Kinetics and Molecular Requirements of *Klebsiella pneumoniae*-Induced Endovascular Infections under Hydrodynamic Shear as a Function of Platelet Activation

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Abstract

Bio-adhesion between invading bacterial pathogens and host cells such as platelets is known to play a key role in the initiation and propagation of bacterial bloodstream infections. The formation of platelet-bacteria heterotypic aggregates allows bacterial cells to evade the innate immune response. Shear forces associated with flowing blood in the human vasculature are known to modulate associations between an invading bacterial pathogen and host cells such as platelets, erythrocytes, and neutrophils. Moreover, blood flow assists hematogenous spread of bacterial infections, and contributes to their severity. This study was undertaken to investigate the effects of shear rate (0-5000 s^{-1}), and shear exposure time (60-120 s) on *Klebsiella pneumoniae*-platelet heterotypic aggregation. Besides, the effect of thrombin mediated activation of platelets upon heterotypic aggregation was studied by treating the platelets with different thrombin concentration. Also, different platelets to bacteria ratios were tested for the selection of the ratio that best resulted in increased heteroaggregates formation. Based on the study, it was found that the hydrodynamic shear modulated the heterotypic aggregation. At higher shear rates, the intercellular interaction levels were vividly immense. Thrombin treatment didn't have a profound effect on heterotypic aggregation for higher shear exposure times. The molecular mechanisms of the bacteria-platelets interaction still lie unfolded.

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Chapter 1

Motivation

Despite the introduction of penicillin in 1941, and vancomycin in 1956, infectious diseases are still a dominant public health problem, especially in developing countries like India. The development of antimicrobial resistance due to overuse of these drugs -- coupled with microbial adaptability -- have led to the emergence of new infections, threatening to lead us back to the pre-antibiotic era. Dissemination of carbapenemase-producing *Klebsiella pneumoniae* (CP-Kp) has caused a public health crisis that can be paralleled with that caused by the spread of MRSA. CP-Kp, being multidrug-resistant, mainly affects patients with severe underlying conditions in the acute-healthcare setting. CP-Kpis responsible for a variety of life-threatening infections including bacteremia and pneumonia [1]. The motivation for this project can be traced to recent studies that report the emergence of multidrug-resistant *K. pneumoniae* producing the enzyme New Delhi Metallo Beta Lactamase (NDM-1) [2-8]. These strains were found to be highly resistant to all antibiotics except to tigecycline and colistin [9, 10]. The potential of NDM-1 to be a worldwide public health problem makes developing novel therapeutic strategies to combat NDM-1 *K. pneumoniae*-induced infections an urgent need.

1.1 Bacteria-induced endovascular infections

Incidences of endovascular infections by antibiotic-resistant microbes are on the increase worldwide, thereby posing a serious threat. Importantly, a significant proportion of bacteria-induced endovascular infections are known to lead to bacteremia, and subsequent metastatic complications. For instance, most patients with liver abscess develop bacteremia and septic metastatic complications including endophthalmitis [11], brain and lung abscesses, osteomyelitis, septic arthritis, psoas abscess [12], necrotizing fasciitis [13, 14], epidural spinal abscess [15], and septic pulmonary embolism [16]. Once inside the bloodstream, propagation of bacteria-induced endovascular infections may be regulated by complex interactions between surface components on the invading organism and host cells like platelets and neutrophils. The interaction between surface components on the invading pathogen and host cells such as platelets plays a key role in the regulation of endovascular infections [17]. For instance, intercellular interactions between

platelets and *Staphylococcus aureus* (*S. aureus*) are known to induce platelet activation and aggregation. Experimental and clinical observations suggest that the ability of *S. aureus* cells to bind to platelets correlates with the capacity of this organism to induce infective endocarditis [17, 18]. It is believed that platelet aggregates may allow bacteria to settle and remain at the site of infection withstanding the hydrodynamic shear forces prevailing in the circulatory system, and thus serving as a positive mediator of the *S. aureus* metastasis.

1.2 Role of platelets

Platelets are the earliest and most numerous cells to accumulate at sites of vascular infections. The concept that platelets contribute to host defense against infection is substantiated by a line of evidence relevant to platelet structure and function [19]. They are the granulocytes known to bridge the innate and adaptive immunity, and also to interact with bacterial pathogens through a wide array of cellular and molecular mechanisms. They are crucial to engaging bacterial pathogens directly and indirectly both in vivo and in vitro. They are known to express an array of constitutive and inducible receptors functioning to recognize and respond to infection. For example, toll like receptors (TLR2 and TLR4) expressed by platelets detect the presence of potentially harmful microbes by sensing the Pathogen Associated Molecular Patterns (PAMPs) [20]. Furthermore, distinct platelet receptors are capable of sensing agonists and binding to ligands on the surface of activated endothelial cells, or exposed sub-endothelium due to infection or tissue invasion. In pathological conditions, platelets have been major contributors to arterial thrombosis, which typically occurs at the sites of artherosclerosis with stenosis of the vessel lumen, where shear stress values are considerably higher than that in normal circulation. For these reasons, and because the complex events that regulate platelet functions are influenced by the flow of blood, the mechanisms supporting platelet adhesion and aggregation under the constraints of elevated shear stress are of particular interest in the study of thrombus formation.

Depending on the matrix protein exposed to blood and the hemodynamic conditions, platelet adhesion requires the synergistic function of different platelet receptors, ultimately leading to platelet activation and aggregation. The extracellular matrix components that react with platelets include collagen, van Willebrand factor (vWF), fibronectin and other adhesive proteins such as laminins, thrombospondins [21].

Expression of platelet toll-like receptor (TLR) has been discovered as the detector of the hallmark signals of bacterial infection. On interaction with bacterial lipopolysaccharide (LPS), the expression of the TLRs increases in vivo, stimulating the release of cytokines, platelet microbicidal proteins, and kinocidins [19]. TLR4 is also capable of detecting cognate ligands in blood, inducing platelet adhesion and subsequent neutrophils adherence at the sites of bacterial invasions and infections.

1.3 Effect of Hydrodynamic shear

Rheological properties of fluid flow in the vasculature have a predominant effect on bacteria-platelet interactions. A major limitation of our current knowledge stems from the fact that all previous studies aimed at investigating platelet-bacterial cell interactions were performed under static conditions [22], which neglect the rheological parameters of fluid flow in the vasculature. As has been appropriately argued in the literature, data obtained in vitro using static binding assays may not be relevant to the fluid dynamic environment encountered in the vasculature [23]. It is now well established that the local fluid mechanical environment of the circulation critically affects the molecular pathways of cell-cell interactions [24-26]. Consequently, the relative contribution of each of the aforementioned molecular constituents in platelet-bacterial interactions under the action of hydrodynamic shear is unknown. The presence of flowing liquid, mucus or blood, for instance, determines adhesion because it generates a mechanical force on the pathogen[27]. Fluid-shear stress is created when adjacent blood layers move past one another or past a vascular surface, and is a measure of the exerted force. In a previous study using *Neisseria meningitidis* as a model, it has been observed that bacterial microcolonies forming on the endothelial cell surface in the vessel lumen are remarkably resistant to mechanical stress offered by the vasculature [28]. Shear stress acting on cells in the circulation stimulates cell signal transduction, gene expression, and affects cell shape and survival [29-31]. Furthermore, shear stress enhances the inflammatory response of monocytes to infection [32]. The exposure of chlamydia-infected monocytes to short durations of arterial shear stress significantly enhances the secretion of cytokines in a time-dependent manner and the expression of surface adhesion molecule ICAM-1. As a functional consequence, infection and shear stress increased monocyte adhesion to endothelial cells under flow and in the activation and aggregation of platelets.

Under the influence of shear, cell attachment depends on the balance between dispersive hydrodynamic forces and the adhesive forces generated by the interactions of membrane-bound receptors and their ligands. Thus interactions of bacterial cells with platelets play a key role in initiation of pathogenesis of blood stream infections. In this work, we have investigated the adhesive interactions between platelets-*K. pneumonia* under shear conditions.

Chapter 2

Literature Review

2.1 *K. pneumoniae* infections

Klebsiella pneumoniae is responsible for hospital-acquired urinary tract infections, septicemia, pneumonia, and intra-abdominal infections [33]. In addition, *K. pneumoniae* can cause severe pneumonia, bacteremia, and meningitis [34]. It is also know to be an important pathogen that causes urinary tract infections (UTIs), pneumonia, and intra-abdominal infections in hospitalized immune-compromised patients with severe underlying diseases. Of the gram-negative bacteria implicated in nosocomial bloodstream infections (BSIs), *K. pneumoniae* is second only to *Escherichia coli* [35].

2.2 Platelet-Bacteria interactions

Bacteria-platelet interactions are characterized by binding of bacteria to platelets either directly via a surface protein on the bacteria [36], or indirectly via a plasma bridging molecule linking bacterial and platelet surface receptors [22]. Either of these interactions may result in platelet activation. Hawiger et al. [37] demonstrated that *Staphylococcus aureus*(*S. aureus*) triggers platelet morphogenesis, aggregation, and degranulation in a pathway involving *Staphylococcal* protein A (SPA), host IgG, and platelet Fc receptors. Considerable work since then has revealed that this organism employs several factors to interact with platelets when it is advantageous to do so. For example, *S. aureus* surface proteins that mediate direct or indirect platelet interactions include clumping factors A and B (ClfA/ClfB), fibronectin-binding proteins A and B (FnbpA/FnpbB), SPA, serine-aspartaterepeat protein E (SdrE), serine-rich adhesin for platelets (SraP), extracellular fibrinogen-binding protein (Efb), and other molecules. A review of these *S. aureus* surface proteins and homologous factors of other pathogens has been compiled by Fitzgerald et al. [38].Herzberg et al. reported thatcertain viridans streptococci such as *Streptococcus sanguis* adhere directly to the platelet surface via a 150-kDa two domain adhesion [39-41]. Zimmerman et al. [42] have shown that *Streptococcus pneumonia* induces platelets aggregation only in the presence of cognate anti-pneumococcal antibody in vitro. As observed in

S.aureus, Ford et al. [43]demonstrated that *S. sanguis* aggregates platelets through a fibrinogen pathway involving platelet integrin GPIIb/IIIa receptor. Similarly, platelet aggregation and degranulation prompted by *S. pyogenes* can be mediated by fibrinogen [44], while Johnson and Bowie [45] reported that some group C streptococci indirectly interact with platelets through the von Willebrand factor.

2.3 Plasma proteins

The platelet-bacteria interaction occurs by direct or indirect binding[46]. For instance, *Streptococcus gordonii* and *Streptococcus sanguinis* are capable of binding directly to platelet gpIb receptor via Hs antigen (Hsa) [47]and Serine rich protein (Srp) [48] respectively.

More commonly, many bacteria have also been shown to bind plasma proteins such as fibrinogen, vWF (von Willebrand Factor), and IgG, which in turn can bind to receptors expressed on the platelets. Among the substrate requirement for normal thrombus formation, vWF is unique for its role in initiating platelet adhesion and sustaining platelet aggregation under conditions of elevated shear stress [49]. Fibronectin, another class of plasma protein is an essential substrate in many fundamental biological processes. When assembled into fibrillar structure, fibronectin may also support initial platelet adhesion either directly or indirectly by association with collagen and/or vWF [21]. *S. aureus* is known to bind platelet gpIb receptor via vWF, which acts as a bridging plasma protein. In addition, *S. aureus* is reported to bind fibrinogen and fibronectin and subsequently binding to gpIIb/IIIa receptors expressed on platelets.

2.4 Thrombin as platelet stimulus

Thrombin is the main effector protease of the coagulation system and is among the most effective activators of platelets. Thrombin formation is initiated by the exposure of tissue factor to plasma coagulation factors after disruption of the vascular endothelium. Thrombin formation takes place on cellular surfaces including that of activated platelets. The local production of thrombin on the platelet surface represents an important mechanism by which activated platelets stimulate coagulatory processes. In addition, it may facilitate activation of platelets by thrombin, which is rapidly inactivated after its formation. Activation of platelets by thrombin is mediated by protease-activated receptors (PARs), which couple to heterotrimeric G proteins. Of the four

protease-activated receptors, PAR1 and PAR4 are present on human platelets, whereas mouse platelets express PAR3 and PAR4. Studies using PAR1 antagonists or antibodies blocking PAR1 or PAR4 activation have indicated that PAR1 mediates human platelet activation at low thrombin concentrations, whereas PAR4 contributes to thrombin-induced platelet activation only at high thrombin concentrations. Fig. 2.1 characterizes receptor mediated platelet activation by thrombin.

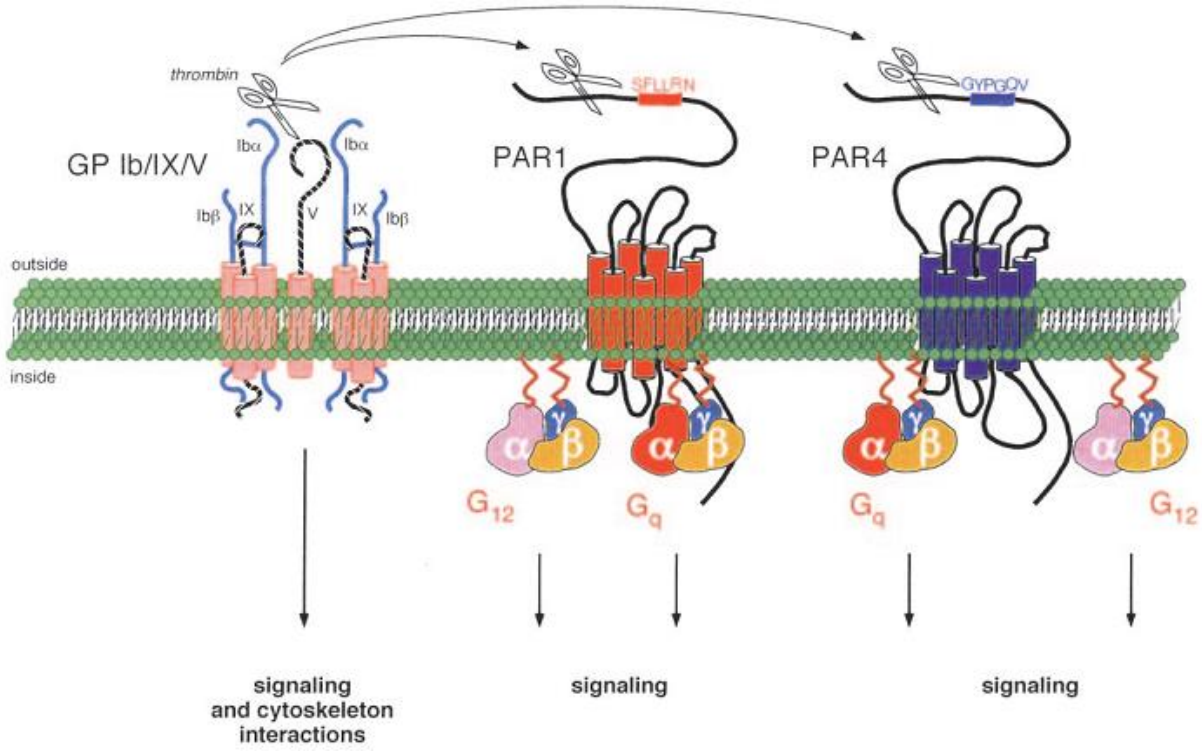

Figure 2.1 Receptor mediated platelet activation by thrombin [50].

Chapter 3

Objectives

Specific aim 1: To confirm if *K. pneumoniae* cells are capable of binding to platelets under the in vitro conditions of the vasculature.

S. aureus has been known to interact with blood cells like platelets and erythrocytes under the hydrodynamic environment of the vasculature. But no previous studies, static or dynamic, have been carried out to predict if *K. pneumoniae* cells are capable of any such interactions.

Specific aim 2: To investigate the kinetics of *K. pneumoniae*- platelet bio-adhesion as a function of hydrodynamic shear.

The commencement and propagation of endovascular bacterial infections are regulated by complex interactions between surface components on the invading organism and host cells like platelets [17]. The data obtained in vitro using static binding assays may not be relevant to the fluid dynamic environment encountered in the vasculature as the local fluid mechanical environment of the circulation critically affects the molecular pathways of cell-cell interactions.

Therefore, rheometric-flow cytometric methodology was used to investigate the kinetics of platelet-*K. pneumoniae*bio-adhesion in physiologically and patho-physiologically relevant shear fields.

Specific aim 3: To elucidate the effect of platelet activation, as a function of thrombin treatment (in optimized concentration) on their ability to recruit *K. pneumoniae* cells.

Platelet activation may be of relevance in enhancing the binding of bacteria to platelets, thereby yielding increased numbers of heterotypic aggregates.

Specific aim 4: To observe the effect of shear exposure time on heterotypic aggregation.

Shear exposure time may have a crucial role to play in bacteria-platelet heterotypic aggregation. High shear exposure times may provide enough duration for the stable hetero-aggregate

formation, as compared to the ones formed at low shear exposures. Conversely, high shear exposure time may also result in breaking of the larger hetero-aggregates into smaller ones, or no hetero-aggregate formation at all for different shears.

So, shear exposure durations of 60, 90 and 120s were used to observe the extent of hetero-aggregation.

Specific aim 5: To probe the extent of heterotypic aggregation by varying the platelets to bacteria ratio.

To predict the cell concentrations required for yielding heterotypic aggregation, different platelets to bacteria ratios were tested; viz. 5:1, 10:1, 20:1 and 50:1. The cell ratios were adjusted based on hemacytometer cell counting protocol.

Specific aim 6: To observe the effect of small bio-molecules present in bacterial supernatant on the levels of homotypic platelet aggregation.

The bacterial mechanisms for protein secretion has become an important factor in pathogenesis due to the observation that key virulence factors of bacterial pathogens are secreted into the culture environment. Based on this, some extracellular proteins released by K. pneumonia can be responsible in causing platelet homotypic aggregation, which may then lead to heterotypic aggregation as well.

Chapter 4

Methodology

4.1 Bacterial growth conditions

Klebsiella pneumoniae (ATCC 27736) strain procured from American Type Culture Collection (ATCC) was used in this study. Glycerol stocks were prepared from growing cultures on nutrient agar and stored at -80°C. Experimental cultures were started from glycerol stocks, and grown at 37°C with constant rotation for 16-18 h Tryptic Soya Broth. Cells were harvested by centrifugation, in Dulbecco's Phosphate Buffered Saline (D-PBS) with Ca^{2+}/Mg^{2+} containing 0.02% sodium azide to stop metabolic activities. Cells were then washed three times with D-PBS to remove excess medium and sodium azide.

4.1.1 Inoculation and incubation

50 ml of sterile medium was inoculated with 20 µl of glycerol stock culture through a micropipette inside the biosafety cabinet. The freshly inoculated flask was taken out from the cabinet and placed in the incubator. The incubator temperature was set at 37°C, shaking speed at 135 rpm and time at 18 hours.

4.1.2 Harvesting of cells

1000 µl of 16-18 h cultured bacterial cell cultures were transferred to each 1.5 ml sterile micro-centrifuge tube and labeled inside the biosafety cabinet. These micro-centrifuge tubes were placed in the centrifuge which will be set at 12000 rpm and spin duration of 2 min. The supernatant was discarded and the pellet was re-suspended in 1 ml of 0.02% sodium azide solution.

4.1.3 Washing of cells

The re-suspended cells were centrifuged at 12000 rpm for 2 min and the pellet was recollected. The pellet was re-suspended in PBS without Ca^{2+}/Mg^{2+}. This process was repeated three times. Finally, the pellet was re-suspended in PBS without $Ca^{2+/}Mg^{2+}$.

4.2 Bacterial Staining

The harvested cells of *K. pneumonia* were stained using 120µg/ml Hexidium Iodide for 1h at room temperature. The stained cells were then washed three times with D-PBS to remove excess dye. The washed cells were re-suspended in the same buffer with $Ca^{2+/}Mg^{2+}$ divalent ions.

4.2.1 Protocol

200 µl of washed cells were suspended in Phosphate Buffer Saline (PBS) with Ca^{2+}/Mg^{2+} into a micro-centrifuge tube and 2.4 µl of Hexidium Iodide i.e.120 µg/ml will be added to the washed *K. pneumoniae* cells. The tubes were placed tubes in incubator for 1 hour at room temperature with constant rotation.

After staining, the washing process was repeated three times and the stained bacterial cells were re-suspended in PBS with $Ca^{2+/}Mg^{2+}$.

4.3 Isolation of platelets from whole blood

Human blood was drawn by venipuncture from healthy volunteers into sodium citrate (0.38% w/v) anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 160 × g for 15 min. Platelet-poor plasma was obtained by further centrifugation of the blood at 1900 × g for 15 min. The final platelet count of the PRP was adjusted to the desired levels with platelet-poor plasma. Specimens were stored at room temperature in capped polypropylene tubes and used within 3 h of isolation.

4.3.1 Platelet preparation

Platelet rich plasma (PRP) and platelet poor plasma (PPP) were prepared by centrifuging the citrated whole blood.

4.3.1.1 Platelet Rich Plasma (PRP)

20 ml of citrated blood was slowly transferred to 10 ml centrifuge tubes and centrifuged. The centrifuge spin was set at 160 x g for 15 min. On completion of centrifugation, the top yellowish layer was collected using the micro-pipette without disturbing the bottom layers. This was the platelet rich plasma.

4.3.1.2 Platelet Poor Plasma (PPP)

The centrifuge spin was set at 1900 x g for 15 min and the same tubes with citrated whole blood were centrifuged. On completion of centrifugation, the top yellowish layer was collected using the micro-pipette without disturbing the bottom layers. This was the platelet poor plasma.

4.4 Fixing platelets to bacteria ratios using Hemacytometer

Hemacytometer is the most widely used type of counting chamber. The cell suspension was introduced into one of the V-shaped wells. The area under the coverslip was allowed to fill by capillary action. The counting chamber is then placed on the microscope stage and the counting grid is brought into focus. The main divisions separate the grid into 9 large squares. Each square has a surface area of $1mm^2$, and the depth of the chamber is 0.1mm. Each square of the hemacytometer (with cover slip in place) represents a total volume of 0.1 mm^3. Cell suspensions were diluted enough so that the cells do not overlap each other on the grid, and were uniformly distributed. For platelets and bacterial cells, large four corner cells and the middle cell were counted. The cell number per ml was calculated as per the following formula:

Cells per ml = the average count per square x the dilution factor x 10^4

4.5 Treatment with thrombin

Thrombin (Sigma Aldrich) was used as a platelet activation stimulant. 0.18ul of thrombin (0.1U/ml as optimized concentration) was added to 180ul of platelet in a microcentrifuge tube. The tube was then placed in incubator at 37^oC for precisely 2 min for thrombin to carry out its activation reaction. Then the platelet sample was loaded onto the rheometer plate.

4.6 Cone-and-plate rheometry assays

Rheometer (Haake MARS III) with 1° cone and plate geometry is used in this study. Rheometry assays were designed to analyze platelet aggregation with three different sets of specimens.

Set 1: Platelets (1.25×10^8 cells/ml) alone

Set 2: Platelets (1.25×10^8cells/ml) and stained Bacteria

Shear rates were varied from 0 to 2000 $s^{-1}$for 60 s, 90s and 120 s and applied on the specimens placed on to the stationary plate of the cone-and-plate rheometer. Static conditions were achieved by setting the shear rate as 0 s^{-1}. Upon termination of shear or static incubation, samples (5 µl) were obtained and instantly fixed with 4% formaldehyde. Specimens were then incubated with a FITC-labeled platelet-specific mAb directed against gpIX (anti-CD42a FITC) for 30 min in the dark at room temperature. The labeling reaction was stopped by further dilution with 1% formaldehyde, and specimens were then subsequently analyzed in a FACSAria III flow cytometer. The 1° cone and plate fixture of the rheometer was set at 37°C during the entire experiment. The cone and plate was pre-coated with 0.2% BSA to prevent loss of cells due to sedimentation or nonspecific binding.

4.6.1 Rheometric assays

The final platelet concentration was adjusted to desired values by diluting PRP with PPP. The 1° cone measuring geometry was fixed to the rheometer head and 180 µl of platelet sample and 20 µl of bacterial sample were then loaded on the center of the plate in the rheometer. The samples were subjected to uniform levels of shear (shear rates ranging from 0, 100, 400, 800, 1200, 2000 and 5000 s^{-1}) for prescribed periods of time.

4.6.1.1 Sample Fixation

After completion of the shear run, 5 µl of sheared sample were collected using a micro-pipette into a 1.5 ml micro-centrifuge tube/falcon tube, and fixed with 1% formaldehyde.

4.6.1.2 Platelet Labeling

1 µl of FITC-labeled platelet specific-mAb (anti-CD42a directed towards gpIX) was added to the fixed specimens. The labeled specimens were incubated for 30 min in dark at room temperature. The labeling reaction was stopped by adding 1ml of 1% formaldehyde to the specimens. The specimens were subsequently analyzed in the FACS AriaIII flow cytometer.

4.7 Flow cytometric analysis

The particle distribution of stable aggregates generated in the rheometric assay is determined by flow cytometric measurements. HI-stained bacterial cells and FITC-labeled platelets were

identified on the basis of their characteristic forward scatter, side scatter, and fluorescence profiles in a FACSAria III flow cytometer. FITC gets excited efficiently at 490 nm by the blue laser of the flow cytometer and its emission is detected by the PMT filter of 515 nm. HI gets excited efficiently at 518 nm by the yellow-green laser of the flow cytometer and its emission can be detected by the PMT filter of 600 nm. Acquisition of 10000 FITC labeled platelets and bacterial cells events will be used to determine the percentage of bacteria-platelet hetero-aggregates in the above mentioned sets of specimens.

The following strategy was used to determine the percentage of platelets aggregated from cytometric measurements. The mean and standard deviation (SD) were computed from the fluorescence histogram of FITC-labeled platelet singlets and HI labeled bacteria singlets. Three times the observed standard deviation provided the range of a single platelet fluorescent event with a 99% confidence. Using this methodology, aggregates comprising of one, two, three, or more adherent platelets and bacteria were detected and enumerated.

Chapter 5

Results and Discussion

5.1 *K. pneumoniae* cells are capable of binding to human platelets

The ability of K. pneumoniae cells to bind to human platelets has been observed for the first time in this study. The intercellular interactions occur in the hydrodynamic shear environment mimicking the conditions of the vasculature.

The kinetics of these interactions, as well as the quantification of heterotypic aggregations has been dealt with in the succeeding sections.

5.2 Hydrodynamic shear modulates the intercellular interactions taking place between *K. pneumoniae* and platelets

The extent of heterotypic aggregation can be quantified both in terms of a) the percentage of platelets present in the hetero-aggregates, b) the percentage of bacteria present in the hetero-aggregates. A heterotypic aggregate might contain a single bacterial cell with greater number of platelets or vice versa.

Controlled levels of shear ranging from 0 to 2000 s^{-1} were applied on bacterial cells and platelets suspended in plasma for 60s in a cone-and-plate rheometer. A ratio of five platelets (1.25 x 10^8cells/ml) to one bacterial cell (2.5 x 10^7cells/ml) was maintained in shearing experiments.

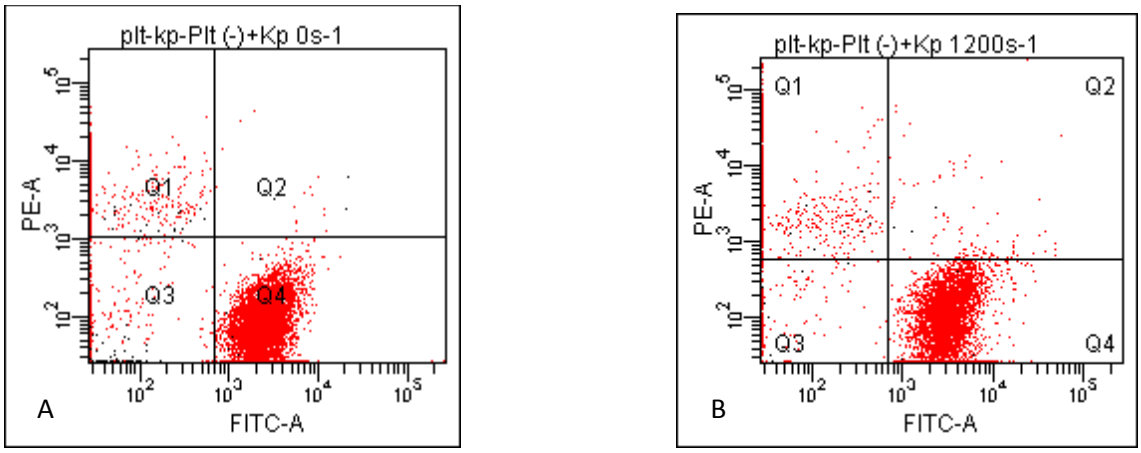

Figure 5.1 Sheared samples of bacteria and platelets. A) At $0s^{-1}$, the extent of heterotypic aggregation, as observed in Q2 quadrant is low. B) At high shear rates of $1200s^{-1}$, an increase in the heterotypic aggregation level was observed.

5.2.1 Quantification of heterotypic aggregation under the influence of hydrodynamic shear

With increasing shear, the percentage bacteria in the heterotypic aggregates were observed to increase, almost linearly. The maximum heterotypic bacterial aggregation was observed at $2000s^{-1}$ (13.09±5.111). The minimum was observed at $400s^{-1}$ (7.67±1.813).

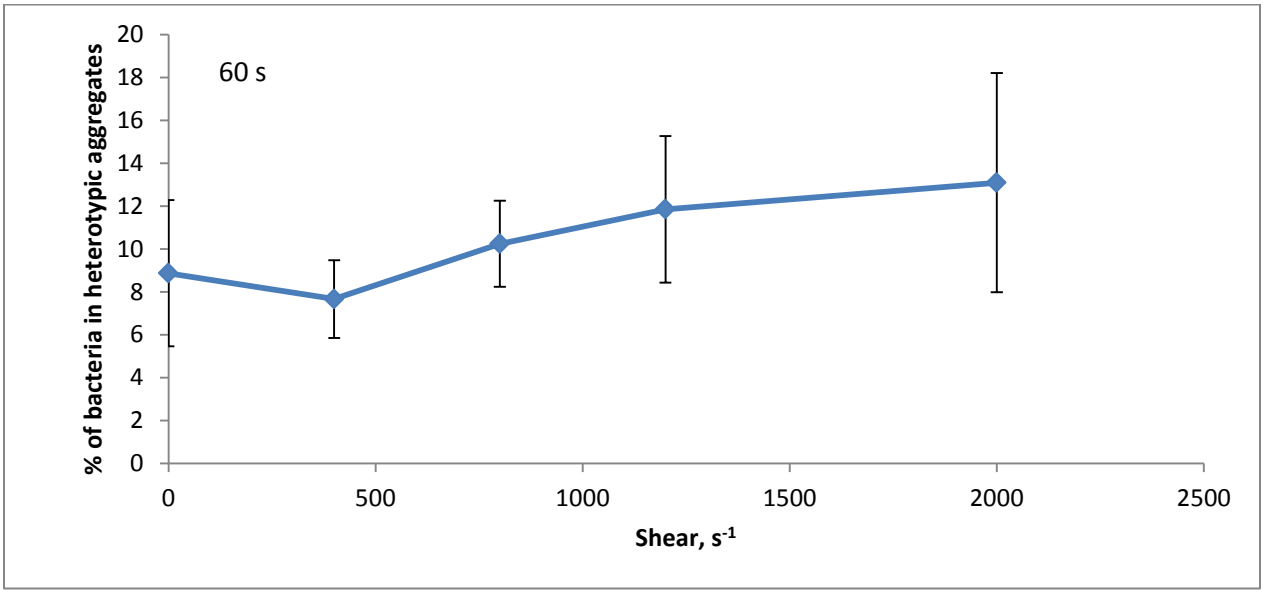

Figure 5.2 Quantification of bacteria in heterotypic aggregates at 60s for varying shear rates

The percentage platelets in heterotypic aggregates were observed to increase with the increasing shear rate from basal levels of 0.697±0171% at $0s^{-1}$ to 4.52±1.636% at $2000s^{-1}$.

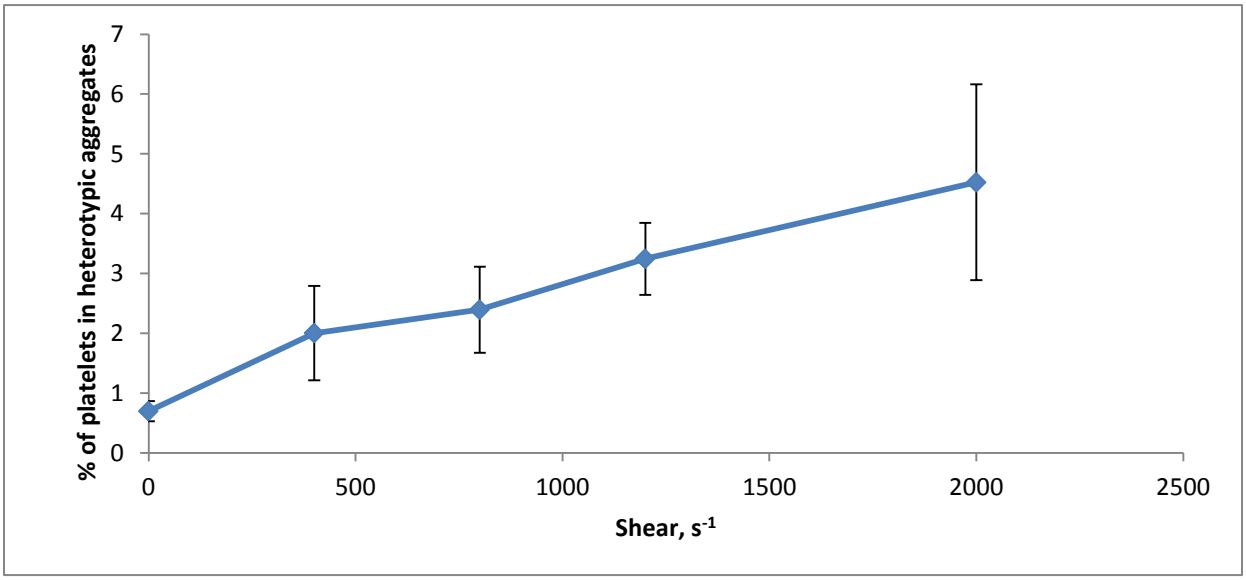

Figure 5.3 Quantification of platelets in heterotypic aggregates at 60s for varying shear rates

Based on these results, the hydrodynamic environment can be considered as a crucial factor in dictating the heterotypic aggregation levels of the two cell types.

5.2.2 Effect of shear exposure time on the *K. pneumoniae*-platelet intercellular interactions

Shear exposure time can be a crucial factor in determining the extent of heterotypic aggregation. It is possible that a prolonged exposure to shear may result in building up of hetero-aggregates. On the other hand, shear for longer durations may weaken the interactions between two cell types resulting in disintegration of the heterotypic aggregates. The samples were subjected to the shear exposure time of 60, 90 and 120 s.

A rough trend was observed for the quantification of bacteria in heterotypic aggregates with increasing shear exposure time of 90 s. Maximum bacterial percentages in hetero-aggregates was found at $1200s^{-1}$. At 120 s, maximum hetero-aggregation was observed at 2000 s^{-1}.

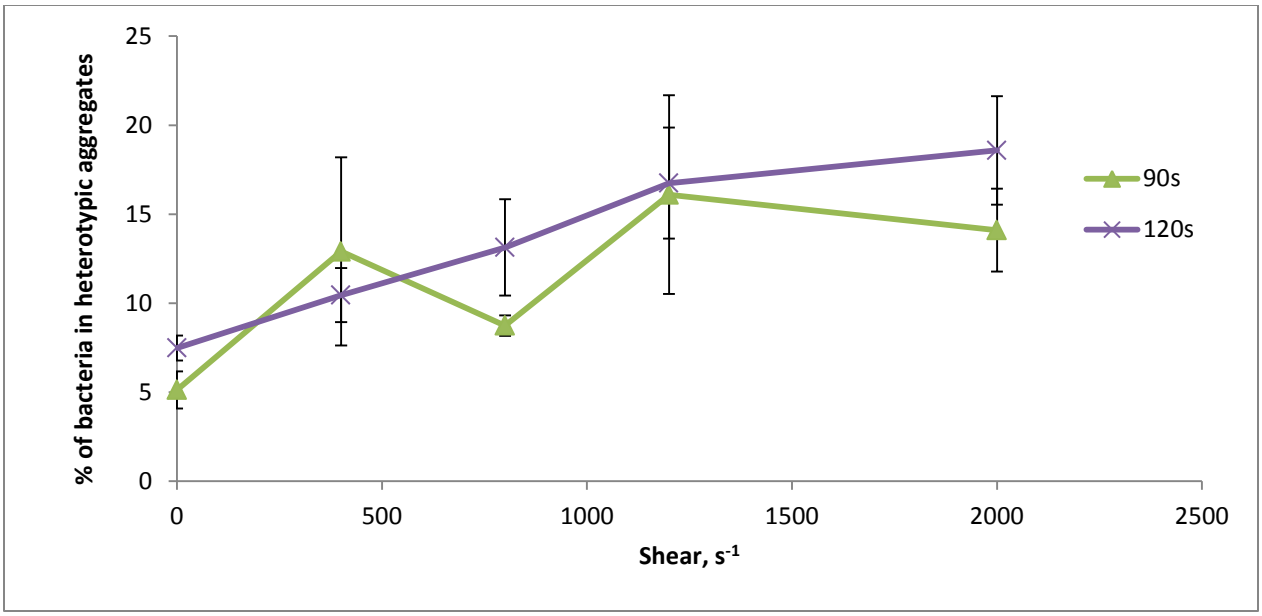

Figure 5.4 Quantification of bacteria in heterotypic aggregates at 90 and 120s for varying shear rates

The level of platelets in hetero-aggregation was seen to increase with increasing shear rates for different durations (90 and 120s). For 90s, the percentage of platelets in hetero-aggregates increased to a maximum of 2.43% at $2000s^{-1}$. At 120s, the percentage platelets in heterotypic aggregates again increased with the increasing shear rate, the maximum being at $2000s^{-1}$ (3.04%).

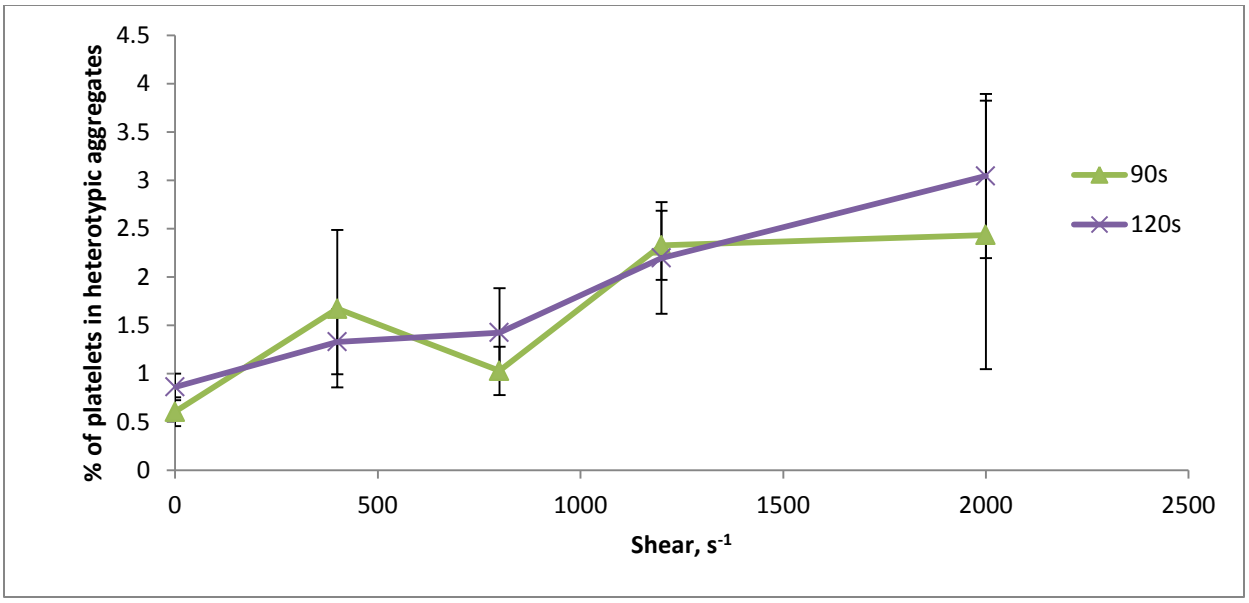

Figure 5.5 Quantification of platelets in heterotypic aggregates at 90s and120s for varying shear rates

5.3 *K. pneumoniae*-platelet intercellular interactions as a function of platelet activation

The effect of platelet activation on the intercellular interaction kinetics may yield an increased heterotypic aggregation levels.

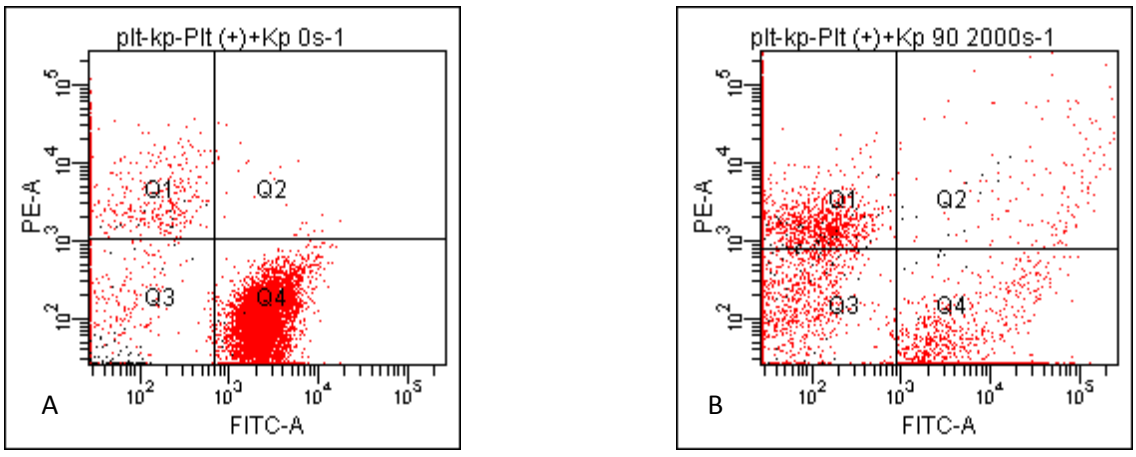

Figure 5.6 Platelet activation (thrombin treatment) affects the heterotypic aggregate formation. A) At lower shear rates, thrombin doesn't have a pronounce effect on the formation of hetero-aggregates, B) With increasing shear, thrombin significantly elevates the heterotypic aggregates formation.

The physiological concentration of thrombin is very low. In order to study the effect of thrombin on platelet activation and aggregation in-vitro, different thrombin concentrations were tested for. A range of 0.05-1 U/ml thrombin concentrations were used to predict the optimum concentration that would minimize homotypic platelet aggregation. The comparative analysis of heterotypic aggregation levels was done between samples treated with thrombin and the ones without thrombin.

On treating platelets with 1 U/ml thrombin for 60s, the basal percentage of bacteria in heteroaggregates increased to 9.79±2.286. But no specific effect was observed at higher shear rates.

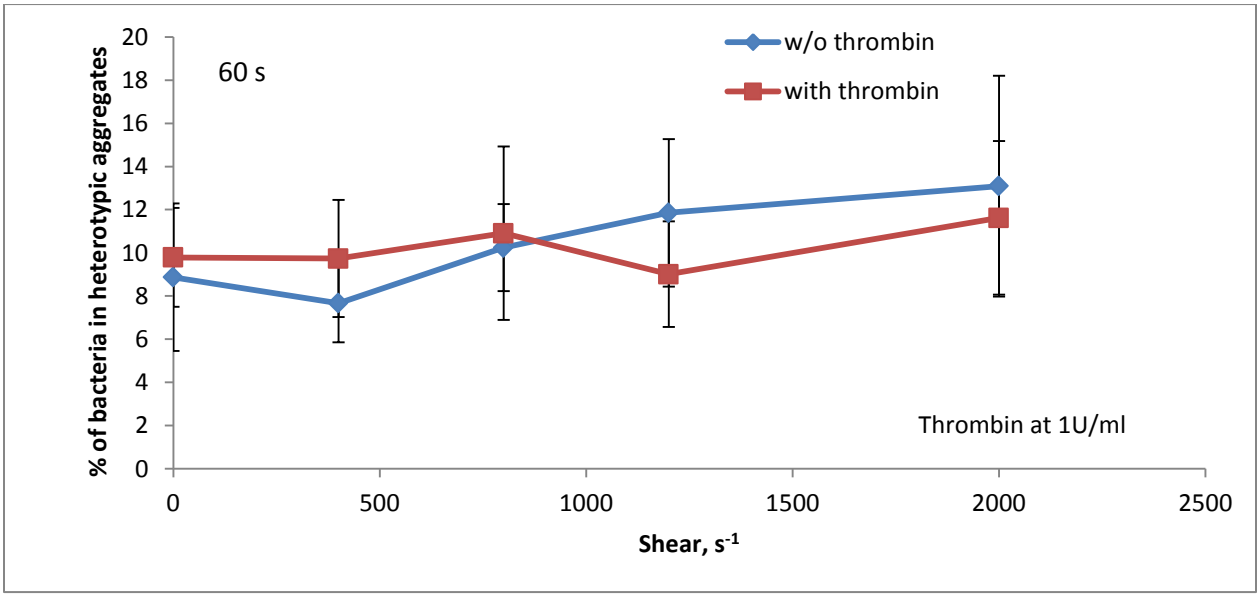

Figure 5.7 Quantification of bacteria in heterotypic aggregates in the thrombin (1 U/ml) treated platelets and control for 60s

In case of platelets percentage in heteroaggregates, a slight increase was observed in thrombin treated platelets for a shear exposure time of 60s.

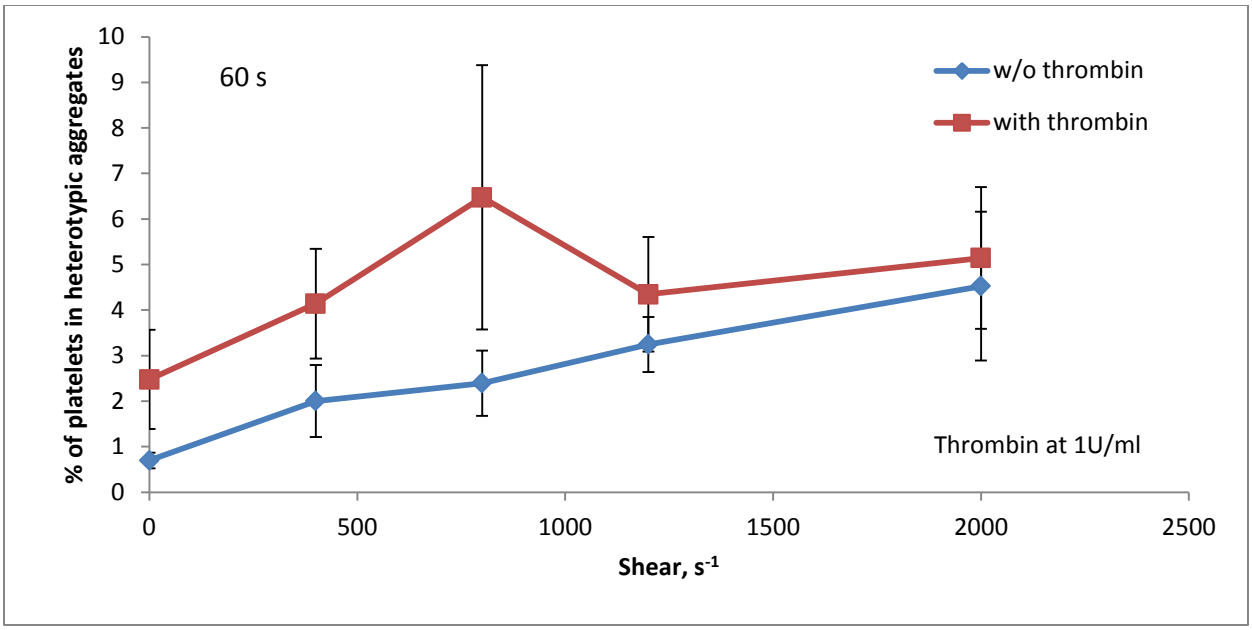

Figure 5.8 Quantification of platelets in heterotypic aggregates in thrombin (1 U/ml) treated platelets and control for 60s

When the samples were exposed to longer shear duration of 90s, the percentage of both bacteria and platelets turned out to be lower in the samples which were treated with thrombin, as compared to the control samples.

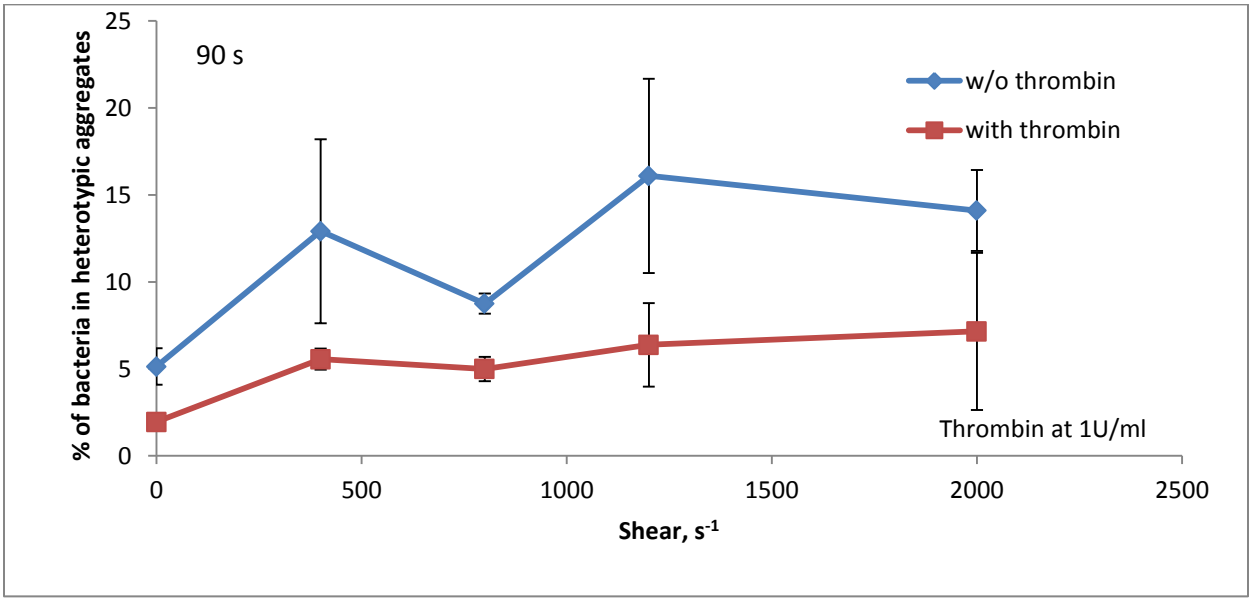

Figure 5.9 Quantification of bacteria in heterotypic aggregates in thrombin (1 U/ml) treated platelets at 90s shear exposure time

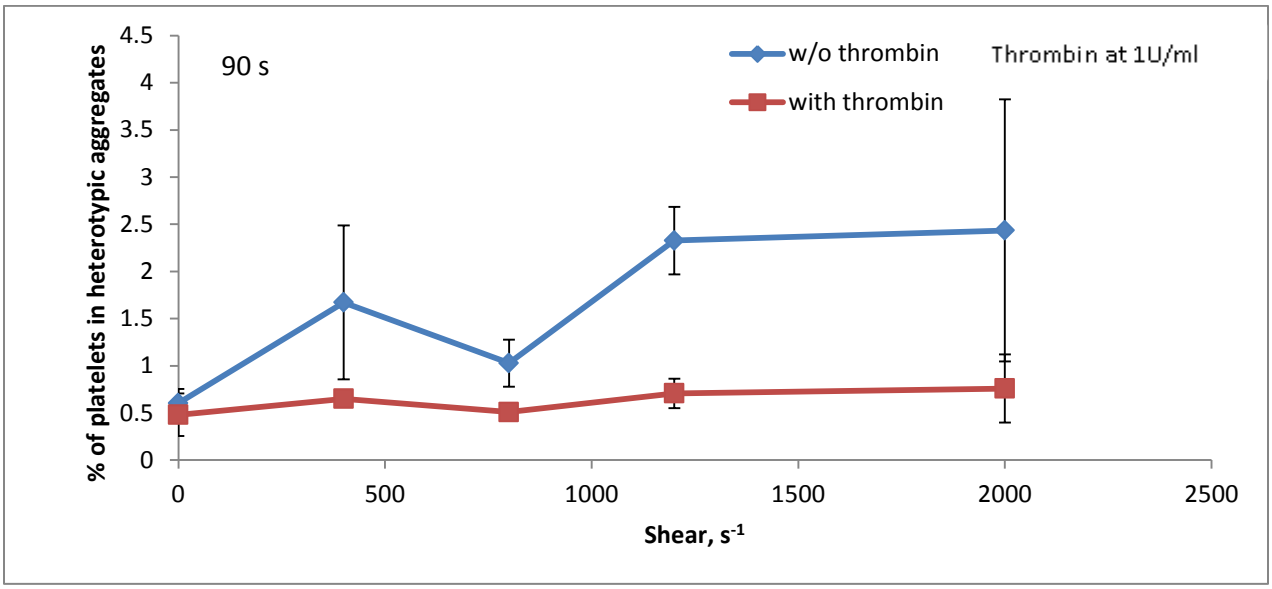

Figure 5.10 Quantification of platelets in heterotypic aggregates in thrombin (1 U/ml) treated platelets at 90s shear exposure time

Similarly, on treating the samples with thrombin at higher shear exposure time of 120s, the percentage of bacteria slightly reduced in hetero-aggregates when compared to control samples.

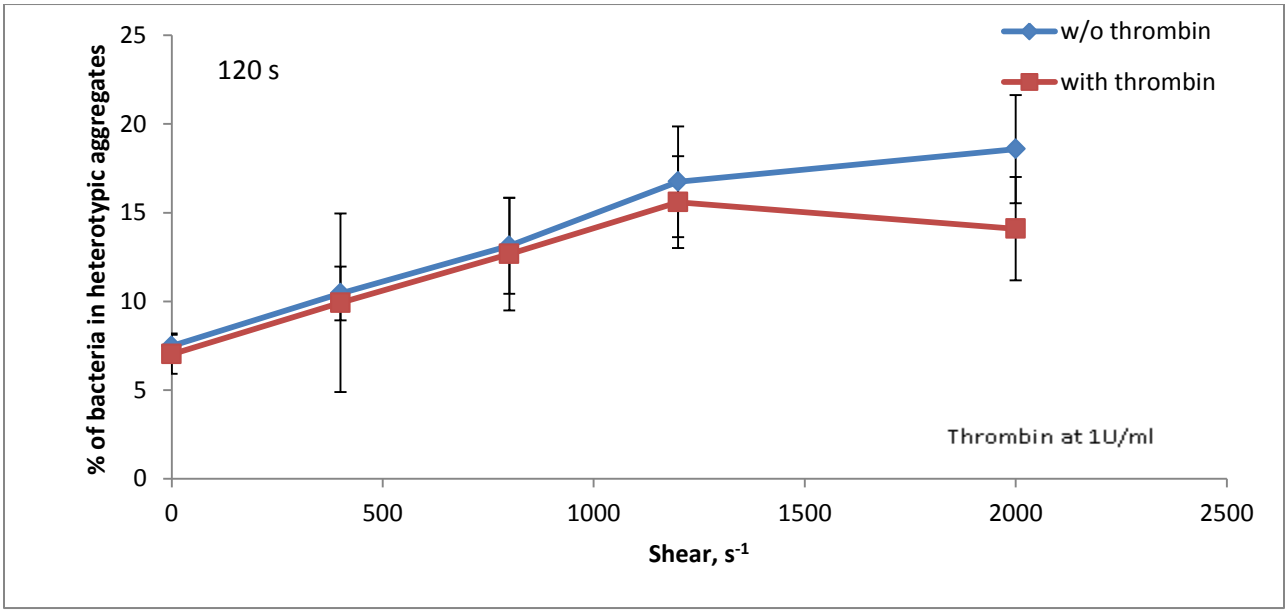

Figure 5.11 Quantification of bacteria in heterotypic aggregates in thrombin (1 U/ml) treated platelets at 120s shear exposure time

But the platelets percentage in thrombin treated sample increased, which can be attributed to the fact that using thrombin could have resulted in platelet aggregation, which then bound to bacteria.

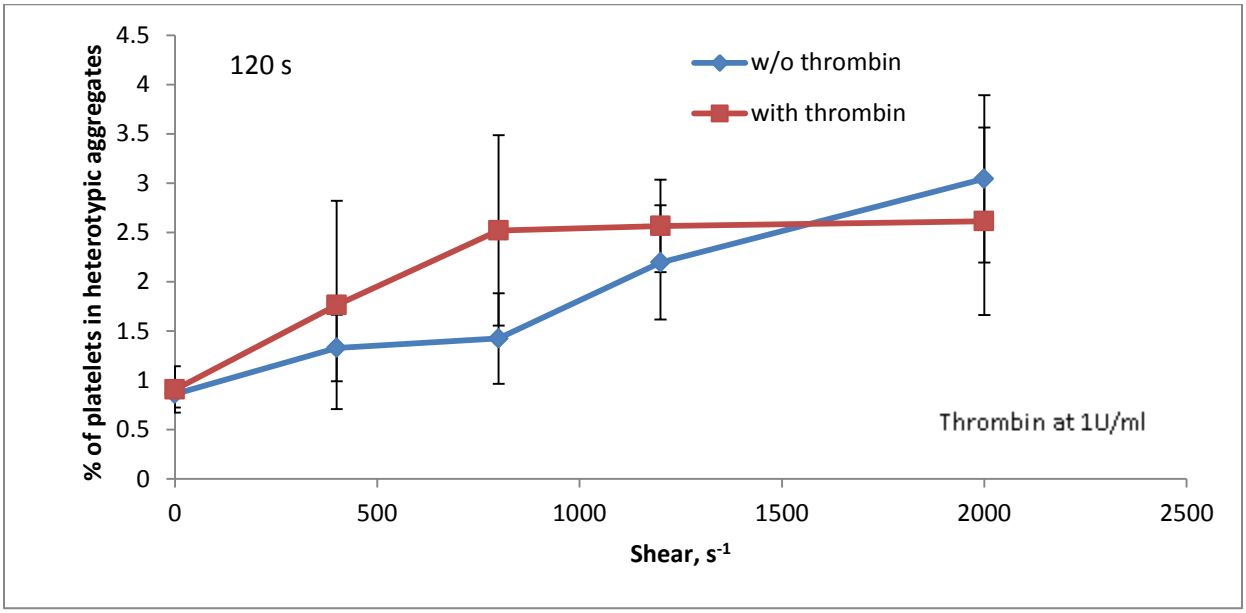

Figure 5.12 Quantification of platelets in heterotypic aggregates in thrombin (1 U/ml) treated platelets at 120s shear exposure time

Since no specific trend was observed in the heterotypic aggregation levels, it was deliberated that using thrombin at a concentration of 1 U/ml may have resulted in increased platelet homotypic aggregation, which might leave little scope for bacteria to participate in heterotypic aggregation. In order to optimize the thrombin concentration, we treated the bacteria-platelet samples with 0.05, 0.075 and 0.1 U/ml thrombin for three different shear rates (0, 400 and 2000 s^{-1}).

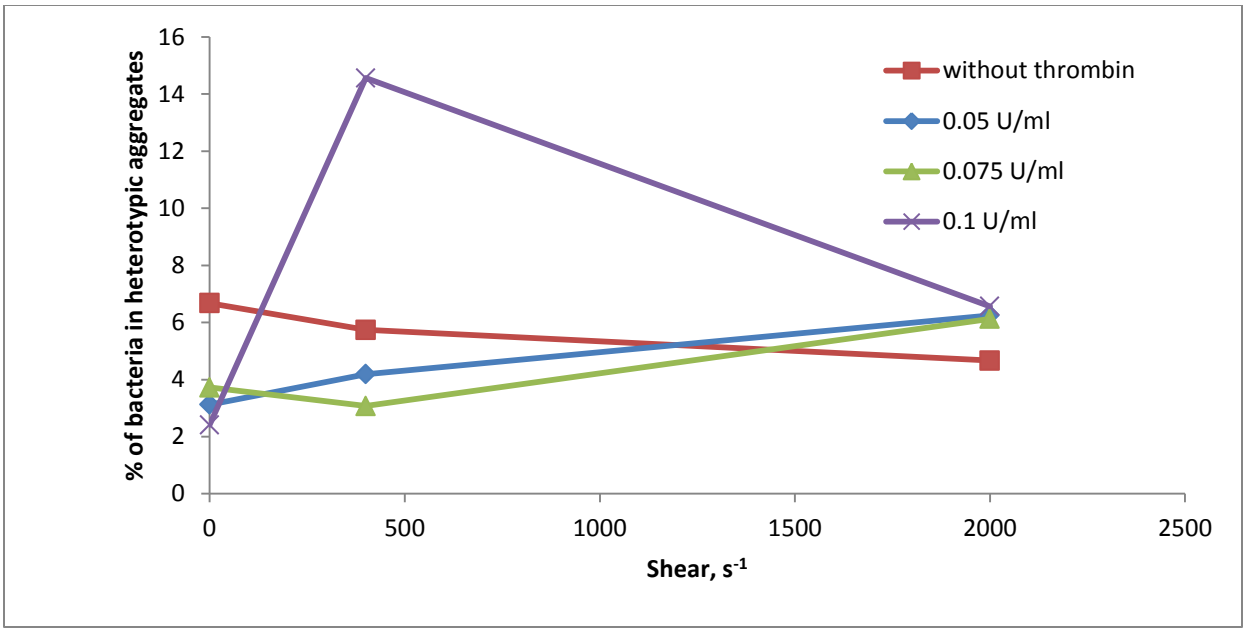

Figure 5.13 Quantification of bacteria in heterotypic aggregates in thrombin treated platelets at 60s shear exposure time, where the thrombin concentration varies in the range of 0.1-0.05 U/ml

After treating the platelets with different thrombin concentrations, it was found that on shearing the sample for 60s, thrombin concentration of 0.1 U/ml gave higher percentage of bacteria in heterotypic aggregates as compared to the percentage observed at 1 U/ml thrombin. So, using 0.1 U/ml of thrombin would be appropriate for carrying out future platelet activation experiments for analyzing heterotypic aggregations.

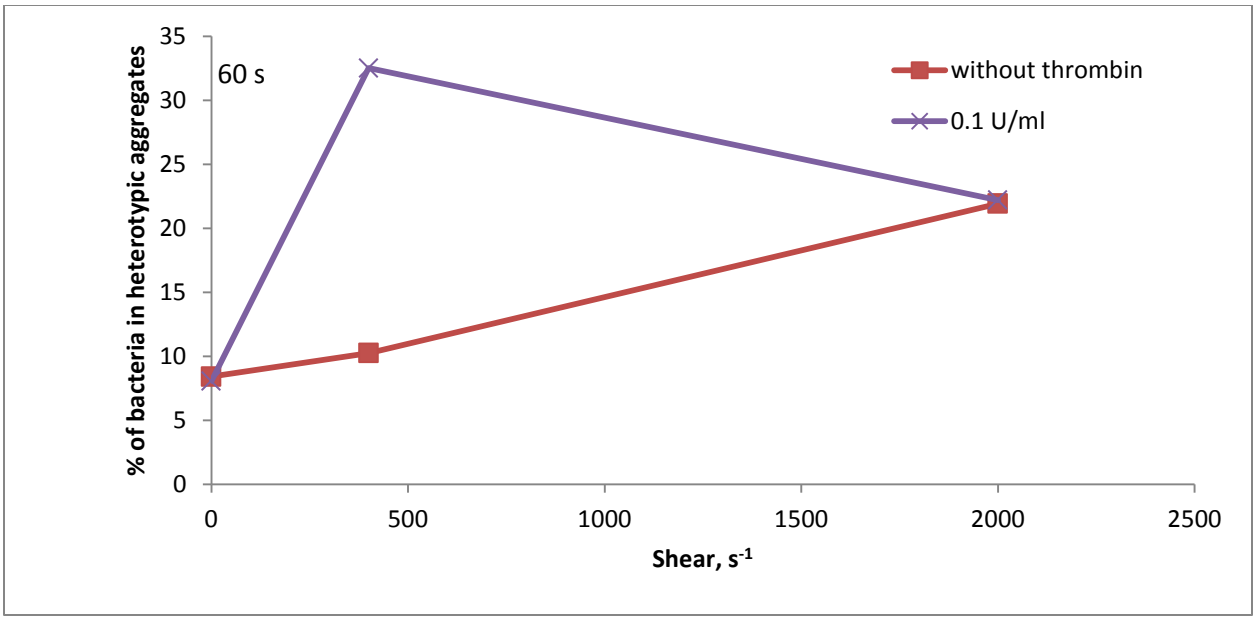

Figure 5.14 Quantification of bacteria in heterotypic aggregates in thrombin (0.1 U/ml) treated platelets at 60s shear exposure time

5.4 Different platelets to bacteria ratios and the effect on heterotypic aggregation

The number of platelets to bacteria may play a crucial role in the heterotypic aggregation phenomenon. A high platelet to bacteria ratio may result in the provision of increased number of platelet surface receptors to bind with the surface or extracellular proteins present on bacteria. For this reason, we tested four different sets of platelets to bacteria ratios (5:1, 10:1, 20:1 and 50:1) to predict the most suitable ratio that may result in better heterotypic aggregation. These different ratios were prepared by

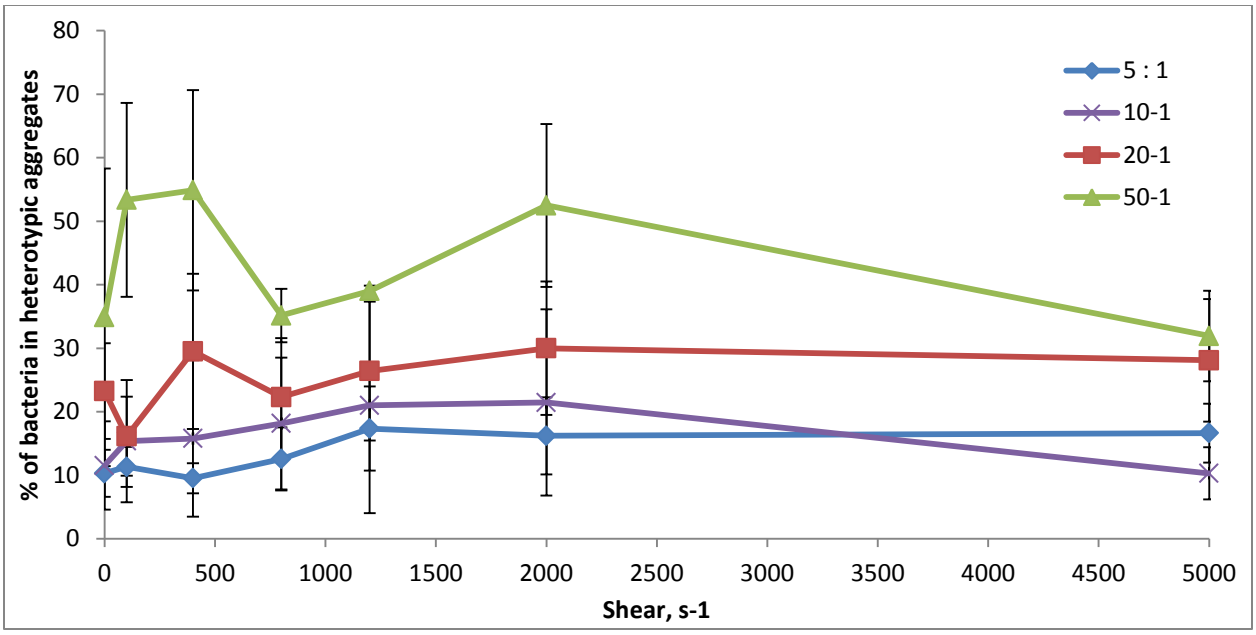

Figure 5.15 Quantification of bacteria in heterotypic aggregates for different platelets to bacteria ratios

Based on the results, it was found that the platelet to bacteria ratio of 50:1 yielded the highest percentage of bacteria in the heteroaggregates. But using this ratio in future experiments will not be a practical option, so 20:1 can be suggested as the most appropriate platelets to bacteria ratio.

5.5 Effect of bio-molecules secreted by growing bacteria on homotypic platelet interactions

Bacterial culture supernatant is enriched with different proteins that are secreted by the live bacterial cells during their growth phase. These proteins may be involved in assisting the heterotypic interactions to occur. The evidence of any such phenomenon is unavailable at present, but the effect of the supernatant was observed on the platelet aggregation incidence.

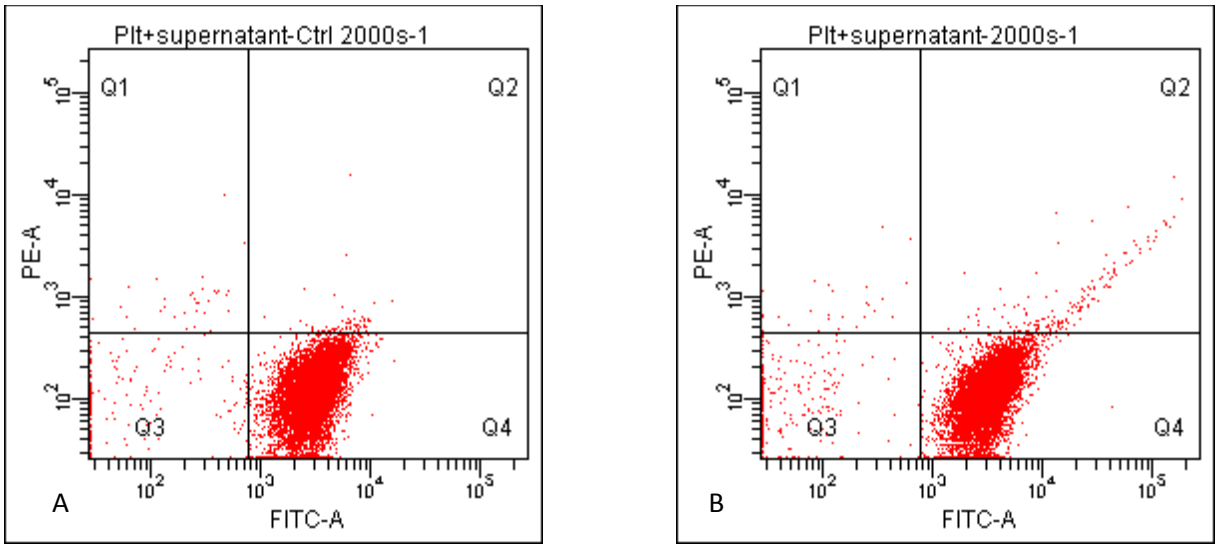

Figure 5.16 Effect of the bacterial culture supernatant on platelet aggregation A) Homotypic platelet aggregation in control at 2000s^{-1}, B) Homotypic platelet aggregation in bacterial supernatant at 2000s-1

5.6 Discussion

Fluid mechanical forces can have intense effects on blood cell function. It is widely accepted that fluid shearing stress triggers platelet activation, causes the release of granular contents in the suspending medium and potentiates platelet aggregation in the absence of exogenously added chemical agonists. Most bacteria can interact with platelets through multiple mechanisms making it difficult to identify the roles of the different proteins (both bacterial and platelet) and is further complicated by interactions that are not only species-specific but strain-specific as well. The exposure of platelets to *K. pneumoniae* cells under hydrodynamic shear initiated platelet activation, resulting in the intercellular interactions between the two cell types.

In the work done so far, *K. pneumoniae* have shown to interact with the platelets in a shear dependent manner. Hydrodynamic shear exerts a great effect on the extent of heterotypic aggregation, and the levels are seen to increase with the increasing shear rates. Thrombin doesn't has a profound effect on the heterotypic aggregation levels as such, but optimizing the thrombin concentration (0.1 U/ml) actually helps in enhancing the intercellular interactions without causing excessive homotypic platelet aggregations, as seen in the results. Similarly, shear exposure time also has an influence on the hetero-aggregation in a shear dependent manner.

Different platelets to bacteria ratios used resulted in different heteroaggregation levels. Of these, 20:1 could prove the optimum ratio to increase the heterotypic aggregation levels up to a substantial amount.

This is the first study to characterize the *K. pneumoniae* interactions with platelets in cell suspensions as a function of the dynamic shear environment and platelet activation. We have observed that *K. pneumoniae* interacts with platelets significantly at high shear rates. The mechanisms of *K. pneumoniae* interactions in activation and binding with platelets are largely unknown. Also, this study provides a preliminary basis to the fact that the bacterial culture supernatant of *K. pneumoniae* might contain certain secreted proteins or other biomolecules which can have a potential role to play in heterotypic aggregation.

In conclusion, the data obtained in the present study suggests that fluid mechanical environment of the circulatory system affects the kinetics of *K. pneumoniae*-platelet intercellular interactions, and molecular mechanisms mediating platelet activation and aggregation. Elucidation of the detailed physical and molecular basis underlying *K. pneumoniae*-platelet conjugate formation may provide insights for the rational development of novel strategies to target antibiotic-resistant strains of pathogenic bacteria, thereby ending the therapeutic dilemma.

Chapter 6

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