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Isolation and Identification of Lactobacillus Species Against Biofilm Forming Klebsiella Pneumoniae Isolated from Food Samples

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Abstract: ***Klebsiella pneumoniae, an important multi drug resistant opportunistic pathogen, it is able to colonise the human intestine and displays a high capacity to form biofilm; which are communities of bacteria embedded in an extracellular matrix. This matrix consists of proteins, exopolysaccharides, DNA, and lipopeptides. K. pneumoniae used these virulence factors for survival and for evade from immune system during infection as well as biofilm formation itself. Type1 and Type3 fimbriae produces biofilm. Biosurfactants produced by Lactobacilli has the ability to reduce adhesion of Klebsiella and inhibit biofilm formation. In this study antibiofilm activity of Lactobacillus was assessed against Klebsiella pneumoniae.***
Keywords: Klebsiella pneumonia , Biofilm, Lactobacillus

I. INTRODUCTION

Klebsiella spp. is normally considered a normal flora in the mouth and gastrointestinal tract. It has many factors enhance its pathogenicity; still it considered as an opportunistic human pathogens besides infecting variety of animals, K. pneumonia has been identified as important common pathogens that can cause urinary tract infection, septicemia, wound infection, hepatic infections, neonatal infection and bacteremia(Abdal et al., 2020). Klebsiella is one of the pathogens able to form biofilm and then to produce Catheter Associated Infections (CAI). The capability to form biofilm can be considered as a virulence factor(Barati et al., 2016). Biofilm are complex communities of microorganisms attached to a surface or interface enclosed in an exopolysaccharide matrix of microbial and host origin to produce a spatially organized three dimensional structure . Biofilms are universal, occurring in aquatic and industrial water systems as well as a large number of environments and medical devices relevant for public health(Maldonado et al; 2012).

Lactobacilli have been extensively studied due to their remarkable ability to inhibit the growth of Other organisms through bactericidal activity and by producing lactic acid as a byproduct of its metabolism(Al-Mathkhury et al., 2012). Beneficial effects of Lactobacilli, including inhibition of Gram-positive and Gram-negative pathogenic and spoilage bacteria have been reported by many researchers . The therapeutic role of lactobacilli in controlling the infections caused by Pseudomonas aeruginosa, Staphylococcus aureus and Salmonella spp. has been reported(Sharma et al; 2017). The formation of biofilms by K. Pneumoniae is thought to protect the bacteria from antibiotics since these entities are highly resistant to such antimicrobial agents. The ability of K. pneumoniae isolates to produce biofilm were evaluated using crystal violet staining technique in pre-sterilized 96-well polystyrene microtiter plates(Mathkhury et al., 2007). The crystalline biofilms formed by Proteus mirabilis can seriously complicate the care of patients undergoing bladder catheterization, the prevention of crystalline biofilms is important to avoid urinary catheter complications. The surfactant of Lactobacillus acidophilus can be interacting with attachment of many microorganisms (Ali et al., 2012).The LAB isolates were first identified based on colony morphology, physiology, and biochemical reactions as per the criteria of Bergey's manual of Systematic Bacteriology. Lactobacillus was characterized by cell morphology, Gram reaction, catalase and oxidase activity, sporulation, and cell motility (Farid et al., 2016).

II. MATERIALS AND METHODS

A. Sub Culturing Of Lactobacillus Species On Mrs Agar

The Lactobacillus was collected from AL Kalamassery, Ernamkulam, India. The obtained Lactobacillus was sub-cultured in MRS agar (De Man, Rogosa and Sharpe agar) after sterilization. The media was prepared by dissolving 1.33g in 20ml of distilled water and sterilised under autoclave at 1210C for 15minutes. Sterilised media was poured to petriplate under aseptic condition and transferred the Lactobacillus after solidification. This inoculated plate was incubated at 37°C for 24 hours.

B. Identification Of Bacterial Cells

Lactobacillus and Klebsiella pneumoniae are subjected to quadrant streaking on Nutrient agar plates to obtain well isolated colonies. Gram staining was done to identify the morphological characteristics of organisms.

C. Determination Of Antibiofilm Activity Against Klebsiella Pneumoniae

The ability of K. pneumoniae isolates to produce biofilm were evaluated using crystal violet staining technique in pre-sterilized 96-well polystyrene microtiter plates. Cultures of Lactobacillus and Klebsiella was added to 96 well plate and incubate for 24-48 hours at room temperature. After incubation, perform crystal violet staining procedure. OD was measured at 540nm using Microtitter plate reader (Humareader HS) . Observe the value and calculate the % of inhibition by the following equation:

% of inhibition = Control OD – Test OD ×100/ Control OD

D. Lactic Acid Estimation

A test solution (50 µl) containing lactic acid was added to 2ml of 0.2% ferric chloride and stirred and absorbance was measured at 390nm against reference solution (2ml of a 0.2% FeCl3 solution). The colour of the solution was stable for 15 minutes.

E. Molecular Identification Of Selected Isolate

The isolate was identified using the molecular technique. The genomic DNA of the isolate was extracted by using the Bacterial Genomic DNA extraction kit according to the manufacturer protocol (QIAGEN, QIAamp DNA Mini Kit) with some modification. The isolated DNA was then amplified using the following PCR mix: 1 µl of bacterial universal 16S rRNA primers forward(5'-AGAGTTTGATCMTGG-3') and 1 µl of reverse primer (5'-ACCTTGTTACGACTT-3') , 2 µl of genomic DNA and 6 µl of PCR grade water were added and the PCR amplification was done. Amplified sequence threads were submitted to the NCBI database and NCBI BLAST (http://www.ncbi.nlm.nih.gov/Blast) was carried out to distinguish the nearest neighbors of the isolates .

III. RESULT

A. Sub Culturing Of Lactobacillus Species On Mrs Agar

Lactobacillus was grown on MRS agar after 24 hours of incubation at 37°C.

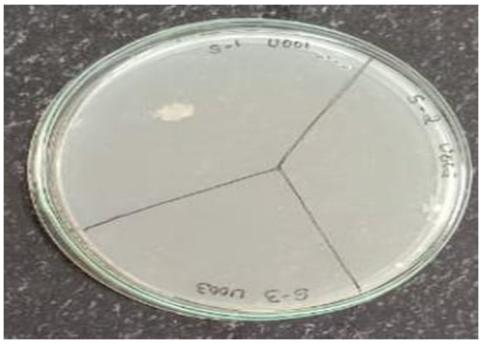

Fig 1: Lactobacillus grown on MRS agar

B. Identification Of Bacterial Cells

Colony morphology was observed on Nutrient agar plate.

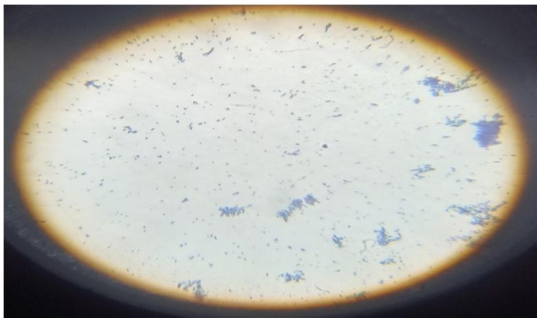

Fig 2: Lactobacillus.

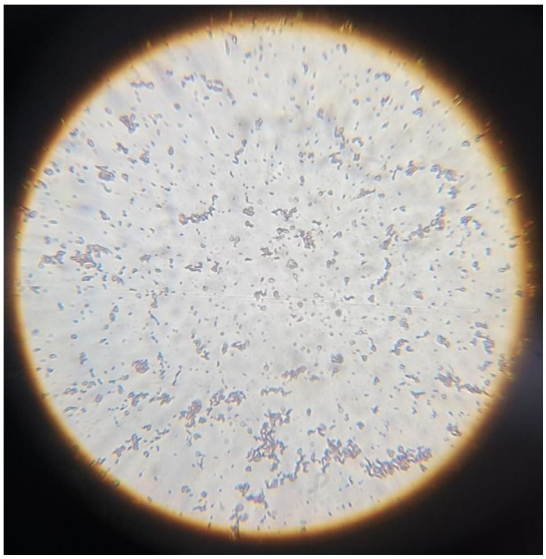

Fig 3: Klebsiella pneumoniae

C. Determination Of Antibiofilm Activity Against Klebsiella Pneumoniae

Control OD :- 0.97

Table no:1

Volume	OD	% of inhibition
10	0.88	9.27
20	0.78	19.58
30	0.61	37.11
40	0.50	48.11
50	0.43	55.67

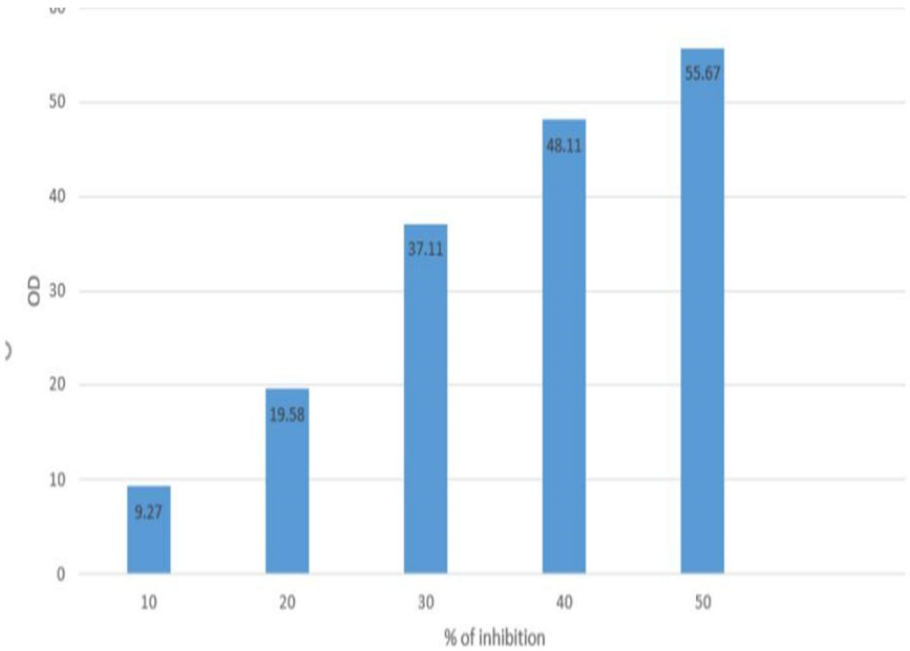

Fig 4: Graphical representation of inhibition of Lactobacillus

D. Lactic Acid Estimation.

The absorbance of test solution was measured at 390 nm against reference solution. The observed value is 0.78.

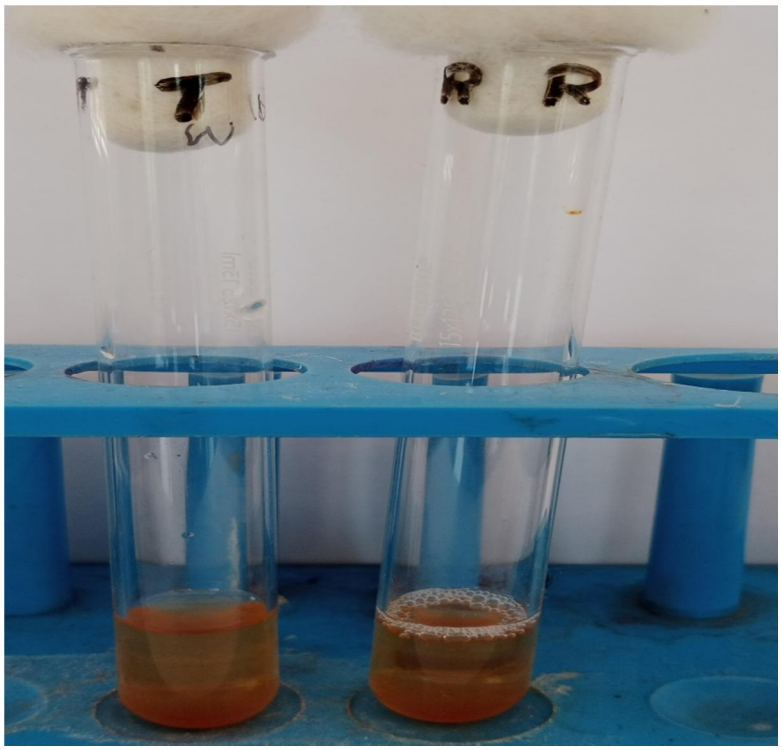

Fig 5: Test solution and Reference solution for Lactic acid Estimation

E. Molecular Identification Of Isolates

1) Isolation Of Bacterial DNA

DNA bands were observed by Agarose gel electrophoresis.

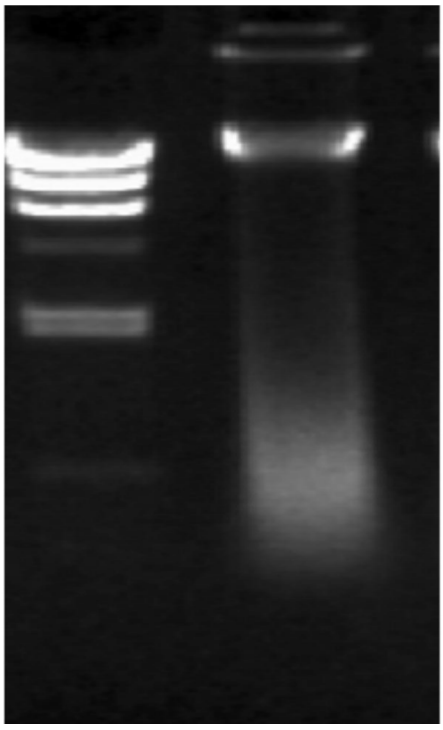

Fig 6: DNA bands were observed by Agarose gel electrophoresis.

2) PCR

PCR products were observed by Agarose gel electrophoresis.

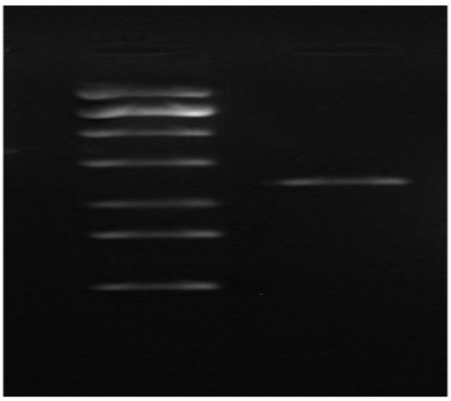

Fig 7: PCR amplification of DNA

3) Sequencing

AGGACGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAACGAACTCTGGTATTGATTGG
TGCTTGCAT

CATGATTTACATTTGAGTGAGTGGCGAACTGGTGAGTAACACGTGGGAAACCTGCCCAGAA
GCGGGGGAT

AACACCTGGAAACAGATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGTTTGAA
AGATGGCTT

CGGCTATCACTTTTGGATGGTCCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACC
ATGGCAATG

ATACGTAGCCGACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCC
TACGGGAGG

CAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGA
AGAAGGGTTC

CGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTTCAGGTATTGA
CGGTATTTA

4) Blast

Lactobacillus acidophilus shows 100% similarly.

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Lactobacillus acidophilus partial 16S rRN...	Lactobac...	905	905	100%	0.0	100.00%	1489	LN869545.1
☑	Lactobacillus plantarum SN13T DNA, com...	Lactiplan...	900	4481	100%	0.0	99.80%	3612790	AP019815.1
☑	Lactiplantibacillus plantarum strain Heal1...	Lactiplan...	900	4485	100%	0.0	99.80%	3265930	CP055123.1
☑	Lactiplantibacillus plantarum subsp. plant...	Lactiplan...	900	3568	100%	0.0	99.80%	3191656	CP053912.1
☑	Lactiplantibacillus plantarum strain CNEI-...	Lactiplan...	900	900	100%	0.0	99.80%	3327596	CP053571.1
☑	Lactiplantibacillus plantarum strain AMT7...	Lactiplan...	900	4480	100%	0.0	99.80%	3227194	CP052869.1

Fig 8: BLAST analysis of Lactobacillus

IV. DISCUSSION

None of the currently available bactericidal-based technologies is completely effective at preventing microbial colonization of medical catheters. It is possible and prudent to improve upon the existing bactericidal technologies by combining them in an effort to increase the frequency in which catheter-related bacterial infections are prevented. An understanding of biofilm biology should reveal important themes about the mechanisms that bacteria employ for microbial adhesion as well as the mechanisms that sessile communities use to survive toxic vicissitudes of the external environment.Biofilm production by Klebsiella pneumoniaeis considered an important determinant of its pathogenicity. A maximum biofilm inhibition of 55.67% of Klebsiella pneumoniae was observed. Antimicrobial compounds in the cell free supernatant were believed to halt the growth of the pathogen and even cause death in the cells, rendering the aggregation of cells to form the biofilm unsuccessful. Thus, identification of such LAB strains that have antibiofilm activity would be essential to include as alternatives to the control of biofilms . In a study reported by Sharma et al .,2020 inhibitory effects of lactobacilli against P. aeruginosa and their biofilm formation were investigated. Dheily et al., 2011, found out that biofilms of marine bacteria grown under dynamic conditions, Pseudoalteromonas sp. Strain 3J6 formed mixed biofilms with Bacillus sp. Strain 4J6 but was largely predominant over Paracoccus sp. Strain 4M6 and Vibrio sp. Strain D01. The supernatant of Pseudoalteromonas sp. 3J6 liquid culture (SN3J6) was devoid of antibacterial activity against free-living Paracoccus sp. 4M6 and Vibrio sp. D01 cells, but it impaired their ability to grow as single-species biofilms and led to higher percentages of nonviable cells in 48-h biofilms.For many years, dairy products have been recognized as valuable products to human health. In recent years, many scientists have isolated and identified LAB and lactobacilli from traditional products worldwide and have evaluated their antagonistic effects against various pathogens. Microorganisms such as lactobacilli and many other bacteria can eliminate pathogens through multiple mechanisms including competitive elimination that results in food safety.

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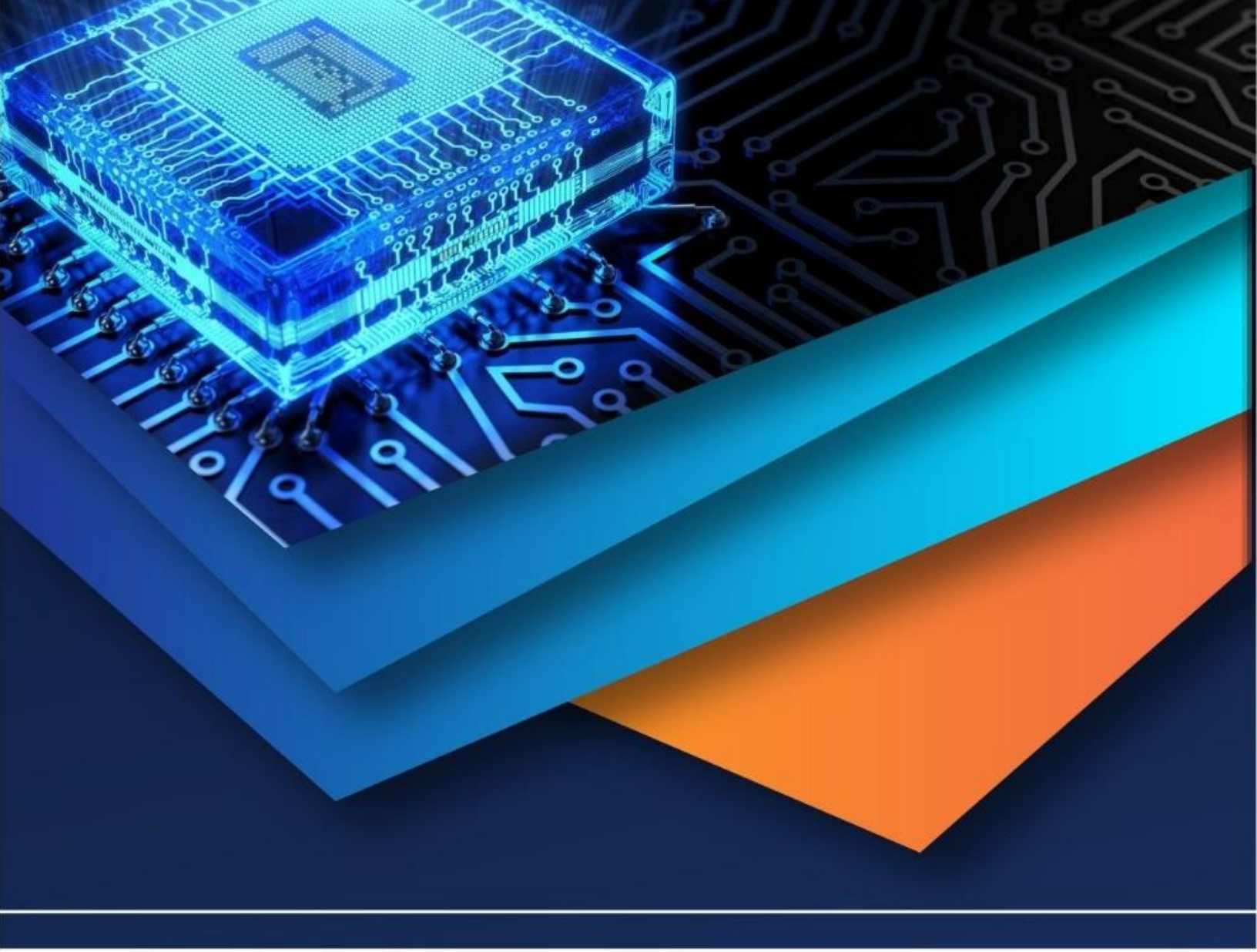

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