≤12	≥16
Imipenem	≤19	≥4
Levofloxacin	≤16	≥2
Meropenem	≤19	≥4
Netilmicin	≤12	≥32
Piperacillin	≤17	≥128
Piperacillin/tazobactam	≤17	≥128/4
Doripenem	≤19	≥4
Tobramycin	≤12	≥16

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