DESIGN AND DEVELOPMENT OF *IN VITRO* BIOASSAYS FOR BACTEREMIA MANAGEMENT

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by

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Submitted

In fulfilment of the requirement of the degree of Doctor of Philosophy

to the

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Dedicated to my family

(Parents, Brother, Swati and Ishan)

CERTIFICATE

This is to certify that the thesis entitled "***Design and Development of In Vitro Bioassays for Bacteremia Management***" being submitted by **Jagtap Pramod Sambhaji Pushpa** for the award of the degree of **Doctor of Philosophy** in Chemical Engineering is a record of bonafied work carried out by him under my guidance and supervision at the Indian Institute of Technology Delhi.

The results contained in the thesis have not been submitted elsewhere, either in part or in full, to any other University or Institute for the award of any degree or diploma.

I certify that he has pursued the prescribed course of research.

Dr. Shalini Gupta

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Pramod Jagtap

ABSTRACT

Bloodstream bacterial infections also known as bacteremia are associated with the release of pathogen-associated molecular patterns (PAMPs) like lipopolysaccharides (LPS) and lipoteichoic acids (LTA). These bacterial endotoxins can trigger an innate immune response in critically-ill patients via the signal-transducing toll-like receptors (TLRs), and cause life threatening conditions like sepsis. We have developed affordable and sensitive *in vitro* bioassays that not only give us fundamental insights into the mode, stoichiometry and kinetics of LPS-TLR interactions but also allow us to screen ligands and design novel point-of-care (POC) technologies that have high clinical utility. To begin with, we have replicated the TLR4 pathway, active during the pathogenesis of Gram-negative bacterial sepsis, into a single tube reaction to study the *in vitro* complexation of recombinant TLR4 (rTLR4) with LPS in the presence of myeloid differentiation-2 (MD2) and cluster of differentiation-14 (CD14) co-receptors in their free (membrane unbound) form. Our results showed that rTLR4-MD2-LPS follow a 1:1:1 stoichiometry and CD14 plays a crucial catalytic role in the complexation process. A bis-biguanide antibacterial agent called alexidine was screened using our assay and it showed good LPS sequestration potential. To understand the nature of this binding, we used NMR, fluorescence and SPR spectroscopy which revealed that LPS and alexidine molecules have a mid-to-high binding affinity (K_D = 38 ± 5.6 µM) and a relative stoichiometry that lies between 1:2 to 1:4. These results were supported by computational studies performed in collaboration with Prof. Hemant Kashyap's group in Dept. of Chemistry at IITD. In the end, we developed a simple, rapid and low-cost POC assay in a flow through format called Septiflo-P for early detection of Gram-positive bacteria in a single drop of blood plasma under 10 min. The principle of the assay involved receptor-less immobilization of LTA on an affinity membrane and its amplification using monoclonal antibody-conjugated gold

nanoprobes. The colorimetric response of the assay was detectable in the clinical range down to 1 pg/mL by the naked eye. The assay was benchmarked against a commercial ELISA and was also clinically validated on 60 patient samples using procalcitonin (PCT) as a host biomarker for sepsis. Further comparison with PCR on 30 clinical samples showed > 90% accuracy, establishing the immense potential of our technology for faster and more cost-effective diagnosis of bacteraemia.

सार

रक्तप्रवाहित जीवाणु संक्रमण जिसे बैक्टीरियोमिया के रूप में भी जाना जाता है, लिपोपोलीसेकेराइड्स (LPS) और लिपोतेइकॉइक एसिड (LTA) जैसे रोगज़नक़ से जुड़े आणविक पैटर्न (PAMPs) के मुक्त करने से जुड़ा हुआ है। ये जीवाणु एंडोटॉक्सिंस गंभीर रूप से बीमार रोगियों में सिग्नल-ट्रांसडेसिंग टोल-जैसे रिसेप्टर्स (TLR) के माध्यम से जन्मजात प्रतिरक्षा प्रतिक्रिया को ट्रिगर कर सकते हैं, और सेप्सिस जैसी जीवन की गंभीर की स्थिति पैदा कर सकते हैं। हमने सस्ती और संवेदनशील इन-विट्रो बायोएसेज को विकसित किया है जो न केवल हमें LPS-TLR इंटरैक्शन के मोड, स्टोइकोमेट्री और कैनेटीक्स में मौलिक अंतर्दृष्टि प्रदान करता है, बल्कि हमें स्क्रीन लिगैंड और डिजाइन नॉवेल प्वाइंट-ऑफ-केयर (POC) प्रौद्योगिकियों की भी अनुमान देता है जो उच्च हैं नैदानिक उपयोगिता। शुरू करने के लिए, हमने TLR4 मार्ग को दोहराया है, जो ग्राम-नकारात्मक जीवाणु सेप्सिस के रोगजनन के दौरान सक्रिय है, एक एकल ट्यूब प्रतिक्रिया में पुनः संयोजक TLR4 (rTLR4) के इन विट्रो परिसर में अध्ययन करने के लिए LPS के साथ माइलॉयड विभेदन -2 की उपस्थिति में (MD2) और समूह विभेदन -14 (CD14) के क्लस्टर अपने मुक्त (झिल्ली अनबाउंड) रूप में सह-ग्राही पाए जाते है। हमारे परिणामों से पता चला कि rTLR4-MD2-LPS एक 1: 1: 1 स्टोइकोमेट्री और CD14 का पालन करता है जो कि प्रक्रिया में एक महत्वपूर्ण उत्प्रेरक भूमिका निभाता है। एलेक्सिसिडिन नामक एक बीआईएस-बिगुआनाइड जीवाणुरोधी एजेंट को हमारे परख का उपयोग करके जांचा गया था और इसमें LPS सीसेस्ट्रेशन की अच्छी क्षमता थी। इस बंधन की प्रकृति को समझने के लिए, हमने एनएमआर, प्रतिदीप्ति और एसपीआर स्पेक्ट्रोस्कोपी का इस्तेमाल किया, जिसमें पता चला कि LPS और एलेक्सिडाइन अणुओं में एक मध्य मजबूत बंधन संबंध है (K_D = 38 ± 5.6 μM) और एक सापेक्ष स्टोइकोमेट्री जो 1: 2 के बीच स्थित है से 1: 4 तक। ये परिणाम IITD में रसायन विज्ञान विभाग में प्रो। हेमंत कश्यप के समूह के सहयोग से किए गए कम्प्यूटेशनल अध्ययन द्वारा समर्थित थे। अंत में, हमने 10 मिनट के तहत रक्त प्लाज्मा की एक बूंद में ग्राम पॉजिटिव बैक्टीरिया के शुरुआती सांद्रता पता लगाने के लिए सेप्टिफ्लो-पी नामक प्रारूप के माध्यम से एक प्रवाह

में एक सरल, तेजी से और कम लागत वाली POC परख विकसित की। परख का सिद्धांत एक एफिनिटी झिल्ली पर LTA के रिसेप्टर-कम स्थिरीकरण में शामिल है और मोनोक्लोनल एंटीबॉडी-संयुग्मित सोने के नैनोकणों का उपयोग करके इसके प्रवर्धन। परख की वर्णमिति प्रतिक्रिया नैदानिक सीमा में 1 pg/mL तक नग्न आंखों द्वारा पता लगाने योग्य थी। परख को एक वाणिज्यिक एलिसा के खिलाफ बेंचमार्क किया गया था और सेप्सिस के लिए मेजबान बायोमार्कर के रूप में प्रोक्लेसिटोनिन (PCT) का उपयोग करके 60 मरीज के नमूनों पर नैदानिक रूप से मान्य किया गया था। 30 नैदानिक नमूनों पर पीसीआर के साथ आगे की तुलना ने दिखाया > 90% सटीकता, बैक्टीरियोमिया के तेजी से और अधिक लागत प्रभावी निदान के लिए हमारी तकनीक हमारी तकनीक उपयोगी साबित हुई और शोध की अपर सम्भावनाये है।

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ABBREVIATIONS

TB: Tuberculosis

ICU: Intensive care units

AMR: Antimicrobial resistance

MDR: Multidrug resistant

IDSA: Infectious Diseases Society of America

CDCP: Center for Disease Control and Prevention

WHO: World Health Organization

GLASS: Global Antimicrobial Surveillance System

PAMPs: Pathogen-associated molecular patterns

LPS: Lipopolysaccharide

LTA: Lipoteichoic acid

TLR: Toll-like receptor

SOFA: Sequential organ failure assessment

PCT: Procalcitonin

NPs: Nanoparticles

PS: Polystyrene

PMMA: Polymethyl methacrylate

CD: Cyclodextrin

CNTs: Carbon nanotubes

POC: Point-of-care

NMR: Nuclear magnetic resonance

SPR: Surface plasmon resonance

CD14: Cluster differentiation-14

MD2: Myeloid differentiation-2

PPG: Polypropylene glycol

Alex: Alexidine

PRRs: Pattern recognition receptors

TIRF: Total internal reflection fluorescence

LBP: Lipoprotein binding protein

CTCs : Circulating tumor cells

PCR: Polymerase chain reaction

LFAs: Lateral flow assays

WBC: White blood cell

CRP: C-reactive protein

MIC: Minimum inhibitory concentration

NTA: Nitrilotriacetic acids

CMC: Critical micellar concentration

K_D: Equilibrium dissociation constant

LIP A: Lipid A

HEK: Human embryonic kidney

ZnS: Zinc sulfide

CdS: Cadmium sulphide

ZnO: Zinc oxide

CT: Computed tomography

AuNPs: Gold nanoparticles

MNPs: Magnetic nanoparticles

MRI: Magnetic resonance imaging

SiNPs: Silica-based NPs

FRET: Fluorescence resonance energy transfer

NIR: Near-infrared

OMVs: Outer membrane vesicles

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

PBS: Phosphate buffer saline

NaCl: Sodium chloride

NaOH: Sodium hydroxide

RT: Room temperature

SD: Standard deviation

MRSA: Methicillin Resistant Staphylococcus aureus

FWHM: Full width at half maxima

T_2: Transverse relaxation time

SNR: Signal to noise ratio

MD: Molecular dynamics

PMB: Polymyxin B sulfate

AUC: Area under curve

ELISA: Enzyme-linked immunosorbant assay

mAbs: Monoclonal antibodies

DLS: Dynamic light scattering

TEM: Transmission electron microscopy

UV-VIS: UV-Vis spectroscopy

ROC: Receiver operating characteristic

PBP: Penicillin binding proteins

NA: Not available

NA: Not available

N: Negative

Y: Positive,

NE: Not elevated

NG: No growth

DOL: Degree of labelling