Detection and quantification of *Legionella pneumophila* using latex agglutination test

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ABSTRACT

Legionella pneumophila (*L. pneumophila*) are ubiquitous in environmental water sources that are responsible for causing sporadic and epidemic cases of atypical pneumonia, called Legionnaires' disease (LD), after inhalation of contaminated water droplets. There is a need for a rapid detection method for *Legionella pneumophila* fluid samples without the need for complex instrumentation. Agglutination assays are widely used in biology and medicine for their simplicity and specificity. However, conventional agglutination tests only provide presumptive results. Here I describe and perform a latex agglutination test (LAT) that can detect and quantify *L. pneumophila* in a sample by determining the degree of agglutination. The latex microbeads (beads) are coated with polyclonal antibodies using established methods. The optimal ratio of the beads to the antibody for the best performance of LAT was found to be 20:3. *L. pneumophila* serogroup 1 is chosen as the target bacteria and *Escherichia coli* K12 as the negative control. Strains of *L. pneumophila* serogroup 1 were cultured and inactivated by heat treatment for safety. The target bacteria displaying the antigenic epitopes were mixed with the antibody-coated beads and have been shown to facilitate agglutination. Microchannels fabricated using adhesive tapes are used to entrap the fluid sample, where an image analysis algorithm quantifies the degree of agglutination using images obtained by a microscope, thereby, resolving issues with complex techniques. Specific binding between antibody and *L. pneumophila* serogroup 1 is investigated by tracking the motion of the beads and bacteria in the images. The number of beads involved in agglutination compared to the total number of beads present in an image determines the degree of agglutination. The sample volume used for detection is 3 µL and the detection

limit of the method is 10^6 cells/ml. Compared to expensive and complex PCR techniques and time-consuming culture methods, this LAT is simple, affordable, and can produce results within hours using only a microscope.

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List of Abbreviations

Ab	Anti- *Legionella pneumophila* antibody
Ag	Antigen
BCYE	Buffered charcoal yeast extract
Bead	Latex microbead
BSA	Bovine serum albumin
CML	Carboxyl modified latex
DNA	Deoxyribonucleic Acid
EC	*Escherichia coli* K12
EDC/NHS	1-ethyl-3-(3-dimethylaminopropyl)/ N-Hydroxysuccinimide
ELISA	Enzyme linked Immunosorbent assay
LAT	Latex agglutination test
LB	Luria Bertani
LD	Legionnaires' disease
LP	*Legionella pneumophila* serogroup 1
LPS	Lipopolysaccharide
MES	2-(N-morpholino) Ethane Sulfonic acid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

List of Symbols

D	Diameter
D_{Bb}	Distance between the bead entity and bacteria
ρ	Density
C	Microsphere surface capacity
S	Surface saturation

PART 1. Introduction

1.1 *Legionella pneumophila*

Legionella pneumophila (*L. pneumophila*) is the most common and notorious pathogenic bacteria responsible for all the infections and outbreaks of Legionnaires' disease (LD). This potentially fatal respiratory disease is acquired through environmental exposure to aerosolized contaminated water from water-containing appliances such as air conditioners, hot tubs, and humidifiers, in North America [1-2]. Numerous large outbreaks of LD have also been reported associated with cooling towers, which distribute aerosolized plumes to relatively large areas [4]. *L. pneumophila* grows and multiply within a building water system and they spread through droplets small enough for people to breathe in. The incubation period is typically 5 to 6 days but ranges from 2 to 14 days following exposure to aerosolized water containing *L. pneumophila*. Elderlies, as well as individuals who smoke or have a weakened immune system, are more vulnerable to infection. Some common types of diseases which are derived from *L. pneumophila* are respiratory failures, septic shocks, and acute kidney failures [5]. The infection can be identified by laboratory testing and takes hours to weeks depending on different parameters such as source availability. This detection time is very important for those affected by *L. pneumophila* to decrease the lifetime of pathogens inside the body. The most common cause of LD is *L. pneumophila* serogroup 1 (LP), responsible for approximately 70%-92% of laboratory-confirmed cases [3]. After analyzing over 200 clinical *Legionella* isolates, it was found that the common feature of LP from other serogroups is the lipopolysaccharide (LPS)-associated epitopes [6]. LPS is the major immunodominant antigen (Ag) of all *Legionella* species contributing to the pathology associated with disease [7]. Studies have reported that the serogroup-specific antigen of *L. pneumophila* is contained in the

LPS portion of the bacterial cell wall such that each serogroup possesses different LPS profiles [8] providing the basis for the classification of *L. pneumophila* into serogroups and subgroups [7, 9].

The number of reported cases has been rising in the United States, Europe, and other developing countries over the past decade [9-10]. In the United States, a total of 63,520 confirmed LD cases were reported to the National Notifiable Diseases Surveillance System (NNDSS) from 2000 through 2017 [12]. In Canada, the average number of reported cases of LD is generally less than 100 per year, but the actual number of cases is thought to be much higher, as many people with pneumonia may not be tested for infection with *L. pneumophila* [13]. Still, several major outbreaks of LD in Canada have been reported which include Toronto in 2005 affecting 135 people and causing 23 deaths [14], and Quebec City in 2012 affecting over 180 people and causing 14 deaths [15].

Legionellae (plural of *Legionella*) species are Gram-negative, non-spore-forming, rod-shaped, aerobic bacteria with a single, polar flagellum. Free-living *Legionellae* are approximately 0.3-0.9 μm in width and 2-20 μm long, depending on the age of the culture. Fresh cultures of *Legionellae* grown in nutrient-rich media have a length of about 2-6 μm, whereas older cultures may produce filamentous forms up to 20 μm long. The movement of *Legionellae* is through one or more polar or subpolar flagella [16].

Detection and monitoring of water-borne pathogens are of most importance to public health which requires the use of fast response, simple and affordable devices to monitor water samples for detecting pathogens. Being able to detect the number of pathogens such as *L. pneumophila* in current water sources, helps individuals avoid inhaling aerosolized water contaminated by *L. pneumophila.* [11]. Several detection methods are currently being used for bacteria sensing during

outbreaks and routine testing. Routine testing for *Legionellae* to prevent LD (i.e., the presence of *Legionella* is detected before cases occur) is implemented on an individual system-by-system basis [17]. The circumstances provided by the Centre for Disease Control (CDC) and the Health and Safety Executive when monitoring facilities for *Legionellae* that may be appropriate are as follows [17]: (1) difficulty maintaining water systems within established control levels; (2) having a prior history of LD; and (3) residents who are at increased risk for LD. According to Public Services and Procurement Canada, the two most widely used and accepted detection methods are the culture-based test and polymerase chain reaction (PCR) test to control *Legionella* in mechanical systems such as cooling towers, air conditioning systems, open water systems, and domestic water systems [18]. The gold standard for *Legionella* testing in water samples is culturing according to the standardized protocol of the ISO 11731:2017 [19]. This ISO method describes the standardized cultivation of *Legionella* on selective culture media. The degree of contamination is determined quantitatively as colony-forming units (CFU) per 100 mL sample. This form of testing is widely accepted as the most reliable detection test for *Legionella* bacteria because of its low detection limit (1CFU/100 mL). However, *Legionella* is slow-growing, and culture results may take 10-14 days. Initial growth may be slow, and genus-level identification normally requires at least one round of subculture involving an additional period of incubation. The presence of naturally occurring bacterial or fungal co-contaminants may require additional subculture or may mask the presence of *Legionella* altogether [18]. Moreover, *Legionella* can enter a viable but non-culturable (VBNC) state to withstand harsh environmental conditions (presence of disinfectants, low nutrients, high temperatures, and low oxygen levels) [17]. *Legionellae* are generally dormant and cannot be cultured within the VBNC state, even though they continue to retain their viability and virulence. This difficulty can lead to false-negative results underestimating the presence of

Legionella in water systems, which have serious public health consequences. Culture test methods allow for the quantification of *Legionella* bacteria, as well as specific serotypes including LP.

However, due to the mentioned limitations associated with culturing, PCR is used as an alternative method to detect *Legionella.* PCR is a rapid test designed to specifically detect nucleic acid sequences, including those for all *Legionella* species [17]. A very large number of PCR-based analyses for *L. pneumophila* as a specific target has been developed. They have the immediate advantage over culture techniques as they can return results in 1-2 days. A major category of PCR-based tests is quantitative PCR (qPCR). qPCR simultaneously amplifies and quantifies a target deoxyribonucleic acid (DNA) sequence of *Legionella* from environmental samples, giving the number of genome units per milliliter (GU/mL). The rapid turn-around time and sensitivity of qPCR are advantageous when compared to traditional culture methods[20]. However, according to a study where 101 spa water samples were tested, *Legionella* was detected and quantified in 14 of 101 samples with measured concentrations ranging from 250 to 3.5 x 10^2 CFU/mL using the culture method. With the qPCR method, *Legionella* was detected and quantified in 42 of 101 samples with concentrations ranging from 1000 to 6.1 x 10^4 GU/mL. An equivalence between the number of GU and CFU has not been established and the results obtained are highly dependent upon the method used and the sample composition [21]. Moreover, qPCR tends to overestimate due to the amplification of non-viable and may persist for extended lengths of time. Therefore, qPCR could be used as a screening tool to rapidly ascertain the absence of *Legionella*, but a positive result involves the need to resort to conventional culture. Although the PCR technique is highly sensitive and able to provide quick results compared to culturing, this technique is expensive (supply cost, machinery fees, and training fees) and requires trained personnel. The above-mentioned challenges in the detection of *Legionella* from environmental samples have led

to the requirement of a simple detection test with less complicated procedures that provide faster results. These methods also lack in that they are not able to specifically detect LP, which is of more significant importance when considering the containment of an outbreak.

1.2. Latex agglutination test

Immunoassays are developed to measure the presence of an analyte (usually proteins) through Ag – antibody interactions. Culturing methods continue to be the 'gold' standard; followed by nucleic acid-based assays such as PCR, ranked 'silver', and immunoassays ranked 'bronze' [22]. However, immunoassay is the most rapid compared to the other two methods as culture methods require longer periods to obtain results and nucleic acid-based assays require technical expertise and a nucleic acid extraction step. Agglutination is a reaction in which Ags, such as proteins present on the surface of bacteria, combined with their specific antibodies to form complexes that come together as visible clumps (also known as agglutinates) [23]. Agglutination tests, categorized under immunoassays, are widely used in biology and medicine for the identification of antibodies on microbial cell surfaces from environmental samples [24] as well as to demonstrate the presence of antibodies in serum [25], where the principle is the same in both applications. They are generally fast and highly specific, yet economical and do not require sophisticated equipment or personnel with specialized skills. Agglutination reactions are classified as direct, indirect (passive), and reverse passive agglutination reactions. Direct agglutination test refers to the assays in which the Ag directly agglutinates with the antibody. On the other hand, an indirect or passive agglutination test involves the coating of Ag on the surface of a carrier molecule (e.g. red blood cells (RBCs), latex microbeads) such that the antibody binds to the coated Ag and agglutination takes place on the surface of the carrier molecule. Over the past several years, RBCs have been replaced by synthetic particles, such as latex microbeads (termed as beads). The use of such synthetic beads

offers the advantages of consistency, uniformity, and stability for the agglutination test [24]. Furthermore, agglutination reactions employing synthetic beads can be read rapidly, often within 15 mins to an hour, when the test sample is sent to the lab and mixed with beads coated with specific Ag or antibody [24]. These agglutination tests using beads as a carrier molecule are called the latex agglutination test (LAT). A reverse passive agglutination is a type of LAT, used in this project, in which the antibody is coated on the beads which detect Ag in the sample as seen in Figure 1.1 [26].

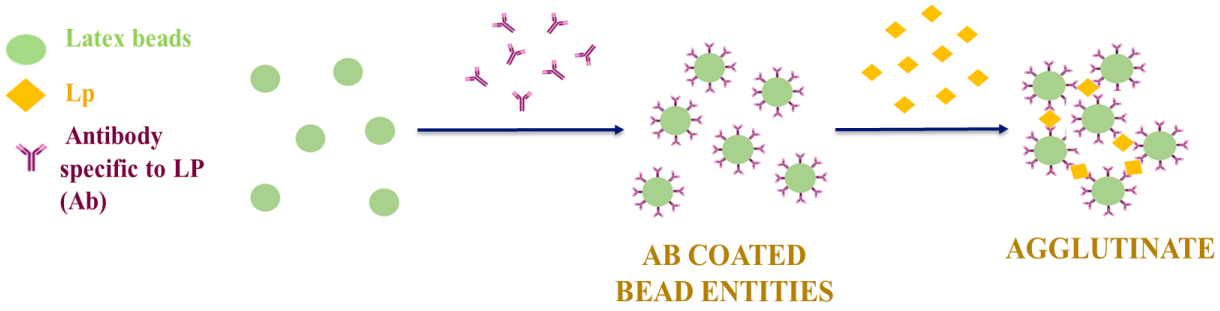

Figure 1.1: Latex beads coated with Ab detect Lp and form agglutinates.

The antibody or Ag molecules can be bound in random alignment to the surface of the beads. Agglutination of beads evident by clumps in the dilutions is considered a positive result. Ag or antibody present in a specimen binds to the combining sites of the corresponding antibody/Ag exposed on the surface of the beads, forming cross-linked agglutinates of beads and Ag/antibody. In the absence of Ag/antibody within the sample, the mixture will keep its appearance as a homogenous suspension of microparticles.

LATs are employed in a wide range of applications from the diagnosis of life-threatening diseases such as Paracoccidioidomycosis and typhoid fever to the detection of antigens such as Candida antigens in the serum of patients with invasive candidiasis [26-28]. They are inexpensive,

relatively stable, and not subject to cross-reactivity with other antibodies. They are very popular in clinical laboratories for detecting Ags to *Cryptococcus neoformans* in cerebral fluid or serum [30]. The sensitivity and specificity using LAT for detecting *Cryptococcal* capsular polysaccharide Ag are 91.1% and 96.0% respectively. LATs using beads coated with anti-CRP antibodies is the most widely used method employed worldwide for the detection of C-reactive protein [31]. One of the limitations of using LATs is that the test requires more analyte (Ag or antibody) to observe a positive result compared to other immunoassays such as ELISA [32]. Although LATs have high LOD compared to ELISA, they could be used for the detection of Ag or antibody as they are rapid and simple to use. LAT kits utilizing suspensions of beads coated in the appropriate Abs have recently become commercially available for the identification of many species of *Legionella* for routine purposes such as *Legionella* latex test due to their simplicity and low cost [33]. However such tests can only be used for the identification of predominant *Legionella* species grown on plate media from patients with suspected LD or environmental sources and cannot quantify *Legionella* [34].

The level of water contamination by *Legionella* must be known to launch an investigation and take appropriate measures to reduce hazardous conditions that allow *Legionella* to grow. There is no known safe level of *Legionella* in building water systems [35] According to the American Industrial Hygiene Association (AIHA), a measured concentration of *Legionella* below 1 CFU/mL is considered to be at the detection limit [36]. *Legionella* amplification could be possible for concentrations between 1 and 10 CFU/mL. Any sample that contains *Legionella* concentration above 10 CFU/mL indicates that amplification has occurred, and action needs to be taken [36]. Conventional agglutination tests such as the *Legionella* latex test do not determine the level of *Legionella* samples, which has inspired significant efforts towards quantification using latex

agglutination. Currently, the degree of agglutination is determined by grading the variation on the clumping ability observed when a sample containing the specific Ag is mixed with its antibody coated on the surface of the bead [22, 35-36]. For example, a positive reaction was graded on a scale of 0 to 4+ based on the degree of agglutination present. A '0' reaction appears negative macroscopically when the solution does not show any agglutination. A '1+' reaction has a grainy appearance macroscopically based on only a few beads participating in an agglutination reaction. A '2+' reaction has discernible agglutinated clumps on a field of the individual or slightly agglutinated beads. A '3+' reaction has several agglutinated beads of moderate size with a relatively clear background. A '4+' reaction has one or more agglutinated bead clumps with no macroscopically free beads [38]. However, this method of visual observation is very subjective as the readings can differ between individuals. Hence LATs lack the feature of determining the degree of agglutination from the results which could have estimated the level of *Legionella* in water samples.

In this work, a new technique has been developed for detecting and quantifying *L. pneumophila* using LAT due to its simplicity and cost-effectiveness. Beads are functionalized with anti- *L. pneumophila* antibodies (Ab). The optimal ratio of beads to Ab required for the functionalization was found to be 20:3. The specific binding between Ab and LP is investigated by tracking the movement of the beads and LP in an agglutinate. The number of beads involved in agglutination can determine the level of water contamination by LP through an image analysis designed to analyze images captured by a microscope and return a quantitative output for the LAT results. Channels were fabricated using adhesive tapes to entrap the water sample for the image analysis. The detection limit of *L. pneumophila* using this method is in the range of 10^6 cells/mL. Although the detection limit is high, this new technique can provide results in 5 to 8 hours compared to the

long duration for culture results. Compared to the PCR technique which requires complex machines, the preparation of specific primers, and trained personnel with heavy technical background required for troubleshooting, this technique only requires a microscope which is less expensive and less time on training comparatively, making this technique cost-efficient for the detection of *L. pneumophila*. Moreover, this LAT-based technique can quickly and accurately quantify the amount of *L. pneumophila* in a sample.

PART 2. Preparation and confirmation of *L. pneumophila* serogroup 1

2.1. Introduction

In this chapter, the preparation of LP is explained. Since LP is responsible for most of the infections and outbreaks of LD, it is taken as the target bacteria for LAT. LP was cultured at standard conditions [39] in buffered charcoal with yeast extract (BCYE) agar plates. Activated charcoal decomposes hydrogen peroxide, a metabolic product toxic to *Legionella* species. Yeast extract supplies the proteins and other nutrients necessary for growth. Buffering the medium maintains the proper pH for optimal growth [40]. In this work, the culture plates are incubated at 36 ± 1°C for up to 14 days and are examined every two or three days. Also, LP is inactivated by heat treatment for the safety of the lab from contaminations leading to infections. In addition, a commercially available LP Latex test, Oxoid Drypot test, was used to confirm that the cultured bacteria are LP.

2.2. Materials

2.2.1. Antigen

Although many of the *Legionella* species or serogroups have been related to human infection, LP is the most frequent agent of LD. LP is also one of the most frequently identified in environmental samples [41]. Therefore, LP obtained from ATCC® 33152™ is used as the Ag for the LAT. The most recognized waterborne opportunistic pathogens, dwelling in tap and groundwater, are *L. pneumophila* and *Escherichia coli* (*E. coli*), where both bacteria are Gram-negative and rod-shaped [42]. In addition, the *E. coli* strain K12 is the most popular commercial strain used in the lab today

due to its fast growth, high-throughput cloning, cloning unstable DNA, and preparing unmethylated DNA [43]. To demonstrate the specificity of the LAT to detect LP, *E. coli* K12 (EC), purchased from New England Biolabs (E4107S) was used as the negative control.

2.2.2. Chemical reagents and media

BCYE agar plates for the culture of *L. pneumophila* were obtained from Cedarlane Labs. Luria Bertani (LB), and phosphate-buffered saline (PBS) solution were purchased from Sigma-Aldrich. The addition of glycerol stabilizes the frozen bacteria, preventing damage to the cell membranes and keeping the cells alive [44]. Glycerol was purchased from Thermo Fisher Scientific. LB broth was prepared by diluting 10 g of the powder in 500mL of distilled and autoclaved water and used as the culture media. 1x PBS used in washing and suspending bacteria was sterilized in an autoclave. 30% of glycerol (v/v) with PBS [45] was prepared by adding 3 ml of glycerol to 7ml of 1x PBS and the solution was autoclaved for cryopreservation of bacteria.

2.2.3. Confirmation of the presence of *Legionella* using Oxoid Dryspot *L. pneumophila* serogroup 1 latex test

Oxoid Dryspot LP Latex Test is a commercial agglutination assay. This test kit provides a fast and simple screening procedure for the pathogenic LP. The Oxoid Dryspot Latex Test uses antibody-sensitized blue latex beads that are dried onto cards as seen in Figure 2.1a. Each test strips consist of a test and control section. The test section contains blue latex beads sensitized with rabbit antibody that is specific to LP dried onto the card (Test reaction area). If one or two drops from the sample containing LP are added to the latex area, a blue precipitate will be formed as seen in

Figure 2.1b. The latex beads will agglutinate in the presence of the specific LP cell wall Ags to form visible clumps [34]. On the other hand, the control section contains blue latex beads sensitized with non-specific rabbit antibodies (the document has not mentioned the target microorganism for the antibodies used in the control section) which do not coagulate with the LP (Control reaction area) and thereby, will show no precipitation if the same sample is tested on the control section (Figure 2b). This assay is intended to be used for the confirmation of the serogroup of LP by dropping one to two drops of the cultured LP onto the blue antibodies-sensitized latex beads. Agglutination can be observed within 30 seconds after the start of applying the drops from the sample.

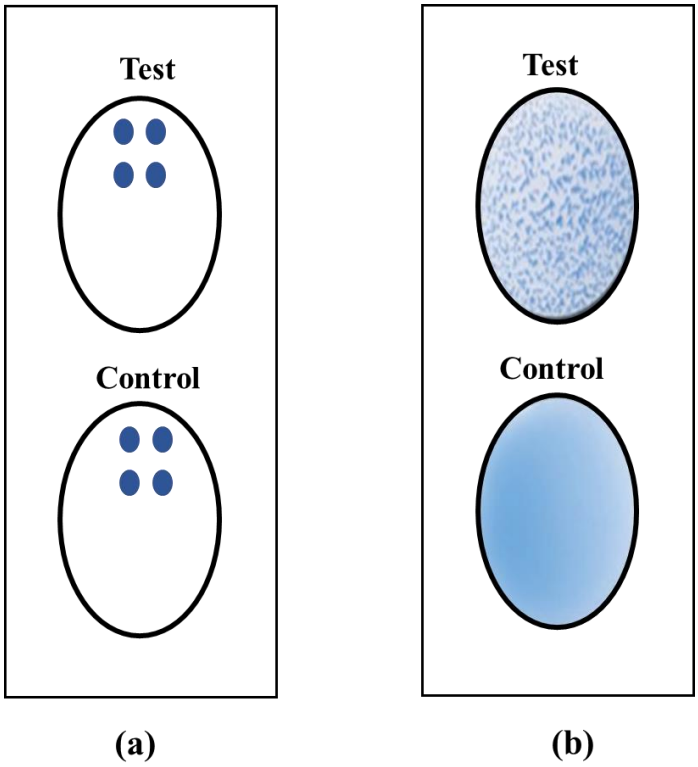

Figure 2.1: (a) A dry spot test for LP bacteria that contains its specific antibody sensitized blue latex beads on the test section and latex beads sensitized with the non-specific antibody on the control section before adding the sample; (b) a drop of the sample containing LP bacteria were added to both sections where a precipitate is formed on the test section (top) due to Ag-Ab specific binding and no precipitate on the control section (bottom) as the latex beads contain non-specific antibody.

2.3. Methodology

2.3.1. Culture of LP and EC

LP was cultured as per the standard procedures [39]. PBS was sterilized and filtered using a syringe filter of 0.22µm. Purchased lyophilized LP (Figure 2.2a) was resuspended in 500µL of sterilized PBS. 2-3 drops from this solution were inoculated onto the BCYE agar plates. A sterile single-use loop is used to streak the bacteria onto the BCYE agar plates. The plates were incubated at 37°C for 48-72 hours. Once the colonies were grown as seen in Figure 2.2b, they were collected using a sterile loop and then were resuspended in PBS with 30% of glycerol v/v. This solution was then filled into vials and stored at -80°C.

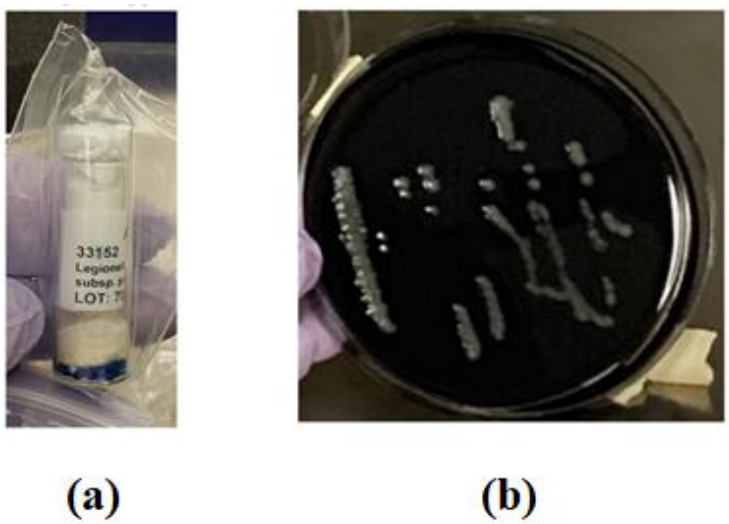

Figure 2.2: (a) Lyophilized LP bacteria purchased from ATCC; (b) LP bacteria colonies grown on BCYE agar plates after 72 hours.

EC is cultured by using LB media which supports a higher density of bacteria and cultures enough bacteria necessary for experimental use. A sterile pipette tip dipped in the EC vial is dropped into a test tube containing 6 mL of LB media. A test tube containing the same amount of LB media

with a blank pipette tip (not dipped in EC vial) is taken as a control to confirm the culture of EC. The test tubes containing the pipette tip are then loosely covered with a cap that is not airtight and incubated in a shaking incubator overnight. The growth of EC is confirmed by the turbidity observed in the media. On the other hand, the media from the control remain transparent as there are no bacteria to culture, free from contaminants.

2.3.2. Inactivation of LP by heat treatment

Failure to control storing samples containing LP has often been associated with disease outbreaks. In high-risk buildings such as hospitals and educational settings, the users in contact with such samples may come into contact with LP in the absence of adequate control measures [46]. Therefore, LP is inactivated by heat treatment to prevent inadvertent laboratory contamination leading to infection with airborne agents and so that it is safe for use in a biosafety level 1 (BSL1) facility. The main objective in this section is to modify bacterial cells in such a way that they are inactivated: in other words, that they lose the potential to multiply.

To inactivate the resuspended LP - a beaker containing water is placed on a hot plate for an hour to reach a pre-set temperature. Temperature and exposure time are the important variables for inactivating the bacteria [48]. In this study, four temperatures (60°C, 70°C, 80°C, and 90°C) and five exposure times (15, 30, 45, 60, and 90 mins) have been used [49]. After inactivation, the tubes with LP samples were kept in the fridge at 4°C. The heat-inactivated LP bacteria samples were plated on the BCYE plate to ensure that LP is inactivated. The absence of the colonies in the plate after incubation confirms that LP is inactivated.

The samples treated at 60°C for all exposure times showed LP growth (orange) indicating that LP is not inactivated and hence, can culture. The samples treated with 70°C and above showed no LP growth (green) for all exposure times because the heat killed the bacteria. Before LP was used for the experiment, the heat-treated samples (70°C for 30 mins) were then tested with the same dry spot test kit used earlier. The samples showed positive agglutination in the test section of the kit, thereby, the bacteria cultured is from LP and is ready to use.

Table 2.1: Inactivation of *L. pneumophila* sg1 bacteria samples at different temperatures for different durations

Temperature (°C)	**Time (mins)**				
	15	**30**	**45**	**60**	**90**
60					
70					
80					
90					

Not inactivated **Inactivated**

PART 3: Functionalization of latex beads with antibodies

3.1. Introduction

The development of latex agglutination assays requires antibodies that can be functionally adsorbed to beads. The protocol followed for the adsorption of immunoglobulins to the bead is as per the protocol provided by Bang Laboratories [50]. The beads used in this study were carboxyl modified latex (CML) beads. These beads are conjugated with Ab via covalent functionalization. Ab, used for this project, reacts with *L. pneumophila* serogroup 1 – 12 [51]. The main objective in this chapter is to determine the ratio of beads to Ab for the optimal performance of LAT.

3.1.1. Latex beads and antibody: Background

Latex is produced by the polymerization of styrene. The synthesis of styrene and other polymer resins has been traced back to 1839 [52] because of the need for synthetic rubber during World War II [53]. Latex beads are prepared by using emulsion polymerization. Polymerized particles are of two types: homopolymers and copolymers. Homopolymers consist of repeated long chains of the same monomer, whereas copolymers consist of more than one type of monomer. Polystyrene is an example of the homo-polymerization of styrene [54].

The antibody, also known as immunoglobulin, is secreted by B-cells in the body in defense of foreign Ag or invading pathogen [24]. The highly specific biorecognition property of antibodies with Ag has made antibodies one of the most indispensable molecules for broad application in the detection of bacterial pathogens. The structure of an antibody is Y-shaped molecules composed of two heavy chains and two light chains in which each tip of the "Y" structure contains a paratope (a structure analogous to a lock) that is specific to one particular epitope (similarly analogous to a

key) on an Ag allowing the antibody and Ag to specifically bind together. The antibody's ability to recognize and bind with high affinity to specific antigenic epitopes is exploited for qualitative and quantitative measurement of the Ags [22].

The antibodies can be classified into two primary types based on how they are created from lymphocytes: monoclonal and polyclonal. Polyclonal antibodies are a heterogeneous mixture of immunoglobulins produced by different B cell clones in the body that can recognize and bind to many different epitopes of a single Ag [55]. Antibodies used for research and diagnostic purposes are often obtained by injecting a lab animal such as a rabbit or goat with a specific immunogen. Within a few weeks, the animal's immune system will produce high levels of antibodies specific to the antigen. These antibodies can be harvested in an antiserum, which is the whole serum collected from an animal following exposure to an antigen. Since most antigens are complex structures with multiple epitopes, they result in the production of multiple antibodies. Antiserum drawn from the animal will thus contain antibodies from multiple clones of B cells, with each B cell responding to a specific epitope on the antigen. The animal is given a secondary and even a tertiary immunization to produce higher titers of antibodies against the Ag [55]. The polyclonal antibodies are then obtained from the serum or purified to obtain a solution that is free from other serum proteins [55]. Monoclonal antibodies are generated by identical B cells which are clones from a single parent cell, thereby, having monovalent affinity and only recognize the same epitope of an Ag [55]. Production of monoclonal antibodies begins with an injection of the desired Ag into an animal. Once the animal develops an immune response, the B-lymphocytes are isolated from the animal and fused with a myeloma cell line, creating a continuously growing B-cell myeloma hybridoma producing antibodies [55].

Today, most antibodies are referred to as being affinity-purified because the affinity-purified antibodies exhibit the specificity and sensitivity that can be obtained from serum [56]. Antibody purification involves selective enrichment or specific isolation of antibodies from serum (polyclonal antibodies) or cell culture supernatant of a cell line (monoclonal antibodies). In affinity chromatography (also called affinity purification), a particular ligand (Ag) is chemically immobilized or coupled to a solid support so that when a complex mixture (containing antibodies) is passed over the column, those molecules having a specific binding affinity to the ligand become bound [57]. After other sample components are washed away, the bound molecule is stripped from the support, resulting in its purification from the original sample. Ag-specific affinity purification purifies only those antibodies in a sample that binds to particular Ag molecules through their specific Ag-binding domains [58]. This purifies all antibodies that bind the Ag, resulting in the purest antibodies with the least amount of cross-reactivity.

3.1.2. Binding of antibody to latex beads

Detergents can decrease or inhibit the binding of proteins to polymeric surfaces [59]. Since latex is manufactured by polymerization processes, removal of the emulsifier is a necessity to obtain a well-characterized surface for protein binding. Washing the beads in a buffer that does not contain phosphate or acetyl (as they can reduce the reactivity of carbodiimides) can remove emulsifiers and extraneous reactants from latex surfaces [59]. 2-(N-morpholino) Ethane Sulfonic acid (MES) buffers are commonly used as activation buffers for carboxyl-modified microspheres [50]. Washing involves diluting a suspension of beads in an appropriate buffer such as MES buffer, which is centrifuged to sediment the beads. The buffer is removed after sedimentation of beads and a fresh buffer is added. Since the pH at which each reaction such as activation of beads, binding

of the antibody to beads, and blocking non-specific sites are important, recommendations provided within the coupling protocols [50] are followed.

3.1.2.1. Adsorption

Adsorption is the simplest technique to coat a ligand on beads. A suspension of beads is made by dissolving the beads in a buffer solution and a suitable ligand is added to this solution to react for times ranging from a few minutes to more than a day.

The diameter of a bead determines the total surface area available for adsorbing ligands [59]. The amount of any protein-ligand adsorbed is proportional to the diameter of the beads. The actual amount of protein adsorbed, however, is not conclusively known. Bangs calculated that approximately 2.5 mg of immunoglobulin will bind per square meter of the surface area [60]. Very rarely should the entire surface of the bead be coated with the ligand. Because of a prozone effect (the phenomenon exhibited by some reactions in which agglutination fails to occur because of either an antibody or Ag excess; a zone outside of optimal proportions for reaction), maximum sensitivity of a latex assay requires less than saturation coverage [60].

Latex is generally hydrophobic and negatively charged. Adsorption of proteins to beads is understood. The current theory is that the mechanism for the adsorption is based primarily on hydrophobic (Van der Waals) attractions between the hydrophobic portions of the adsorbed ligands and the polymeric surface of latex [50]. At this point, the latex coating process may be complete and ready for use in latex agglutination assays, or additional preparation such as blocking unreacted sites can be accomplished.

Adsorption initially increases rapidly, then plateaus following a typical bimodal adsorption isotherm [61]. In the bimodal isotherm, with the addition of small amounts of protein, a protein

monolayer forms, latex surface charges are neutralized, and beads tend to flocculate spontaneously.

3.1.2.2. Covalent Coupling

Coupling is either a direct reaction between protein and bead or through an active ester intermediate formed with the bead's carboxylate groups. Covalent bonding provides greater stability for the protein-bead binding over time and provides a specific orientation of the protein on the bead [59]. Therefore, covalent coupling of proteins to beads can be used if simple adsorption is ineffective or the treated beads do not function well in the assay.

There are many covalent coupling methods available for attaching proteins to beads. One such method is by using CML, where beads are modified to have carboxylic acid groups on the surface [50]. The primary amine of the antibodies is permanently attached to the carboxyl-modified surface of the bead to form a peptide bond. This characteristic of CML beads is a great advantage for the development of LAT as proteins in an antibody are coupled directly with CML beads which are faster and easier. The superactive layer (carboxyl groups) of CML beads is a three-dimensional layer that increases the colloid stability of the beads and provides a 'soft landing' for the antibodies while binding to the bead's surface. Hence, there is less distortion of the antibody than if it were physically adsorbed to a rigid surface [62] Carbodiimides are also used as coupling agents which react with free amine groups and carboxylate groups of protein and bead, respectively. For this surface, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-Hydroxysuccinimide (NHS) are two commonly used carbodiimides for the activation [50].

In the EDC/NHS activation chemistry used for covalent bonding, EDC is used to activate the carboxyl group on the surface of the bead to create a crosslinker. The resulting intermediate can

bind to primary amines on the antibody but is unstable and susceptible to hydrolysis. Therefore, NHS is added with EDC to create a more stable anime-reactive intermediate, which will bind to the primary amines on the antibody. In this way, fewer antibodies are needed to maximize sensitivity, reducing the overall cost of the assay. Moreover, the covalent conjugates offer increased stability in difficult sample matrices and harsh buffering conditions.

3.1.3. The ratio of latex beads and antibody

The amount of protein bound to the beads was determined by the method suggested by Bang Laboratories [50]. Most adsorption applications start with a monolayer of protein bound to the microspheres. To ensure the correct spatial orientation and decrease the likelihood of nonspecific binding, adding protein in a 3 – 10X (where X is the estimated amount of antibody according to Eq. (1)) excess of the calculated monolayer is generally recommended. However, some applications such a LATs, seem to work best with less than a monolayer of coverage. The monolayer amount can be derived from the following equation:

$$S = (6 / \rho D) \times (C) \quad (1)$$

where S = amount of representative protein needed to achieve surface saturation (mg protein/g of microspheres); C = capacity of microsphere surface for given protein (mg protein/ m^2 of polymer surface); 6 / ρD = surface area / mass (m^2/g) for microspheres of a given diameter (ρ= density of microspheres, which for polystyrene is 1.05 g/cm^3) and D = diameter of microspheres, in microns.

Since LATs do not require a monolayer of protein, 0.1X, 1X, 2X, and 3X of the calculated amount of antibody will be used to find the optimal ratio of beads to the antibody for the highest reactivity of LAT. 2 - 3X excess of antibody, which is more than the monolayer, are used as there are possibilities of losing some of the antibodies during the functionalization of beads with antibody.

From the results of LAT (based on their quantitative outputs), a saturation point will be reached after which the performance of LAT remains the same (due to unavailability of antibody binding sites for the Ags) or starts to decrease (due to the prozone effect). The amount of beads and antibody used at that saturation point will determine the optimal ratio of beads and Ab for LAT. However, if this saturation point is not seen within 3X of antibody, then 4X, 5X and 10X excess of antibody will be used in order to reach the saturation of the LAT performance. If the saturation point is still not seen within 10X excess of antibody, some modifications should be made for the functionalization protocol such as increasing EDC and NHS concentrations or the size of the beads because using an excess amount of antibody (beyond 10X) can increase the overall cost of the assay.

3.1.4. Blocking agents

Binding ligands to polymer surfaces for latex agglutination assays often require blocking of unreacted sites with molecules that do not affect the assay. Blocking agents are often coated on the beads (via adsorption) after the antibody coupling reaction. These compounds are used to minimize nonspecific interactions between the coated bead and non-target molecules in the sample. Blockers such as bovine serum albumin (BSA) are often used in varying amounts, standard concentrations being from 0.05% to 0.1% (w/v) [50]. Non-ionic surfactants are often used in combination with another blocker with a common ratio of 1% blocker: 0.05% surfactant. Low concentrations of non-ionic surfactants such as Tween 20 can reduce non-specific hydrophobic interactions between analyte and bead surface and prevent analyte from binding to container walls [50].

3.2. Materials

CML beads (4% w/v, 0.3 µm in diameter), Tween20, EDC, PBS, BSA, and MES were purchased from Sigma-Aldrich. According to theory, polyclonal antibodies are ideal reagents in agglutination reactions due to their ability to recognize different epitopes of a target molecule, leading to better detection [55]. Therefore, polyclonal antibodies for LP (ab20943), purchased from Abcam, were used for this project. NHS was purchased from Thermo Fisher Scientific. The blocking agent to block the unreactive sites of the latex particles is prepared with 1% BSA and 0.05% Tween 20 in PBS. The washing buffer used for the experiment is 0.1M PBS solution (pH 7.2). The storage buffer was prepared with 0.1M PBS buffer (pH 7.2) with 0.1% BSA.

3.3. Methodology

Beads are cleaned before Ab coating by centrifugation washing. A 15 mL test tube containing 2mg of beads is mixed with 0.025M MES buffer, bringing the total volume to 1 ml. The bead suspension was mixed several times manually by inversion before washing. The test tube containing the bead suspension is centrifuged for 5 to 15 mins at 13,000 rpm in a microcentrifuge. During this time, 0.1M NHS (11.5mg) and 0.4M EDC (75mg) are weighed and transferred to a 1.5 mL vial, after which 1 mL of MES buffer is added to the same vial containing EDC/NHS. This solution is allowed to incubate for 15 minutes with continuous mixing using the vortex. The supernatant of the bead suspension is aspirated from the test tube without disturbing the latex pellet (formed by the beads). The EDC/NHS solution prepared in MES buffer is added to the latex pellet and the beads are resuspended by vortex mixing. Washing with MES buffer is performed twice before the beads are suspended at 1mg/mL concentration. Several aliquots of 100 µL (100 µg) bead suspension are prepared for different concentrations of the Ab.

The monolayer amount of Ab required for the adsorption on 1g of microspheres with a mean diameter of 0.3 µm is carried as follows:

C ~ 2.5 mg/m^2, so from Eq. (1):

$$S = (6 / 1.05\ g/cm^3 \bullet 0.3\mu m)\ x(2.5\ mg/m^2) = \sim 50\ mg\ of\ Ab$$

The amount of beads used for the experiment is 100 µg. Therefore, the monolayer amount of Ab for the functionalization of 100 µg of beads will be 5 µg, and the amounts of Ab that will be used to find the optimal amount of Ab are 0.5 µg (0.1X), 5 µg (X), 10 µg (2X), 15 µg (3X), 20 µg (4X), 25 µg (5X) and 50 µg (10X). Each aliquot of beads is treated with different Ab amounts (calculated above). Latex–protein mixture is incubated with gentle mixing at room temperature for 2 - 4 hours. The resulted protein-labeled beads are centrifuged to separate from unbound protein and are resuspended in the blocking agent. This washing step is repeated two times and the final latex is suspended in storage buffer giving a final latex concentration of 0.2 mg/mL and they were stored at 4 ºC. The preparation of the blocking agent and storage buffer is mentioned in *3.2. Material* section.

PART 4: Quantitative analysis of latex agglutination test

4.1. Introduction

In this chapter, a latex agglutination test is performed to discuss the application of the prepared Ab-coated beads for the detection of heat-inactivated LP in different concentrations. The beads used for LAT are carboxyl modified latex (CML) beads that consist of carboxyl groups present on the bead surface. These beads are conjugated with Ab via covalent functionalization. Since Ab purchased from Abcam reacts with *L. pneumophila* serogroup 1 – 12 [51], they can detect heat-inactivated LP suspension prepared from the stock. In this section, the ratio of beads to Ab (in weight) required for an optimal latex agglutination reaction is also investigated.

4.2. Methodology

4.2.1. LAT for bacteria detection and quantification

An ideal test to detect LP should be able to confirm the presence of this bacterium in the water sample. Moreover, the test should distinguish between different water-borne pathogens present in water samples such as *L. pneumophila* and *E. coli*, both thriving commonly in aqueous environments.

Agglutination reactions occur after mixing the LP suspension and Ab-coated beads for 20-30 minutes. In this project, the use of beads in LAT provides an advantage of observing the specific binding of LP with Ab on the bead's surface by forming agglutinates. Agglutinates are the large lattices formed when LP antigens on its cell wall bind to Abs from different beads, thereby, bridging the beads. These agglutinates can be observed easily using a microscope due to the large size of the beads. Therefore, the presence of any agglutinates after 30 minutes of continuous

mixing (on a roller) of the sample with Ab-coated beads can be interpreted as a positive result (i.e., the sample contains LP). A negative result will show no agglutinates and the milky appearance of the beads remains unchanged throughout the test.

The goal of this project was not only to detect LP but also to quantify the LP concentration by determining the degree of agglutination. The degree of agglutination in each sample can be determined by producing a quantitative output that can be compared between samples with different concentrations of LP. If an image of the final mixture (LP and Ab-coated beads after 30 minutes) under the microscope is captured, one can easily identify the agglutinated bead entities in an agglutinate and non-agglutinated bead entities as seen in Figure 4.1. If two or more bead entities are bridged by LP and seen together as a cluster of beads, they can be identified as agglutinated bead entities. Non-agglutinated bead entities are individual bead entities that are not connected to any other beads. The fraction of agglutinated bead entities out of the total number of bead entities in an image can be interpreted as the degree of agglutination. Samples with a higher concentration of LP will clump most of the beads, as more LP antigens are available on its cell wall for the Abs on the beads to bind when compared to samples with a lower concentration of LP. Therefore, the number of agglutinated bead entities will increase with higher concentrations of LP. The fraction of agglutinated bead entities in an image is a quantitative output that can be compared between samples and indicate the level of water contamination due to the presence of LP.

The degree of agglutination can only be determined from the image of the final mixture containing the Ab coated beads and heat-inactivated LP observed under a microscope. All the beads and LP in the sample move with random velocities due to Brownian motion and the heat produced from the light source of the microscope. A single image to find the agglutinated beads is not promising because the two-dimensional (2D) image can be captured when unbound bead entities and LP

come together, for instance on top of each other, while moving in a three-dimensional (3D) volume of sample. Hence, the bead entities should be confirmed that they are part of agglutinates before categorizing them as agglutinated ones. If the beads can move freely in random velocities, the bead entities in an agglutinate should move in correlation as they are linked to each other due to specific LP Ag- Ab binding. Therefore, a set of images (10 images in this project) of the sample should be taken in which every image is taken after 5 ± 1 seconds. In this way, the agglutinates identified in the first image can be observed over time to check if the bead entities in the agglutinate are truly agglutinated. All the bead entities that remain in the agglutinate till the last image of the set are categorized as the agglutinated bead entities and the rest of the bead entities as the non-agglutinated ones which can therefore determine the degree of agglutination.

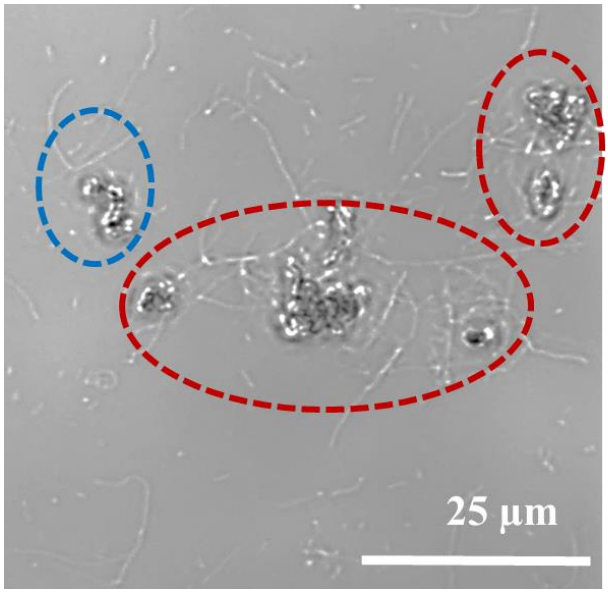

Figure 4.1: Agglutinated and non-agglutinated bead entities identified by visual observation where the agglutinated beads are circled in red and non-agglutinated beads in blue.

An interference experiment should be performed to test the specificity of this new quantitative analysis technique. Therefore, three kinds of samples are prepared in PBS for the LAT: (1) Positive control, containing heat-inactivated LP and Ab-coated beads; (2) Blank control: containing LP and beads with no coating of Ab; and (3) Negative control, containing EC and Ab-coated beads, where both the bacteria should have the same concentration and the Ab-coated beads should contain the same amount of beads for all the samples. The degree of agglutination can be compared among the three kinds of samples to confirm that this quantitative analysis of LAT assay is specific to LP.

The sensitivity of a test is the minimum amount of analyte (LP in this case) that can be detected. It is also defined as the limit of detection (LOD). The sensitivity of this new technique is tested by diluting LP for different concentrations. The heat-inactivated LP and EC vial from the culture stock are measured using a spectrophotometer by measuring the optical density at 600 nm (OD_{600}). OD_{600} is commonly used in spectrophotometry for estimating the concentration of bacteria or other cells in a liquid as the 600 nm wavelength does little damage to the cells. The vials of LP and EC contain 10^8 bacteria cells/mL from which three solutions are prepared through 10-fold serial dilutions with concentrations ranging from 10^7 to 10^5 cells/mL. The different concentrations of LP are treated with Ab-coated beads (positive controls) and with beads that have no coating of Ab (blank controls). The number of beads for the positive and blank control samples should be the same. The concentration of LP in the positive control with minimal difference in the degree of agglutination compared to that of the blank control is the LOD. Any concentration of the LP bacteria below LOD will not be enough to agglutinate the beads and therefore, the degree of agglutination will not be statistically different from its control.

Reproducibility, which brings high reliability for this technique, is the ability to provide identical responses for multiple experiments performed under similar conditions. Therefore, the experiment will be repeated three times using the same protocol for detecting and quantifying LP.

4.2.2. Microchannel

The final mixture containing the Ab coated beads and heat-inactivated LP can be pipetted onto a glass slide as a drop which allows the observation of the random movement of the beads and LP. The Ab-coated beads and LP move in random velocities which helps us to investigate the bridging between beads by bacteria. However, this advantage of observing movement can also be a disadvantage. It may be more difficult to take images as the beads or LP bacteria may swim vertically in the water and therefore move in and out of focus due to the large height of the drop which can differ every time the sample is pipetted onto the glass slide. Moreover, the heat from the light source causes water to evaporate more quickly. Therefore, a microchannel is designed to avoid evaporation and control the dimensions of the sample and other imaging conditions.

The microchannel consisted of a 3M double-sided adhesive tape which is patterned and cut into 20mm × 20mm piece with a slit of 4mm using a Cricut cutter as seen in Figure 4.2a. This patterned tape is attached to a diced glass slide with the same dimensions which is then attached over another glass slide (Figure 4.1b). The slit in the tape makes the microchannel. The height of this microchannel is approximately 40µm which is the thickness of the adhesive tape as seen in Figure 4.1c. From the mentioned dimensions, the volume of the microchannel is found to be 3.2µL. Therefore, the volume of any sample pipetted into the microchannel is 3µL. The flow of liquid through the microchannel is primarily due to wetting and capillary forces. The microchannel reduces evaporation, and the small height of the microchannel makes the beads and LP difficult to

swim vertically. The sample entrapped in the microchannel can, thereby, be used to study the movement of agglutinates for the quantitative analysis. The glass slides are washed and rinsed in ethanol before use to avoid any contaminants.

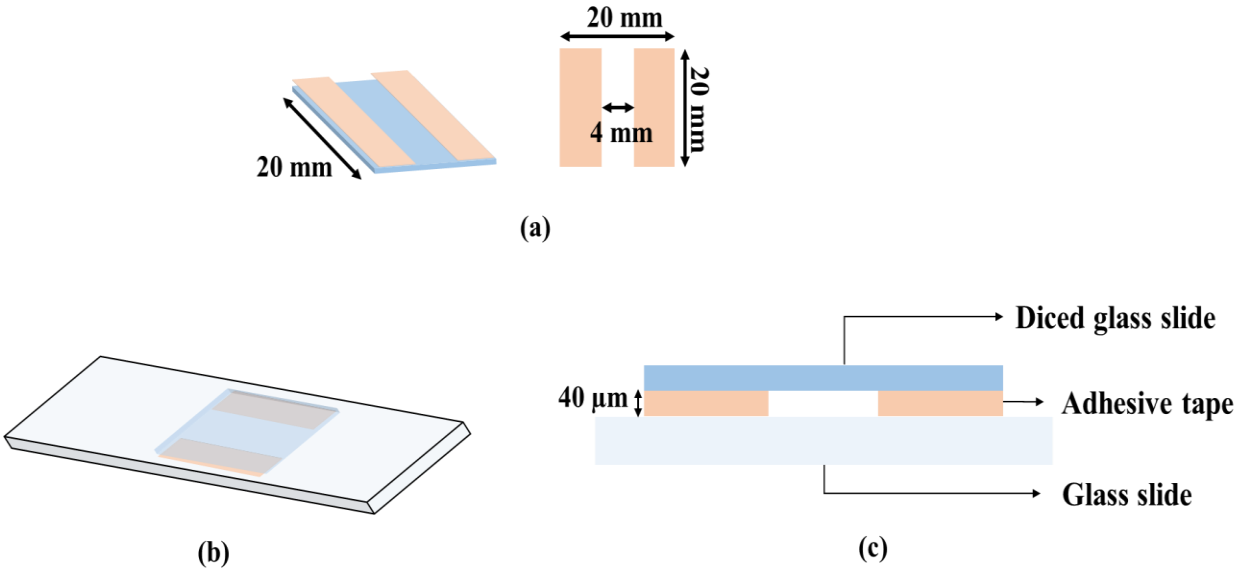

Figure 4.2: (a) Dimensions of the patterned adhesive tape which is placed onto the diced glass slide; (b) microchannel consisting of a sandwich of a microscope slide, patterned adhesive tape, and a thin coverslip; (c) the side view of the microchannel.

4.2.2 Image analysis using ImageJ

The agglutinated and non-agglutinated bead entities can be identified from the set of images by monitoring their movement. However, it is hard to track the movement of all the bead entities from the mesh of LP and time-consuming by manually observing the images alone. Hence, the images need to be analyzed in such a way that only the bead entities are visible in an image and find the positions (X and Y centroid coordinates) of the beads to track their movement using post-processing software such as ImageJ. So, once a set of images from a sample are captured at a

random fixed position, these steps of image analysis can be followed using ImageJ: (1) the images are turned to grayscale, (2) these grayscale images are thresholded to binary images where only the bead entities in the images are visible, and (3) analyze the bead such that the count, as well as the X and Y centroid positions of each bead entities visible in each image, can be found. This way, the average distance of any agglutinate can be found over time to confirm if the bead entities are agglutinated or not. Hypothetically, if the bead entities in an agglutinate move in correlation, their average distance should not change as they move over time. As a control, if a set of images from a sample containing only Ab-coated beads (and no LP) are analyzed similarly, the average distance of the bead entities will fluctuate significantly as these beads are not bridged due to the absence of LP and move in different directions over time.

After the bead entities in the agglutinate, present in all the images of the set, are confirmed to be agglutinated, they will be counted as the agglutinated bead entities. As the final step, the total number of beads and agglutinated beads present in the last image of the set is found to determine the degree of agglutination in that sample. Three sets of images are taken when any sample is observed under the microscope where each set is taken at different locations of the microchannel. The steps are summarized in the flowchart seen in Figure 4.3.

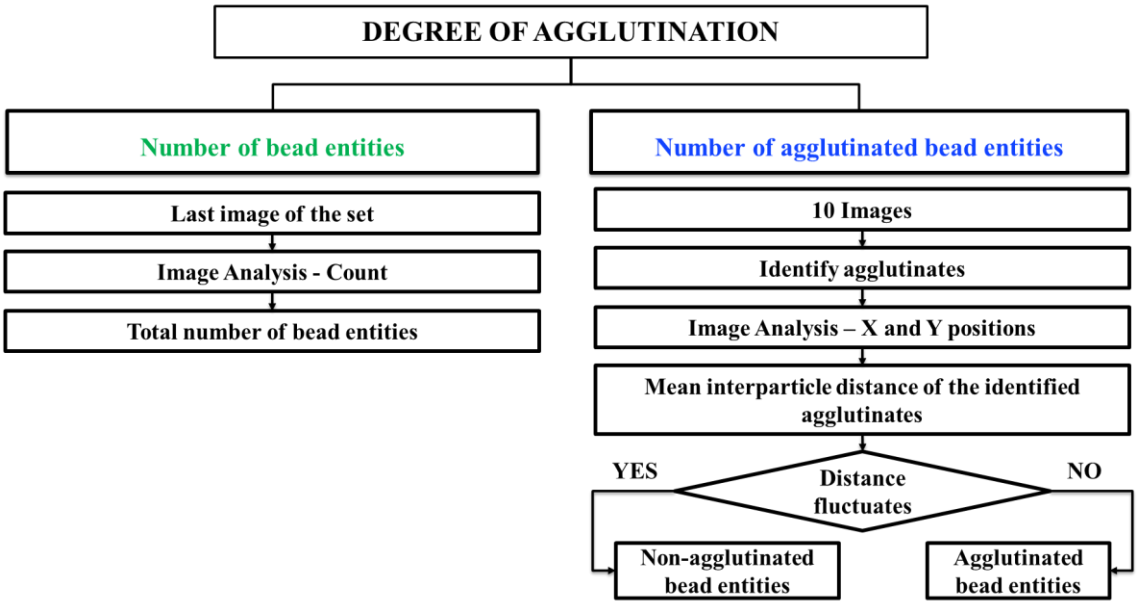

Figure 4.3: Flowchart summarizing the steps to find the number of agglutinated bead entities out of total bead entities to determine the degree of agglutination.

4.2.3. Optimisation of antibodies for functionalization of the latex beads

The degree of agglutination will increase with the amount of Ab coated on the beads when plotted because more binding sites of Abs will be available for LP, thereby, agglutinating more beads. However, there will be a point where the degree of agglutination saturates and remains at that same level because there will be no more available binding sites on the bead surface for the Abs and thereby, removing the excess unbound Abs during centrifugation. That amount of Ab where the degree of agglutination starts to saturate is the optimal amount of the Ab required for the functionalization of the beads.

To find the optimal amount of Ab required for the functionalization, 100 µg of beads are treated with different concentrations of Ab (0.1X, 1X, 2X, 3X, 4X, 5X, and 10X). The concentrations for Ab were selected based on a method by Bang Laboratories which is further explained in *Part 3*.

50 µL from each prepared Ab-coated bead are mixed with 100 µL of heat-inactivated LP. As a control, the same volume of heat-treated LP is added to 50 µL of a solution containing only 100 µg of beads (with no Ab). All the solutions are incubated for 30 mins. The solutions were then washed twice by centrifugation to remove any unbound proteins, after which they were injected into the microchannel and then observed under the microscope.

4.3. Results and Discussion

4.3.1. Visualization of agglutinates

Visualizing agglutinates using a microscope was performed to count the fraction of agglutinated beads in an image of the sample. The microscope used for this project is a Zeiss Inverted Fluorescent microscope (Inverted microscope Axio Observer 7; Item no: 491917-0001-000) with 20X magnification which captures images of the sample entrapped in the microchannel using a bright field. Conventional microscopes image a two-dimensional (2D) cross-section of a three-dimensional (3D) sample in a single snapshot. The area of the 2D cross-section window of the sample observed under the microscope calculated using ImageJ is 0.15 mm^2. Depth of field essentially provides the thickness of this cross-section. The depth of field of the sample observed under the microscope was found to be 10.8 µm using Zen Lite software connected to the microscope. Therefore, the volume of each image of a sample observed under the microscope is 1.6×10^{-3} µL. With the glass slide at a fixed position, ten images of the sample entrapped in the microchannel are captured at a particular focal plane (within the depth of field) by the microscope.

The agglutinates are identified from the first image of a set from the positive control and converted to grayscale, as seen in Figures 4.4a, by converting the red, green, and blue intensity values of each pixel, in a range of possible values from 0 to 255. Zero is taken to be black and 255 is taken to be

white. Values in between make up the different shades of gray. The grayscale images are turned into binary images to outline the beads in the image from the background using ImageJ. The threshold tool in ImageJ segments the grayscale image into binary images (Figure 4.4b) in which the beads are seen in white and the background in black by manually setting the lower and upper mean gray values of beads (which will be explained in the next section). ImageJ scans these binary images with only beads, seen in white, and numbers the white objects by finding their edges, giving us the total number of beads in an image of a sample (Figure 4.4c). The white objects in the binary images are confirmed that they are beads and not LP or EC by two parameters: 1) morphology and 2) mean gray values.

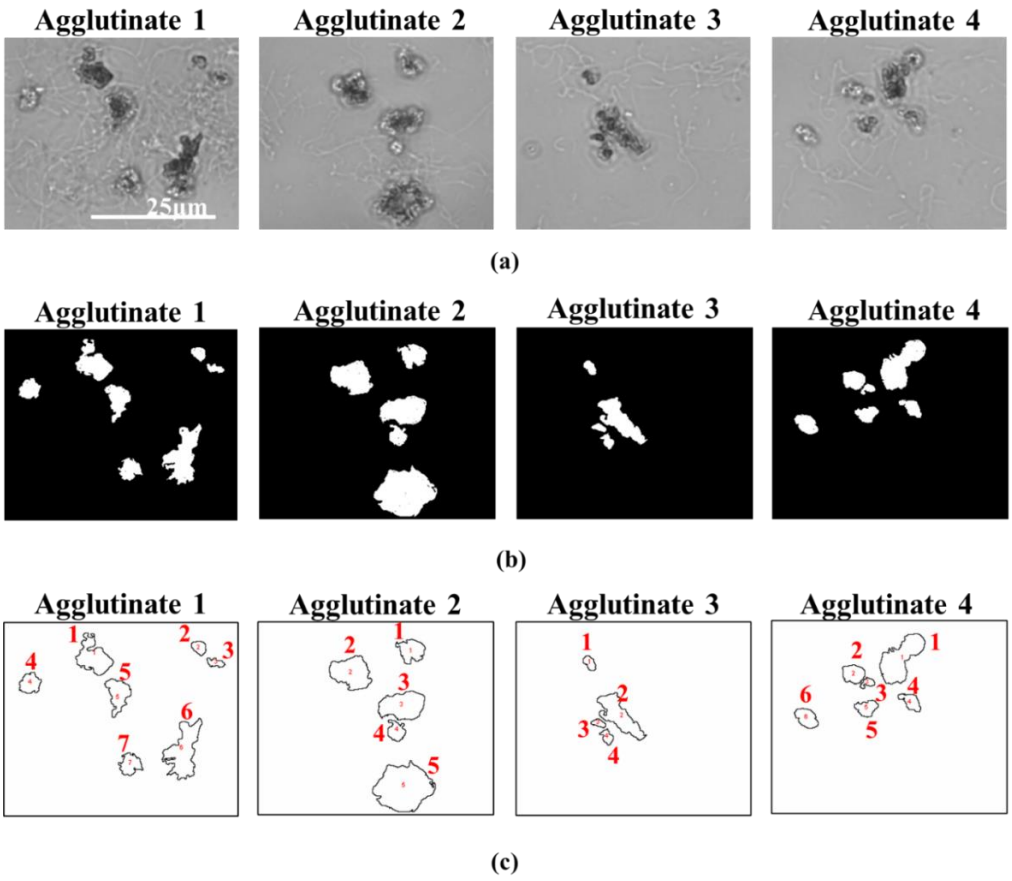

Figure 4.4: (a) Grayscale image of the identified agglutinates from the first image of the set of the sample containing Ab-coated beads and heat-inactivated LP; (b) binary image of the agglutinates where the beads are seen in white; (c) the number of the beads in the agglutinate.

The white objects in the binary image need to be confirmed that they are beads as the focus is on the correlated movement of the beads. One of the parameters used for the differentiation of beads from bacteria (LP and EC) is morphology. Since the beads are manufactured as emulsified micelles with a uniform diameter, the shape of the beads after functionalizing with Ab is nearly spherical and irregular in many cases as seen in Figure 4.5a. Compared to beads, LP and EC bacteria are thin, rod-shaped bacteria as seen in Figures 4.5 b and c. Therefore, the white objects can be differentiated as the beads from bacteria based on their morphology. However, the LP bacteria cannot be distinguished from EC due to the similar morphology of bacteria. As seen in Figures 4.5 b and c, some LP structures look similar to EC.

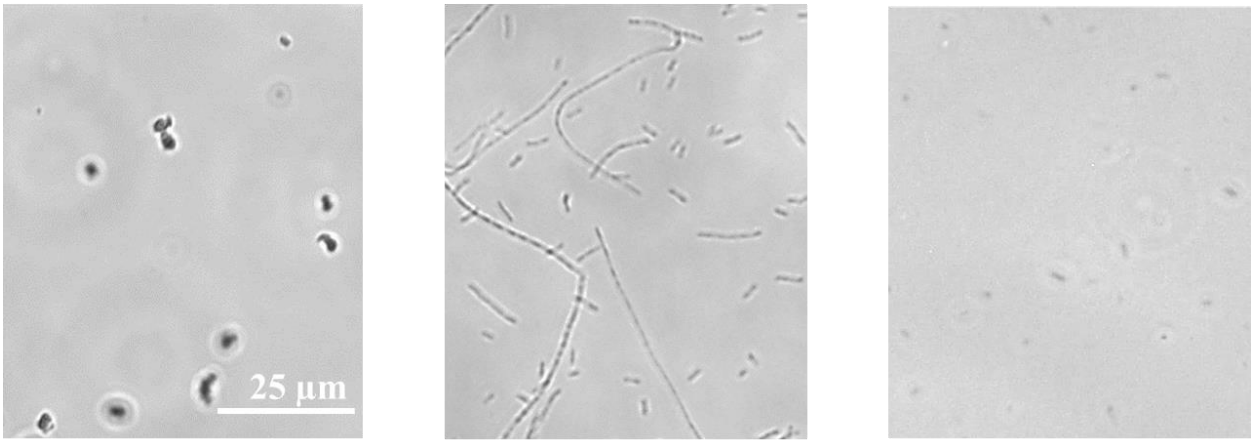

Figure 4.5: (a) Sample containing only Ab-coated beads; (b) sample containing only the heat-inactivated LP and (c) sample containing only EC.

The mean gray value is the average gray value of all the pixels in the selected region. The gray value indicates the brightness of a pixel. Zeiss Inverted Fluorescent microscope used in this project is an interference microscope that enhances the contrast in unstained samples. As seen in Figure 4.6, the unpolarized light, emitted by the light source of the microscope, is polarized at 45° which

is then split into two beams of light by the first prism. These paired beams are polarised at 90° to each other, the sampling and reference beams of light, travel close to one another. These two beams are then focused by the condenser for passage through the sample after which the pair will be recombined by a second prism. The second prism polarizes the pair of beams at 135° leading to the interference of the beams. If the pair of beams passed through the same material in the sample, they would not interfere with each other when recombining and therefore appear gray [63]. However, at an edge of a structure (bead or bacteria) in the sample, one beam of the pair passes through different material from its partner. The two beams will experience different optical lengths because the two materials that the beams passed through have different refractive indexes. This causes a change in the phase of one beam relative to the other. When these beams are recombined by the second prism, they will interfere either constructively producing a bright pixel or destructively producing a dark pixel. This phase difference (or optical path difference) of the two beams is characterized as the intensity of that pixel known as the gray value. These gray values of the pixels in an image can be found using ImageJ.

The mean gray value cannot be determined in a binary image as they only have a gray value of 255 (white objects) and 0 (black background). So, the outlined white objects in the binary image give the mean gray value of those pixels in the original grayscale using ImageJ.

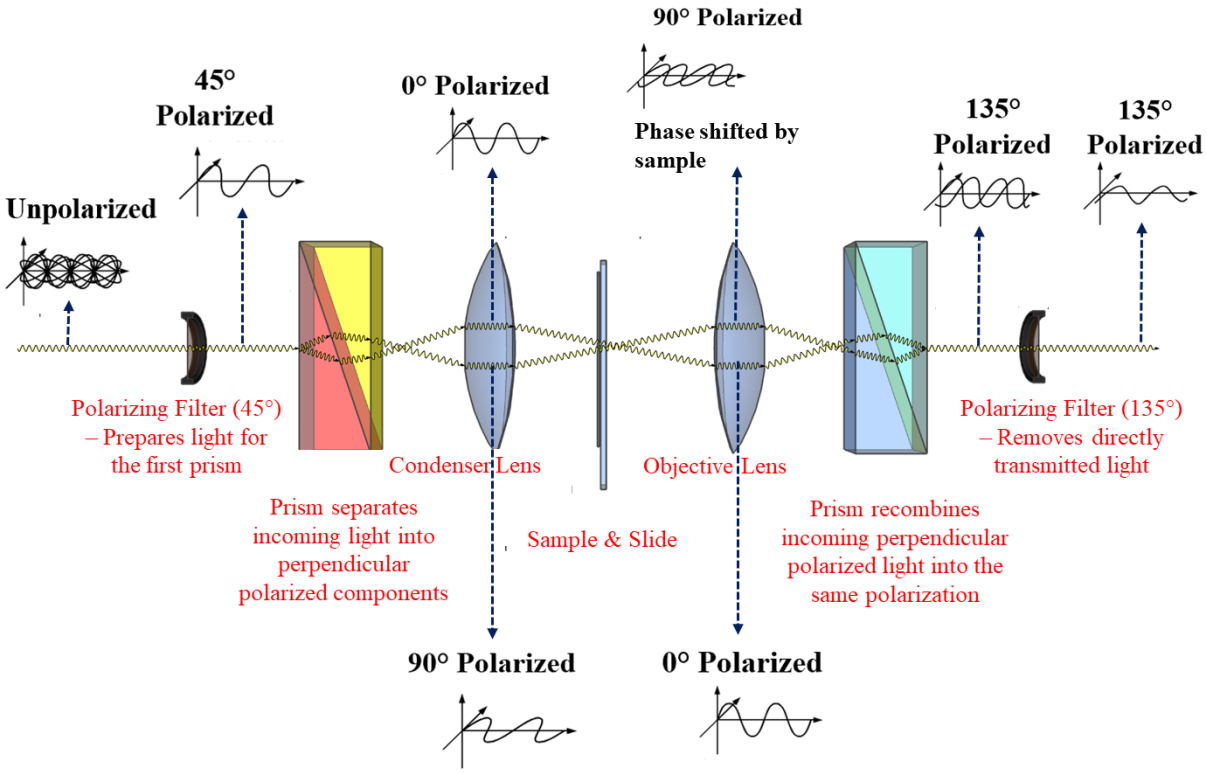

Figure 4.6: The path of light through differential interference contrast microscope adapted from [1]

The mean gray values of ten bead entities selected randomly from a solution containing only Ab-coated beads were found out using ImageJ. Similarly, ten random heat-inactivated LP and EC structures were analyzed from their suspensions and their mean gray values were found. As seen in Figure 4.7a, the mean gray values of the beads vary from 99.46 to 118.6 whereas the mean gray values of bacteria (LP and EC) come in between 150 to 190. This large discrepancy in the mean gray values between beads and bacteria is enough to distinguish the beads from the white objects. Among the two bacteria, the mean gray value of LP is slightly higher than EC as seen in Figure 4.7a due to the difference in their refractive indexes. Polystyrene (n = 1.6), from which beads are made, have a higher refractive index compared to LP (n = 1.39) [64] and EC (n = 1.38) [65]. This

means that the speed of light slows down by a factor of 1.6, 1.39, and 1.38 when passing through the bead, LP, and EC, respectively. Hence during interference, there will be a larger difference in phase (or optical path) for beads than bacteria resulting in lower mean gray values of beads compared to bacteria. To investigate whether this discrepancy in mean gray values differs when beads and bacteria are from the same solution, images from positive control samples (Ab–coated beads and heat-inactivated LP) and negative control (Ab-coated beads and EC) were analyzed. These samples were prepared three times (total number of samples = 2 × 3 = 6) and images from each sample were analyzed to check if the mean gray values are consistent between samples. As seen in Figure 4.7b, the mean gray values of beads, LP, and EC are found to be 119.4 ± 19.7, 178.13 ± 5.64, and 159.63 ± 6.77, respectively. The low error bars (standard deviation) of beads, LP, and EC make their mean gray values consistent without any overlap.

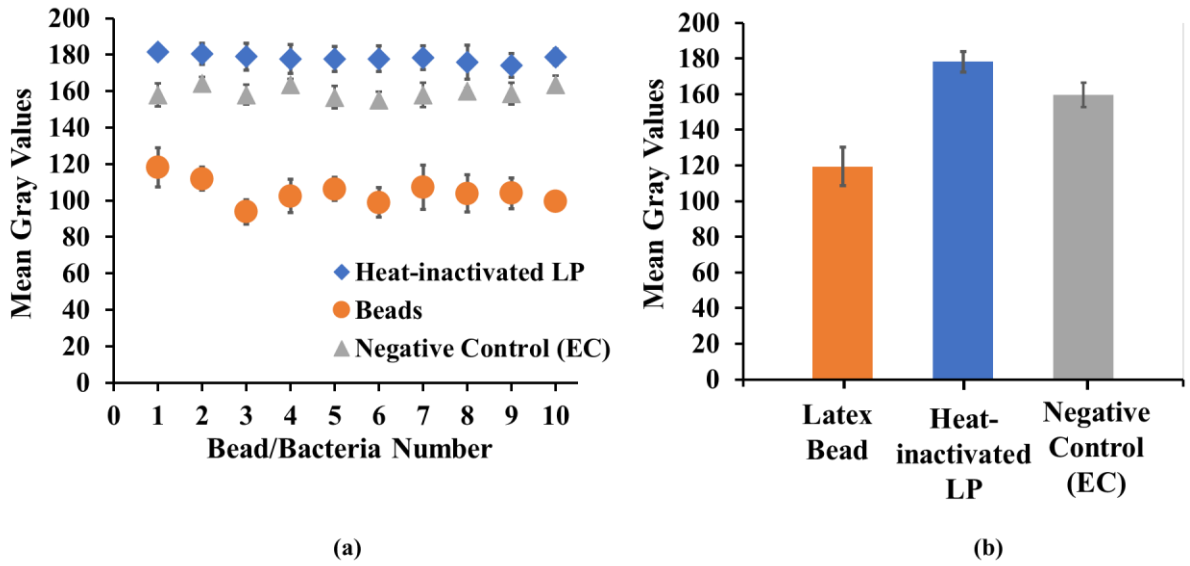

Figure 4.7: (a) Mean gray values with the standard deviation as the error bar of the objects from a sample containing only Ab-coated beads (orange), (2) sample containing only heat-inactivated LP (blue), and (3) sample containing only EC (color gray); (b) mean gray values of beads, heat-inactivated LP and EC from three sets of images of the six samples in which three positive control samples and the other three negative control samples. The mean gray value of beads, LP, and EC is 119.4, 178.13, and 159.63 respectively with a standard deviation error of 19.7, 5.64, and 6.77.

To conclude, the grayscale images are thresholded to binary images with bead entities as white objects by setting the mean gray value range of beads as the threshold set. These white objects are proved again to be the beads based on the morphology and the mean gray value when compared to that of LP and EC. Any objects that do not have a rod-shaped morphology or spherical shape with mean gray values different from the range of beads, heat-inactivated LP bacteria, and EC bacteria (such as dirt and debris contaminants) are omitted.

The X and Y centroid positions of the beads in each agglutinate are found to calculate the average distance of the agglutinate using ImageJ. The X and Y centroid of a bead entity/white object is the average of the X and Y position of all pixels in that white region. As seen in Figure 4.8a, the average distance of the four agglutinates from the positive control sample were found to be 29.0 ± 0.71μm, 39.3 ± 0.67μm, 37.2 ± 1.82μm and 46.9 ± 1.26μm. The small standard deviation is due to different orientations of the bead entities in an agglutinate as they move (Figure 4.9). But this change is minimal compared to the average distance of four clusters of bead entities from the negative control which is 126.9 ± 20.84 μm, 121.9 ± 14.62 μm, 183.6 ± 17.42 μm and 211.5 ± 25.81 μm. The minimal change in the average distance of the agglutinated bead entities from the positive control sample confirms that the beads move in correlation as they are bridged by LP. The distance for each agglutinate differs depending on the number of bead entities in the agglutinate and the size of the bead entities which ranges from 1.22 μm to 99.8 μm (perimeter). On the other hand, the average distance of the bead entities from the negative control changes significantly over time because EC is nonspecific to the Ab on the beads, thereby, no bridging between beads. Therefore, the bead entities move in different directions in the negative control. This way, the bead

entities from the identified agglutinates were confirmed to be agglutinated and counted as the agglutinated bead entities (Table 4.1).

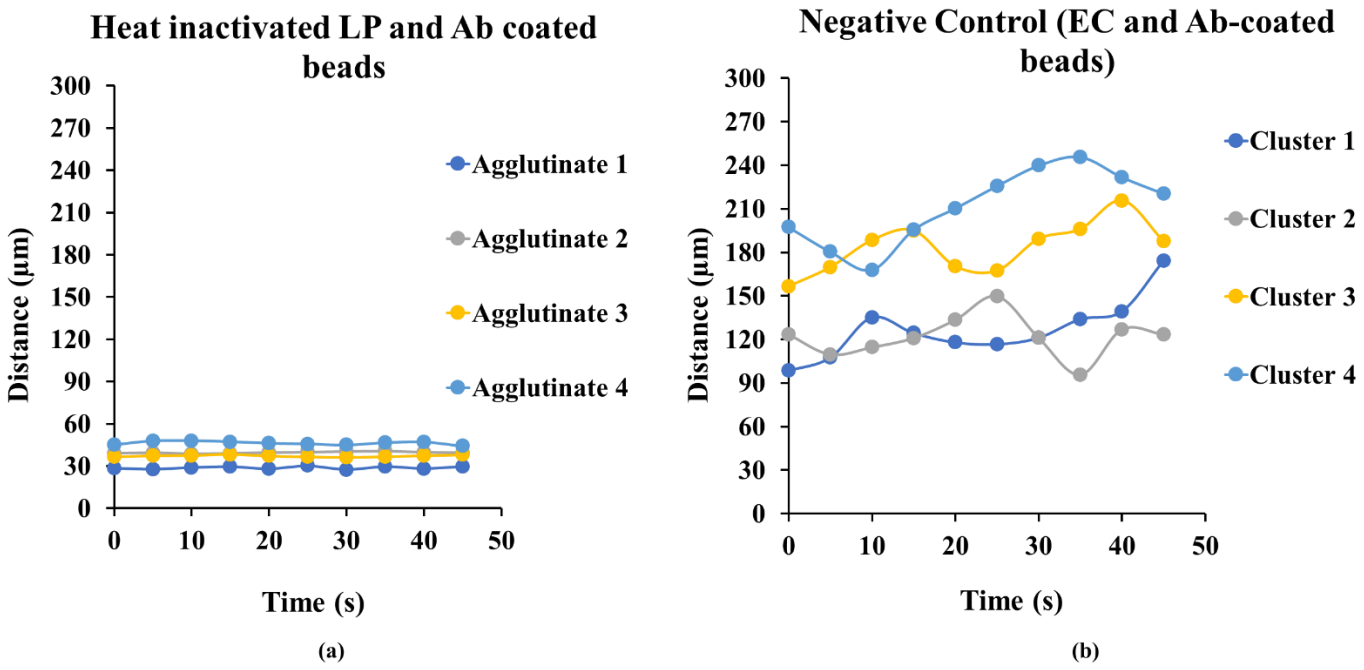

Figure 4.8: (a) The average distance of each of the four agglutinates identified from positive control sample containing heat-inactivated LP and Ab-coated beads; (b) the average distance of four clusters of beads identified from a negative control sample containing EC and Ab-coated beads)

Table 4.1: Average distance and number of agglutinated beads of the agglutinates

Agglutinate no.	Average distance (µm)	Standard deviation	No. of agglutinated beads
1	29.04	0.71	7
2	39.29	0.67	5
3	37.19	1.82	4
4	46.92	1.26	6
Total no. of agglutinated beads			**22**

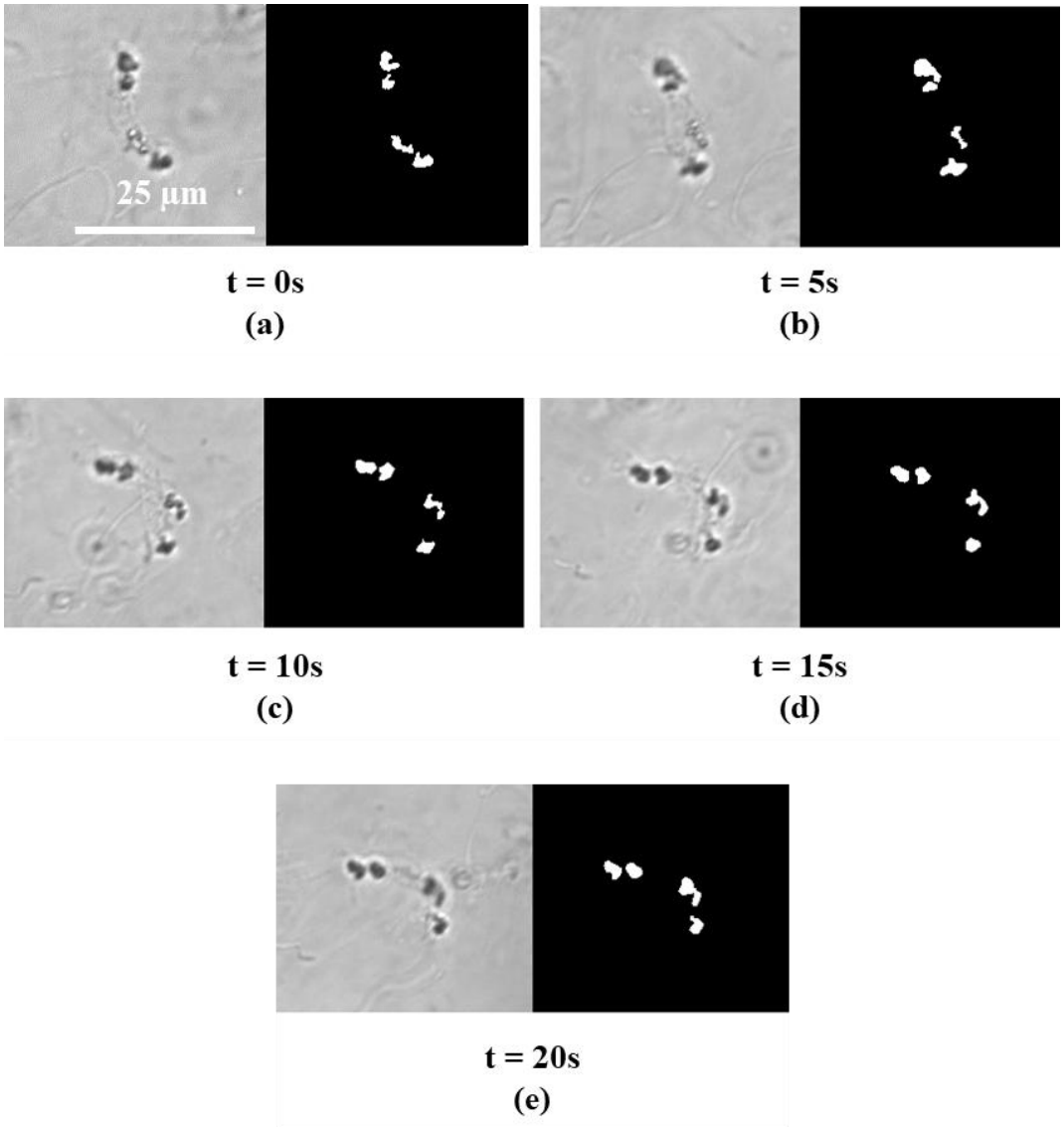

Figure 4.9 (a - e) Agglutinate found from the positive control sample containing heat-inactivated LP and Ab-coated beads in the first five images of the set changing its orientation as it moves.

4.3.2. Specific binding between LP and Ab-coated latex microbeads

The working principle of a LAT is the specific binding of the Ab functionalized over the surface of the beads with the surface Ags on the bacterial cell wall. It is hypothesized that the agglutinated beads will move in the same direction since they are cross-linked by LP. However, these agglutinated beads must be confirmed that they are bound to the LP because there is a

possibility of a case where some of the beads may move in the same direction without any LP linking between them. This can be confirmed by detecting the bound LP that connects the beads in an agglutinate during image analysis.

During the formation of binary images in ImageJ, the threshold is changed by setting the mean gray value range of both beads and LP as the threshold set such that the binary images contain LP and beads in white which can be differentiated based on their morphology and mean gray value (Figure 4.10 a and b). The distance between the bound LP and the identified agglutinated bead entity, termed as D_{Bb}, can be found using their X and Y centroid positions in ImageJ. This method can investigate if all the agglutinated bead entities identified in the previous section are always bound to the bacteria in the agglutinate.

From Figure 4.10c, outlined object labeled as 2 and 3 is a bead entity and LP structure respectively based on the shape and mean gray values of these objects. The shape of object 3 is rod-shaped compared to the nearly spherical shape of object 2. In addition to this, the mean gray value of objects 2 and 3 is 115 and 176, respectively. Since object 2 has a lower mean gray value compared to object 3, objects 2 and 3 are, thereby, confirmed to be the bead and LP respectively. The small change in the value of D_{Bb} between bead (object 2) and bacteria (object 3) over time in Figure 4.10d confirms the binding between the bead and LP. The orientation of the bacteria can change as they flow in PBS which explains the minimal change in D_{Bb}. The negative control sample was used to confirm the specific binding between LP and its Ab. As evident in Figure 4.10d, the D_{Bb} of the bead entity and EC changes over time as they are not bound to each other, thereby, confirming the specific binding between LP and Ab. A total of 336 images of the agglutinates were analyzed, of which 94.2% of the images showed the presence of LP bacteria among the identified agglutinated beads proving that the beads are only agglutinated due to the specific binding between

the bacteria and its Ab on the beads. Some of the beads did not contain enough antibodies or none during functionalization which implies the remaining 5.8%.

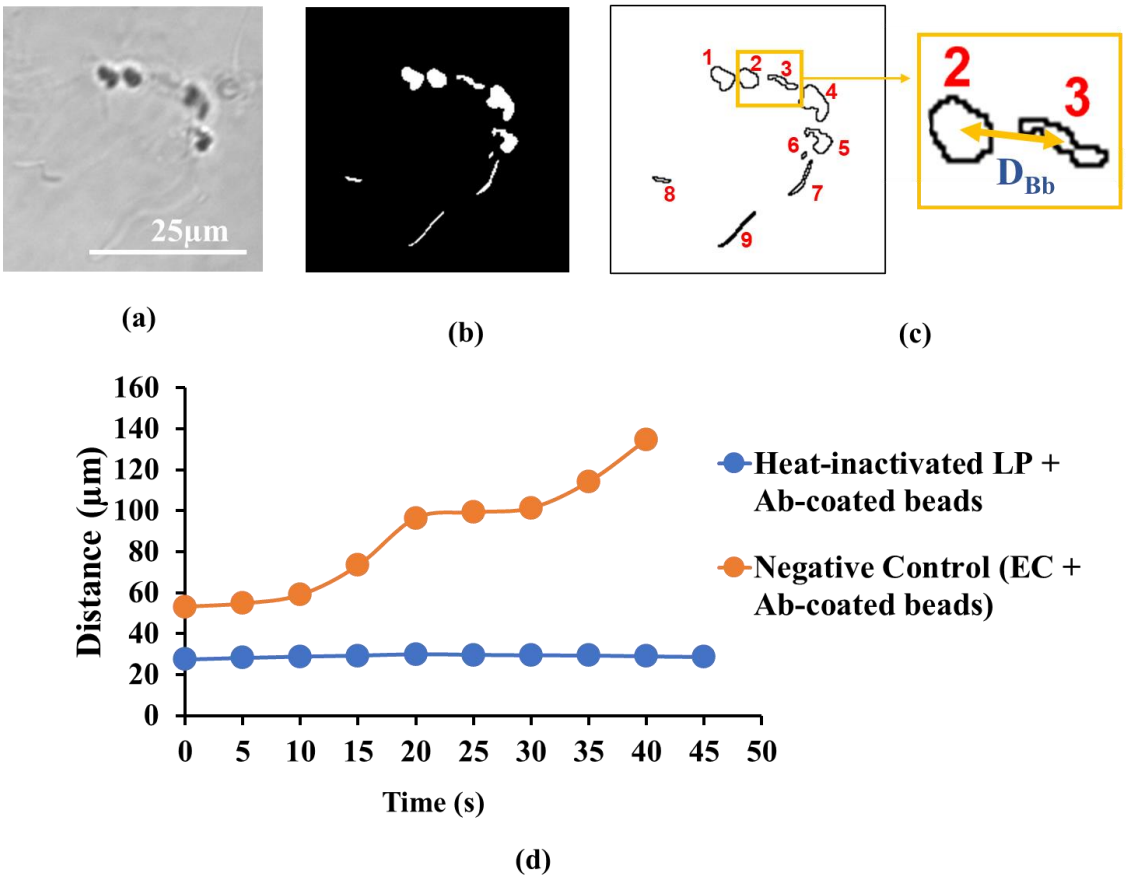

Figure 4.10: (a) Grayscale image of the agglutinate from the positive control sample containing Ab-coated beads and inactivated LP; (b) binary image of the agglutinate where the bead entities and bacteria are seen in white; (c) outline of the bead entity and LP; (d) D_{Bb}, the distance between bead entity and bacteria, in the positive control sample containing heat-inactivated LP with Ab-coated beads and negative control containing EC with Ab-coated beads.

4.3.3. Degree of agglutination

Quantitative analysis of LAT is performed by determining the ratio of all the agglutinated bead entities that appear in all the images of the set to the total number of bead entities counted from the last image of the set of a sample. From the image analysis of the four identified agglutinates

from the positive control sample (heat-inactivated LP and Ab-coated beads), the bead entities were confirmed to be agglutinated by calculating their average distance as they move. 22 out of 33 beads from the last image of the set were found to be agglutinated using ImageJ (Table 4.1).

Therefore, the degree of agglutination of this set of images gives a value of 0.667 (or 66.7%). Three such sets of images of the same sample entrapped in the microchannel are captured at different locations (selected at random) to determine the degree of agglutination of the sample which gave a value of 69% with a standard deviation of 11%.

4.3.4. Sensitivity and specificity

The different performance metrics such as sensitivity and specificity for the quantitative analysis of LAT are investigated. The number of agglutinates formed rises with the concentration of LP, increasing the degree of agglutination rapidly. As more LP is introduced in the sample, bacteria continue to bind to its specific Ab on the bead's surface, thereby, forming more agglutinates. However, the number of available binding sites for LP starts to decrease as the concentration of LP increases, corresponding to saturation as seen in Figure 4.11. The system approaches equilibrium when the formation of agglutinates is no longer possible. The unbound LP were washed away during the washing step leading to a saturation level. As seen in Figure 4.11, the agglutinates are formed in samples with LP concentration of 10^6 cells/ mL and above. For samples with concentrations below 10^6 cells/ mL, the degree of agglutination is the same as that of the controls, indicating that insufficient amounts of LP (any concentration below 10^6 cells/mL) cannot be detected by this technique.

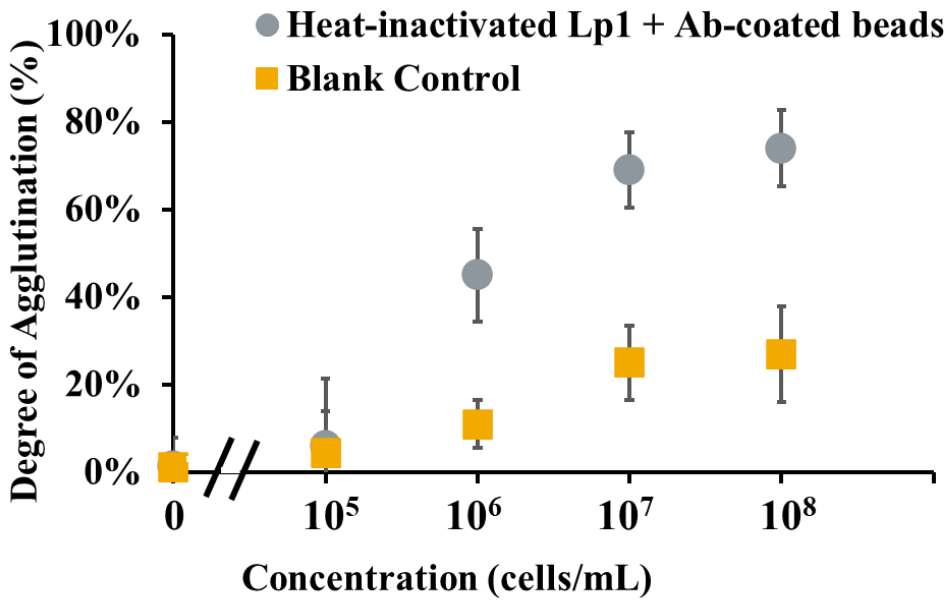

Figure 4.11: Degree of agglutination for different concentrations of LP with Ab-coated beads in comparison with the blank control containing LP with the same amount of beads that have no coating of Ab. This experiment was repeated three times and the error bar indicates the standard deviation showing the variance in the value from the results of the three experiments.

Considering the negative control with EC, the degree of agglutination was calculated for the same concentration of LP to confirm the specificity of this assay. The degree of agglutination for negative control (EC and Ab-coated beads) and blank control (LP and beads with no Ab) was found to be 8% and 25% respectively which is very low compared to that for LP (68.5%), confirming the specificity of this technique (Figure 4.12). Although agglutination occurred in the control samples, the size of the agglutinates was larger for the positive control sample compared to the control samples as the agglutinates in the positive control sample contained more bead entities (between 20 to 30 beads) as LP were binding to most of the available Abs of different beads. On the other hand, the number of bead entities in agglutinates from the control samples is very low (between 2 to 4).

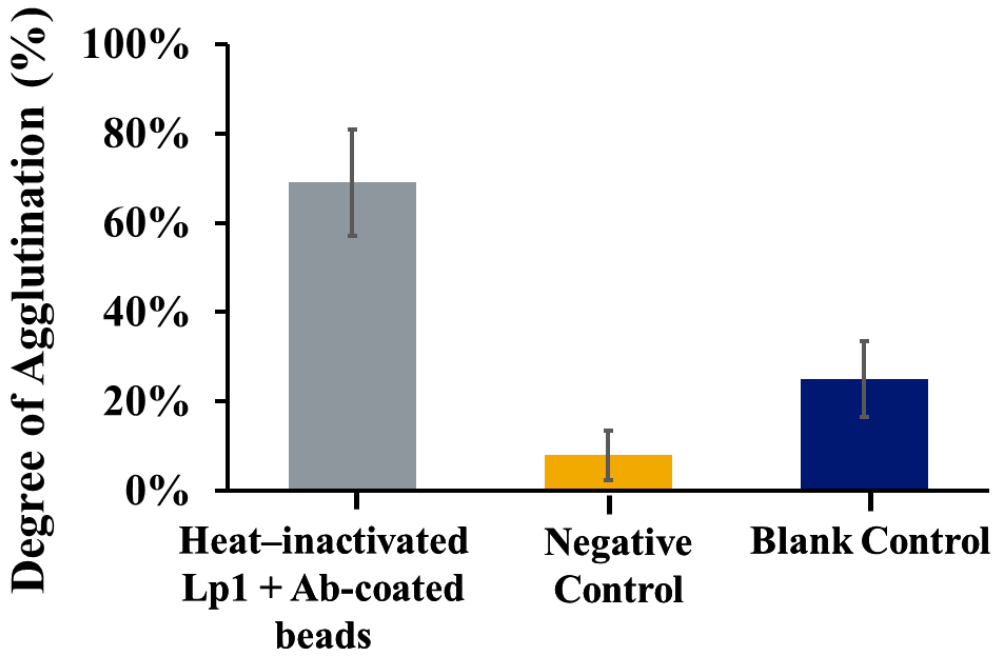

Figure 4.12: Specificity of the assay by comparing the degree of agglutination of positive control samples (LP and Ab-coated beads), negative control samples (EC with Ab-coated beads), and the blank control sample (heat-inactivated LP and beads with no coating of Ab). The concentration of LP and EC was 10^7 cells/mL. This experiment was repeated three times and the error bar indicates the standard deviation showing the variance in the value from the results of the three experiments. The degree of agglutination for the positive control, negative control, and the blank control samples is found to be 0.69, 0.08, and 0.25 respectively with a standard deviation of 0.12, 0.08, and 0.085.

4.6.4. Optimization of antibody amount for functionalization of latex beads

The amount of beads used for every sample is 100µg. Therefore, the theoretical value for the optimal amount of Ab required is 5µg. As seen in Figure 4.13, the degree of agglutination is zero when no Ab is used for the preparation of the bead solution. This is expected as there is no reactive site for LP to react on the bead surface. The only active groups are the carboxyl groups which are masked by BSA and Tween20 (surfactant). When the optimal amount of Ab (as per the calculations) is added, the degree of agglutination increases to 0.24. However, 5µg is not the

optimal amount of Ab because when excess Ab is added, the degree of agglutination continued to increase instead of showing saturation. This observation could be due to the loss of Ab during the washing step. Moreover, 3 – 10X excess of the optimal amount of Ab is recommended for LATs as this ensures a good driving force for adsorption, and crowded, upright positioning of antibodies [50]. As an excess amount of Ab is added, LP is exposed to more binding sites to react, thereby increasing the degree of agglutination linearly i.e. more beads became part of agglutinates. The increase in the degree of agglutination continues till 15µg of Ab, after which the degree of agglutination increases minimally reaching a saturation. This indicates that 15µg is the optimal amount required to create a monolayer around the beads. Any excess Ab added would not have enough space to couple with the carboxyl groups of the beads. Thus, they become unbound antibodies which will be washed away during the washing step, which is why there is no significant change in the degree of agglutination after 15µg of Ab. In two out of three experiments, the degree of agglutination started to decrease when excess Ab was added because of the prozone effect. This is a phenomenon exhibited by some reactions in which agglutination fails to occur due to excess Ab or Ag. When the Abs are in excess compared to LP in a sample, a greater number of non-agglutinated beads are present compared to agglutinated beads due to insufficient LP. Therefore, the fraction of agglutinated beads decreases as the excess amount of Ab is added which is seen in Figure 4.12. The antibodies cannot bridge due to insufficient LP and no agglutination (formation of agglutinates) will occur, thereby decreasing the degree of agglutination. To conclude, 15µg is the optimal amount of Ab required for the functionalization of 100µg beads.

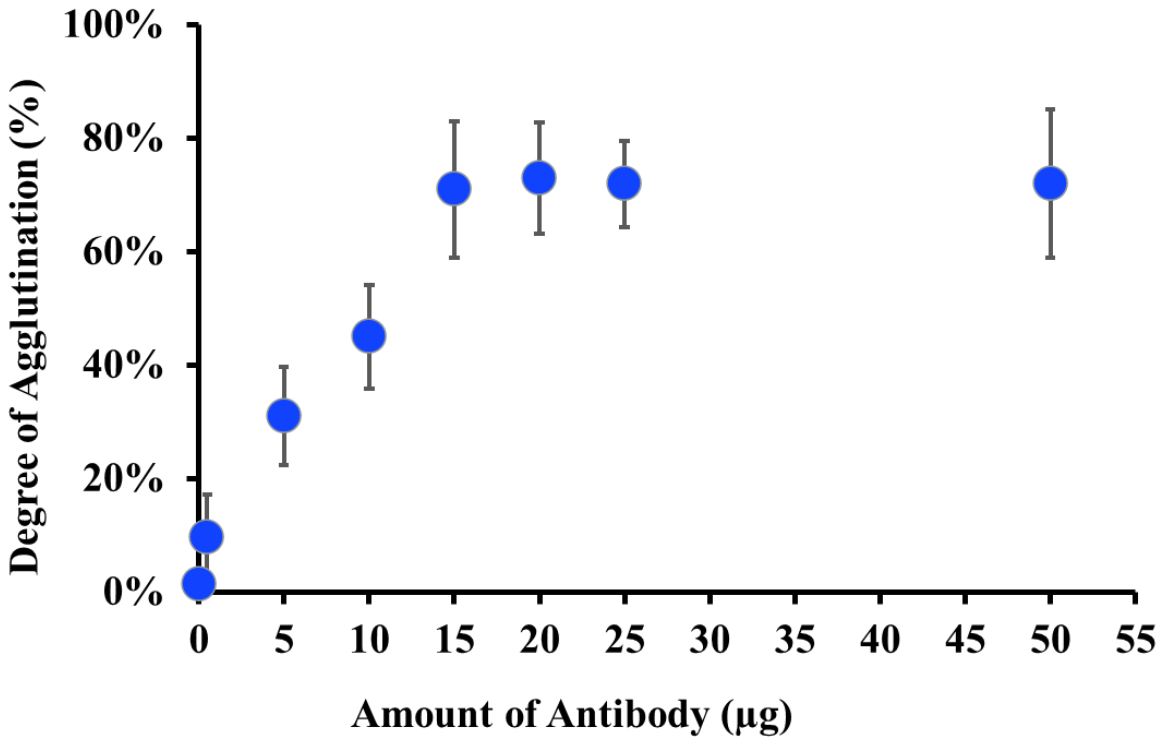

Figure 4.13: Degree of agglutination for a different amount of Ab for 100 µg of beads. This experiment was repeated three times and the error bar indicates the standard deviation showing the variance in the value from the results of the three experiments. The optimal amount of Ab required for functionalization of 100 µg of beads is found to be 15 µg.

PART 5: Summary and future work

5.1. Conclusion

To summarize, a quantitative LAT has been developed for detecting LP using a simple and affordable method that can be applied in most laboratories using only a microscope. Compared to the expenses and complications of PCR machines, this test is affordable and only uses a microscope as the main equipment which does not require heavy technical knowledge. This test can detect LP bacteria up to a detection limit of 10^6 cells/mL in 3 µL of the sample using 100µg of beads coated with 15µg of Ab within 5 to 8 hours. The main advantages of this method rely on its simple performance and the ability to quantify LP in samples.

Compared to current detection methods for LP, this method has a low detection limit. Moreover, this technique cannot differentiate between different serotypes of *Legionella* because the antibody used in this experiment is specific to *L. pneumophila* serogroups 1 – 12. Therefore, the samples may contain other *Legionella* serogroups which can lead to cross-reactions between certain serogroups[34]. As mentioned in the previous chapter, some Ab-coated beads that were not bound to LP moved in correlation confirming that they were agglutinated, hence, leading to false results. Therefore, results derived from this method should be interpreted with further image analysis. Moreover, the degree of agglutination should be considered regarding the size of the agglutinates because the number of beads in an agglutinate differ significantly between positive and negative and blank control samples even though agglutinated beads are identified in both samples.

5.2. Future work

Future work is required for the results seen in section 4.4. as this detection method needs to achieve a lower limit of detection. The degree of agglutination in 10^5 cells/mL concentration of LP and below was the same as that of the control. Therefore, further study on these concentrations would provide a better understanding as to why there was no change between the positive control sample and the blank control.

This problem may be solved when more amounts of beads (> 100 µg) and Ab are introduced which have to be further investigated. Although the values were lower compared to positive control samples with LP, the blank control (with no Ab) samples showed a reasonable output for the degree of agglutination. This could be due to the inefficient amount of BSA and Tween20 in the blocking agent to block the remaining binding sites on the beads. With a further understanding of what happens on the surface of the beads when Ab is introduced, optimization of other parameters such as the blocking agent, pH of buffers, duration for the functionalization can lead to a better specificity. Moreover, the size of the agglutinates can be graded based on the number of beads in the agglutinate as they are different in the positive control and the blank and negative control samples. Another challenge to address is the time required for optimal agglutination in a sample as low concentrations of bacteria agglutinates slower compared to high bacterial concentrations. Future work should also involve another method of determining what is happening when bacteria is introduced to the Ab-coated beads.

Detecting and quantifying LP with LAT is a simple technique that may be used for on-site testing of environmental samples in the future. For example, an LAT assay kit with a portable microscope can be designed where the LP containing sample and Ab-coated bead suspension is mixed and

observed by the microscope. A supporting software can be made where the motion of the beads is tracked to find the number of agglutinated beads.

5.3. Summary

The main goal of this research was to study the potential of using latex agglutination tests to develop a simple and cost-effective detection platform capable of detecting LP. The distance between beads and their bound LP calculated from the images taken after every 5 seconds proved that the beads are bridged by LP forming agglutinates and these agglutinates move together in correlation. By using EDC/NHS as a crosslinker to attach Ab to the carboxylic groups present on the surface of the beads, we can provide a platform for binding between Ab and Ag. Immobilizing Ab with beads confirmed that an optimal amount of 15µg of Ab was required to functionalize 100µg of beads and any excess amount of Ab would only lower the agglutination reaction.

Quantitative analysis of the latex agglutination test is performed by finding the fraction of agglutinated beads in an image of a sample. However, a single image cannot guarantee a cluster of beads are agglutinated because the beads are moving in a random direction (due to Brownian motion) and the image may have been captured when the beads came together at random. Therefore, a set of 10 images were taken of the sample entrapped in the microchannel to allow the observation of latex microbead movement. Once an agglutinate was found in the first image, they are observed in the remaining set of images to confirm that they move correlatedly, identifying agglutinated beads from non-agglutinated. This was mathematically proved by calculating the average distance of the agglutinates as they move in which the distance did not fluctuate due to crosslinking of beads by LP. As the last step, the ratio of all the agglutinated beads to the total number of beads in the image can determine the degree of agglutination for the quantitative analysis.

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