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Development of Bacteriophage Based Detection Technique for Food Safety and Environment Monitoring

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DEVELOPMENT OF BACTERIOPHAGE BASED DETECTION TECHNIQUE FOR FOOD SAFETY AND ENVIRONMENT MONITORING

A Dissertation Presented

by

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DEDICATION

To my mother, for being my first teacher who taught me goodness and kindness;

To my father, for pointing the direction of my academic career;

To my grandparents, for giving me a happy childhood;

And to all of my friends, for your love and support throughout my life.

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ABSTRACT

DEVELOPMENT OF BACTERIOPHAGE BASED DETECTION TECHNIQUE FOR FOOD SAFETY AND ENVIRONMENT MONITORING

SEPTEMBER 2016

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Foodborne and waterborne pathogens which are responsible for numerous worldwide outbreaks of disease have caused serious health problems resulting in enormous economic cost. Development of new bacterial detection technologies with high sensitivity and specificity is of great importance for food safety and public health. However, traditional bacterial detection technique based on culturing and colony counting is a time-consuming process which takes several days to obtain results. This has highlighted the significance of developing rapid and effective techniques for pathogen screening. In the recent decades, bacteriophage has been indicated as a valuable tool for specific bacteria identification due to the high specificity for targeted microorganism as well as the rapid infection between phage and its host cell. The other natural characteristics including low cost, robustness, fast and easy production plus high tolerance under extreme conditions make phages superior for bacteria detection at resource-

limited settings. The aim of this thesis is to explore a bacteriophage mediated detection system for foodborne or waterborne bacteria monitoring.

Bacteriophage as a novel recognition probe has been applied in various biosensor by immobilizing phage particles on solid sensor surface. In the first part of this dissertation, we focused on utilizing biotin- streptavidin interaction system for phage immobilization on biosensor surface. The immobilization efficiency of the bio-recognition element on micro-magnetic beads was investigated followed by evaluating the capture efficiency of targeted bacteria (generic *Escherichia coli*). The fusion of biotin acceptor peptide (BAP) gene and biotin ligase (BirA) gene to phage capsid protein gene enabled the display of BAP ligand and the expression of protein BirA during the replication cycle of phage infection. Then produced phage progenies could be biotinylated *in vivo* and immobilized on the streptavidin coated magnetic beads. Compared with wild type phage, the recombinant phage showed a significantly higher immobilization efficiency of 82.8% on the magnetic bead and the resulting bio-probe showed a capture efficiency of 86.2% of *E.coli* within 20 min. This phage based biomagnetic separation coupled PCR detection provided a detection limit of 10^2 CFU/mL bacteria without additional pre-enrichment. We believe this assay could be further developed to detect other bacteria of interest by applying host-specific phages which will be particularly useful for detecting bacteria that are difficult to cultivate or grow slowly by traditional method.

The second part of this thesis focused on utilizing host specific phage for pathogenic bacterial detection, such as *Escherichia coli* O157:H7 from practical samples. The carboxylic acid functionalized magnetic beads were conjugated with *E. coli* O157:H7 specific phage to which viable host cells could be captured and pre-concentrated. The effects of reaction time, phosphate buffer concentration, pH values and temperature on the bio-magnetic separation were investigated and the optimal reaction condition was as following: 0.01M PBS, pH 7.0 and

20 min of reaction at 37 °C. The capture efficiency of this phage based assay was about 20% higher than that of antibody based magnetic separation assay when the reaction was under extreme conditions. Then captured bacteria on the resulting bead-bacteria complexes were quantitatively monitored by following real-time PCR (qPCR) with a total assay time of less than 2h. The specificity and selectivity of the phage assay system were evaluated as well and no cross-reactivity was detected when non-targeted pathogens, including *Salmonella typhimurium*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* were tested in artificially inoculated agricultural water sample.

In the last part of this thesis, an engineered alkaline phosphatase phage based colorimetric detection assay was developed on membrane filter which offered a convenient and rapid way to identify general *E.coli* from field water on-site. Compared with the common membrane filtration assay for *E.coli* detection in water recommended by U.S. Environmental Protection Agency (EPA), our method could reduce the total assay time without sacrificing the sensitivity and the limit of detection could reach to 100 cells/100 mL water sample. This phage mediated detection also provided a convenient and quantitative detection of bacteria number as the colored signal formed from the enzyme reaction of alkaline phosphatase and BCIP/NBT produced a precipitated dot corresponding to the located bacterial colony, unlike other colorimetric assay which are usually qualitative and require to be measured by spectrophotometer in lab.

In summary, this dissertation demonstrated the practical application of bacteriophage as a promising tool for rapid foodborne and waterborne bacterial detection. And the results suggested that phage as a unique bio-probe offered many advantages over traditional cultural based method and could be coupled with various detection assays or platform with high sensitivity, low operation cost and in short time periods.

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CHAPTER 1

INTRODUCTION

The outbreak of foodborne or waterborne infectious is reported continuously worldwide and has even been rising in certain arears. (1) Meanwhile, public concerns on food safety and environmental health have increased tremendously. Most conventional bacterial monitoring from food or environmental samples is based on culture technology which usually need several days for laboratory analysis (2), during which time potential contamination may render consumers at healthy risk. (3) In addition, limitations such as lack of specificity and difficulty in detection of slow-growing or viable but non-culturable (VBNC) microorganisms also exist. (4) In this sense, exploring more rapid, sensitive and specific methods or technologies are essential and of great importance.

Nowadays various new assays aimed at reducing total assay time to less than 24 h have been reported. (5-12) In general, these methods consist of two steps: 1) target isolation, in which the bacteria of interest are separated, labeled or enriched to distinguish from other material in the sample matrix; 2) signal detection, in which optical or electrochemical technologies are used to quantify the target. (13) Immunomagnetic separation (IMS) technology which is based on antibody conjugated magnetic nanoparticles (MNP) or microbeads specific to bacterial pathogens is applied widely for target separation (14) as the isolation step can be shortened largely compared with selective cultural enrichment. (15, 16) Many pathogenic bacterium, including *Listeria* (17, 18), *E.coli* O157:H7 (19, 20), *Salmonella* (21, 22) and *Campylobacter jejuni* (23, 24) have been identified by IMS coupled methods. In addition, when IMS is combined with polymerase chain reaction (PCR) technique, the analysis time can be reduced to several hours. For example, as few as 10 *Mycobacterium avium* subsp. *paratuberculosis* was reported to be consistently detected from milk and fecal samples by IMS

coupled real-time PCR assay. (25) Rijpens and coauthors had reported the sensitive detection of an average of 5.9 stressed *Salmonella* cells in 25 g of food product using IMS and PCR following a pre-enrichment of 16 h. (26) Gooding and Choudary reached a detection limit of 1 cfu *E. coli* O157:H7/g food in less than 10 h by an IMS-PCR coupled method. (27) Although nucleic acid technologies are the most versatile one, limitation in distinguishing between viable and non-viable cells is still a concern, as the presence of detectable target DNA from a sample does not indicate the exact presence of viable organism with the ability to induce an outbreak or cause a disease. (5)

In contrast, bacteriophage as a virus that only infects bacteria allows the differentiation between live and dead bacteria. They are natural components of the micro-flora and ubiquitously present in our environment, such as soil, water, food, sewage, and other environments that contain corresponding host bacteria. (28) It was reported that more than 5500 kinds of phages had been found and there could be about 10^9 phages per milliliter in freshwater environment. Additionally, *E. coli* phages found from fresh meat, ground beef, raw vegetables and deli food can reach up to 10^4 PFU/g. (29) Generally, lytic phages that attach to host bacteria and utilize the host resources to reproduce new phages (30) can be used to eliminate harmful pathogens as they cause bacterial lysis upon the release of progenies. Thus phages have been applied in controlling bacterial contamination by food industry in food process, named biocontrol. (31) Moreover the high specificity to bacterial host at a strain level or species level has also provided phage as an ideal tool for bacterial targeting. (32)

Recently, many researches have shown the application of immobilized phages on solid surface to form an effective biosorbent. (33, 34) These phage-based probes were indicated to be capable of capturing bacterial antigens and the captured targets could be quantified in real time by following PCR, electrochemical or colorimetric techniques. (35) Biotin, also called Vitamin H is

usually used to label proteins or biomaterials for the immobilization of labeled probes on streptavidin-coated surfaces through the strong binding between biotin and streptavidin. This biotinylation process can be performed by using chemical reagents that specific for a certain type of functional group. For example, capture efficiency of a biosorbent formed from chemically biotinylated *Salmonella* phage on streptavidin-coated magnetic beads was found to be five-fold of that of the control. (36) However chemical biotinylation at multiple sites may result in inactivation by the biotinylating reagent directly or indirectly to the functional groups that are critical for protein. (37) In addition, as chemical biotinylation typically produces a heterogeneous reaction mixture, batch-to-batch reproducibility will become a big challenge. Biotin protein ligase (BirA) found in *Escherichia coli* can catalyze a highly specific formation of a bond between the amino group of lysine residue from biotin acceptor domain and the carboxyl group of biotin, known as enzymatic biotinylation. (38) Unlike chemical reagents, enzymatic biotinylation ensures the uniformity of the reaction products which also in a bioactive form. (39) This property can be applied to create a biotinylable fusion protein such as phage coat protein fused with biotin acceptor peptides (BAPs). When the BAP Tags are labeled onto recombinant proteins and co-expressed with BirA, they can be efficiently biotinylated in *E. coli*.(40) For example, Edgar and et al. have reported a rapid and simple method that combines in vivo biotinylation of engineered host-specific bacteriophage and conjugation of the phage to streptavidin-coated quantum dots. The method could detect 10 CFU/mL with approximately a hundred fold amplification of the signal over control in 1 h. (41) Tolba and his coauthors also showed the introduction of BCCP on T4 phage capsid protein by phage display technique could result in oriented immobilization of phage T4 on the streptavidin magnetic beads. (42) And the phage-based biosorbent coupled with real-time PCR allowed a detection limit of 8×10^2 CFU/ml *E. coli* in 2 h.

Novel phage mediated IMS assay based on bacterial enzymes or DNA liberated from the captured cell after phage-induced lysis has been reported more frequently. Engineered phage with affinity tags of interest can be introduced on the capsid protein by phage display technique as discussed above. (43) Usually phage based detection consists of four main steps: (1) immobilization of specific phages on magnetic particles for corresponding targeted bacteria; (2) capture and pre-concentration of target cells by IMS; (3) infection of captured target bacterium by the phage (wild or engineered); and (4) detection of signals resulting from the released enzymes, DNA or other biomarkers. Favrin et al. reported an IMS–bacteriophage assay that could detect an average of 3 CFU of *Salmonella. enteritidis* in 25 g or ml of food sample and could be adapted to detect *Escherichia coli* O157:H7 in ground beef within about 20 h. (44)

Besides IMS, colorimetric detection of bacteria has also received considerable attention in bioassay due to its simple procedure, short-time testing, low cost, nonhazardous reagents and high sensitivity compared with other available methods. (45) Although the sensitivity of colorimetric methods might not be the highest, simplicity of this type assay utilizing visual detection makes it the most suitable method for in situ testing. Burnham and et al. reported a rapid assay for generic *E. coli* detection in water sample by monitoring the released β-galactosidase after phage induced cell lysis. This method provided a detection limit of 40 CFU/mL within 8 h when wild phage T4 was used. (46) Similar detection by using other enzyme labels, such as alkaline phosphatase (ALP) and horseradish peroxidase (HRP) with their corresponding substrates have also been reported (47, 48) and can be further coupled with phage mediated diagnostic analysis which will be more affordable compared with antibodies. By using a colorimetric substrate such as 5-bromo-4-chloro-3’-indolyphosphate and nitro-blue tetrazolium(BCIP/NBT), *E.coli* alkaline phosphatase needs no additional bio-labeling thus simplifies the detection scheme.

So, to achieve a rapid and sensitive detection and identification of bacteria, three phage based assay were developed and optimized. 1) Preparing biotinylated phage particles through phage display technique (using model phage T7 select kit for phage engineering and packaging) and achieving *in vivo* biotinylation in phage host bacterial cell as well as characterization and oriented immobilization of the biotin tagged phages on streptavidin coated magnetic beads to increase the capture efficiency of target bacteria from sample matrix. 2) For pathogenic bacterial detection from practical water samples, a novel biomagnetic separation assay was developed by immobilizing *E. coli* O157:H7 specific phage particles on magnetic beads via the EDC-NHS coupling chemistry (by amide bond). In addition, the capture efficiency of the phage probe was compared with that of antibody coated magnetic beads and the specificity of the phage based assay was examined as well. To reduce the total assay time, real-time PCR technique was coupled with the phage based MS for bacterial quantification analysis. 3) A membrane filter based colorimetric detection coupled phage assay was also investigated for bacterial monitoring in agriculture water sample. The use of membrane filter technique enables the performance of viable counts on these products by using physical separation of bacteria from the sample matrix to be tested.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Bacterial foodborne and waterborne pathogens cause significant human diseases and are a major threat worldwide. Especially in food industry, monitoring and careful control of foodborne pathogen in time is critical to ensure food safety and public health. Thus, analysis methods have to be sensitive enough because even a single pathogenic organism may cause an infection. For example, the infectious dosage of *E. coli* O157:H7 and *Salmonella* is less than 10 cells along with zero tolerance in food products. (49) Traditional culture based methods for bacterial detection usually requires 5-6 days to get a positive result which are time consuming and always laborious. Immunomagnetic separation (IMS) as a popular approach provides a rapid way of detecting bacteria from different sample matrices as once captured, the isolated bacteria can be concentrated and enriched for downstream detection techniques without the need of cultivation. The capture efficiency (CE) of IMS mainly relies on the antibody that immobilized on the beads as the probe to capture targets. However, antibodies might encounter with consistency issue as there is batch to batch difference of the same product and the production of antibody is not cost-effective. (50) On the other hand, bacteriophage possessing high specificity to its target organism would circumvent these problems as well as minimize the probability of false-positive results generating from the cross-reaction between the antibodies and non-target analytes. In this case, applying phage based MS to reduce the total assay time would have significant potential in the study of rapid bacterial detection and realistic application.

It has been shown that phage based bio-probes can be generated on different solid surface to form a biosensor for various targets. (51) Generally, there are three different phage immobilization ways. The first way is through affinity binding to introduce specific binding ligands to phage head; the second one is through chemical binding such as EDC/NHS reaction and the third one is through simple physical adsorption. For the first immobilization method, different affinity tags have been developed via phage display technique to display protein of interest on its outside including specific antibodies/complementary peptides. Besides the standard affinity tags (His-tag), avidin-biotin system is also used widely as the binding of biotin to streptavidin is a very strong interaction which is advantageous for efficient immobilization. (52) More specifically, biotin protein ligase BirA could activate biotin to form biotinyl 5' adenylate and transfer it to biotin acceptor peptide BAP which named biotinylation. (53) This biotinylation reaction introduces biotin tags and allows for following streptavidin-biotin interaction. As biotin ligase protein BirA and biotin are present in all living cells, biotin molecules can be labeled on engineered phage surface displaying BAP through in vivo biotinylation. Unlike chemical reagents, this reaction which happens intracellularly would assure reaction products immobilized in a uniform, bioactive orientation. (40) The biotinylated phage particles can further be applied on various solid surface for corresponding bacteria detection. Thus, enzymatic biotinylation offers a better way to introduce biotin tags on phage capsid proteins and can be coupled with magnetic separation for rapid and simple bacterial separation.

Quantitative real-time polymerase chain reaction (qPCR) is a commonly used laboratory technique for DNA molecule detection and has been used increasingly in the recent past. (12) Unlike conventional endpoint PCR, qPCR allows monitoring of sample at exponential phase and provides accurate quantification of target nucleic acid. Compared with standard PCR requiring post-PCR analysis (e.g. agarose gel electrophoresis) which is time-consuming and non-

automated, qPCR eliminates these needs. (54) As the entire process (amplification and analysis) is performed in a single tube without further analysis, there is a reduced possibility of product contamination. Especially for Taqman qPCR system which contains a specific set of primers and internal probe (reporter), it is highly specific without the need to add other reagents. (55) Therefore, qPCR can be more efficient and time saving.

Although qPCR is more sensitive and less laborious intensive, there is still a limitation of this assay as it detects and quantifies DNA from both live and dead pathogens as long as the target nucleic acid is present. By combining bacteriophage-based assay to qPCR, positive signal yielded only from viable bacteria would minimize the probability of false positive result. For example, magnetic bead-phage complex can be prepared by the interaction between biotin (labeled on phage surface) and streptavidin (coated on beads) or by EDC/NHS carbodiimide chemistry and the biosorbents can be used to capture viable target cells before downstream PCR analysis. Factors such as the immobilization time, phage density, number of bead-phage complexes, reaction time and washing steps which can influence the immobilization efficiency and capture efficiency could be optimized to increase the capture percentage of bacteria. For quantification, the cell numbers can be estimated from the means of threshold cycles (CT) of amplification curves as the CT value is proportional to gene copy number of the target initially present in a sample.

As Figure 2.1 shows, food industry, clinical and water & environment quality control account for the major portion of all research regarding to pathogen detection. (56) To better apply phage as a bio-probe in bacterial detection for food safety and environment monitoring, a comprehensive study is essential. In this review, the foodborne and waterborne bacterial diseases as well as modern detection methods will be summarized. The characteristic of phages and their specific applications in bacterial pathogen detection will be discussed.

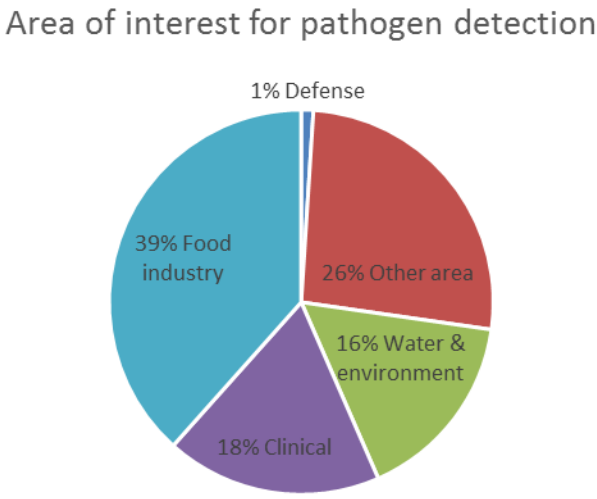

Figure 2.1 Distribution of application of the percent of researches published in literature on detection of pathogenic bacteria.

2.2 Bacterial pathogens

2.2.1 Foodborne pathogens

Bacteria, fungus and other microorganisms widely exist in our natural environment, they can be easily found in soil, foods, the intestinal tract of animals, or surface water contaminated with animal fecal. (7) On the one hand, microbes are crucial to the ecosystem in nature to carry out essential activities like nutrient recycling. They are also applied in biotechnology including traditional food products and beverage preparation such as brewing, baking, wine making, pickling and dairy-making process. (57) On the other hand, a small proportion of harmful microorganisms have harmful effects on animals and humans by causing infectious disease or even death. The World Health Organization (WHO) defines foodborne illnesses as illness caused by bacteria, viruses, parasites or chemical substances (toxic) entering the body through contaminated food. (58) As bacteria can spread easily and rapidly through

contaminated food or water, microbial diseases account for the major cause of death in many developing countries worldwide. (59) The Centers for Disease Control and Prevention (CDC) has reported that one in six Americans get sick from contaminated foods or beverages annually and 3,000 die. (60) Among these, diarrhoeal diseases are the most common illnesses that cause 550 million people to fall ill and 230 000 deaths globally every year. (58)

Due to the overuse and misuse of antimicrobials, a growing number of bacteria get the resistance to antibiotics thus become new foodborne/waterborne pathogens. (61-63) Although most strains of *E. coli* are harmless, some strains such as enterohaemorrhagic *E. coli* (EHEC) can cause intestinal infection or abdominal cramps, bloody diarrhea and even haemolytic uraemic syndrome. (64) For example, *E. coli* O157:H7 is considered to be one of the most dangerous foodborne pathogens due to its production of Shiga-like toxins which is usually transmitted to human by consumption of contaminated foods, including raw or undercooked meat products, raw milk and fresh vegetables.(65, 66) Waterborne transmission (from fecal contaminated water sources) and cross-contamination will also lead to infection. (67)

Another example of a common and widely distributed foodborne disease is Salmonellosis which is caused by the bacteria *Salmonella.* They are widely distributed in eggs, poultry and other food animals. It is a dangerous foodborne pathogen and causes serious public health concern as all serotypes can cause disease in humans and some strains have emerged resistant to a range of antimicrobials since the beginning of the 1990s. (68-71) It is estimated that *Salmonella* causes one million foodborne illnesses in the United States every year, with 19,000 hospitalizations and 380 deaths. (72)

Listeria is as another serious foodborne pathogen which is able to grow at refrigerator temperature (even as low as -1.5 C) and survive well in frozen foods thus make chilled foods also at safety risk. (73) According to CDC's report, approximately 1600 illnesses and 260 deaths due

to listeriosis occur in the United States every year. (61) And it accounts for as high as 25 to 30% of mortality rate associated with foodborne bacterial infections and is responsible for the majority of food recalls due to bacterial contamination. (74) Some of the common foodborne pathogens and the corresponding foodborne illness are listed in Table 2.1.

Table 2.1 Pathogenic microorganisms responsible for foodborne illness(75)

Microorganism	Infective dose[a] V (no. of organisms)	Incubation period[b]	Name of the disease
Campylobacter jejuni	400–500	2 to 5 days	Campylobacteriosis
Salmonella spp.	15-20	12 to 24 h	Salmonellosis
E. coli	< 10	2 to 4 days	Hemorrhagic colitis
L. monocytogenes	< 1000	2 days to 3 weeks	Listeriosis
Bacillus cereus	> 10^6/g	30 min to 15 h	Bacillus cereus food poisoning
Clostridium botulinum	< nano grams	12–36 h	Foodborne botulism
Clostridium perfringens	> 10^8	8–22 h	Perfringens food poisoning
Vibrio vulnificus	< 100	< 16 h	Syndrome called “primary septicemia”
Shigella	< 10	12–50 h	Shigellosis

a Infective dose: the amount of agent that must be consumed to give rise to symptoms of foodborne illness.
b Incubation period: the delay between consumption of a contaminated food and appearance of the first symptoms of illness.

2.2.2 Waterborne pathogens

Water sanitation and hygiene also have important impacts on both public health and disease prevention. Inadequate drinking-water, sanitation and hygiene are estimated to cause 842 000 diarrhoeal disease deaths worldwide per year. (76) Moreover, it has been estimated that 10% of total hospital patients in the USA contract diseases due to poor water sanitation and

the overall lost productivity due to waterborne diseases is estimated to be $20 billion per year. (77)

Waterborne diseases are usually caused by pathogenic microorganisms (including viruses and bacteria) in untreated or contaminated fresh water. Table 2.2 lists examples of some emerging waterborne pathogens which are also listed on the latest US Environmental Protection Agency's (EPA) contaminant candidate List (CCL-3). (78) According to the current USEPA water quality standards, the limit for drinking water is <1 colony/ 100mL; for body-contact recreation is <200 colonies/100 mL; for fishing and boating< 1000 colonies/100 mL; and limitation for domestic water supply <2000 colonies/100 mL.

Traditionally, indicator micro-organisms have been used to indicate the presence of pathogens as they are usually present in small amounts which is impractical for direct monitoring. Among these, *E. coli* has been extensively selected as an indicator for water quality monitoring as they are relatively simple and inexpensive to test. Another key factor is the development of improved testing methods for *E. coli* which also lead to the trend toward the use of *E. coli* as the preferred indicator of fecal contamination. So it is cost-effective to develop the detection method of *E. coli* for better protection of the public's health.

Table 2.2 Example of potential waterborne bacterial pathogens

Pathogens	Major disease(s)
Campylobacter jejuni[a]	Gastroenteritis
Escherichia coli O157[a]	Gastroenteritis, haemolytic uraemic syndrome
Helicobacter pylori[a]	Chronic gastritis
Legionella pneumophila[a]	Legionellosis
Mycobacterium avium[a]	Lung infection
Shigella spp.[a]	Shigellosis
Salmonella enterica[a]	Gastroenteritis, typhoid

a Pathogen on the US EPA CCL-3 (http://www.epa.gov/ccl/contaminant-candidate-list-3-ccl-3)

2.3 Bacterial detection techniques

2.3.1 Traditional detection method

Culturing and colony counting method as a conventional bacterial detection technique, is the gold standard detection method. In general, conventional methods include pre-enrichment, selective enrichment/plating and following biochemical or serological confirmation. They are sensitive and can offer quantitative/qualitative information of the microorganisms based on particular medium. These selective media include MacConkey sorbitol agar for *E. coli* O157:H7, rainbow agar for *Salmonella* and charcoal-based selective medium agar (CSM) for *Campylobacter* detection. However, they are confronted with time-consuming issue due to the time required for bacterial growth to visible colonies. For example, it takes 4–9 days to get a negative result and about 14 -16 days to verify a positive result for *Campylobacter* (79) which is inadequate for many practical application, especially for the food industry.

2.3.2 Immunology-based methods

2.3.2.1 The enzyme-linked immunosorbent assay (ELISA)

A broad range of immunoassays for bacteria detection have provided many analytical tools for the detection of various targets. Based on the immunological techniques, the enzyme-linked immunosorbent assay (ELISA) is the most widely used one and has been applied extensively. It is a test that combines antibody and enzyme induced color change to identify a substance, thus is sensitive and rapid. Figure 2.2 indicates the principles of a typical sandwich-ELISA for antigen detection that contains five steps: 1) prepare a solid surface to immobilize the capture antibody; 2) block the nonspecific binding sites; 3) add test sample to the prepared

surface and the antigen is captured by antibody followed by washing to remove excess antigen; 4) expose the plate to an enzyme conjugate antibody followed by washing to remove unbound conjugates; 5) add colorimetric substrate to induce enzyme reaction and the colored product can be detected directly indicating the presence of antigen.(5) The quantity of the antigen can be determined by measuring the optical density which is specific, versatile and can be used reliably for testing sample in large numbers.

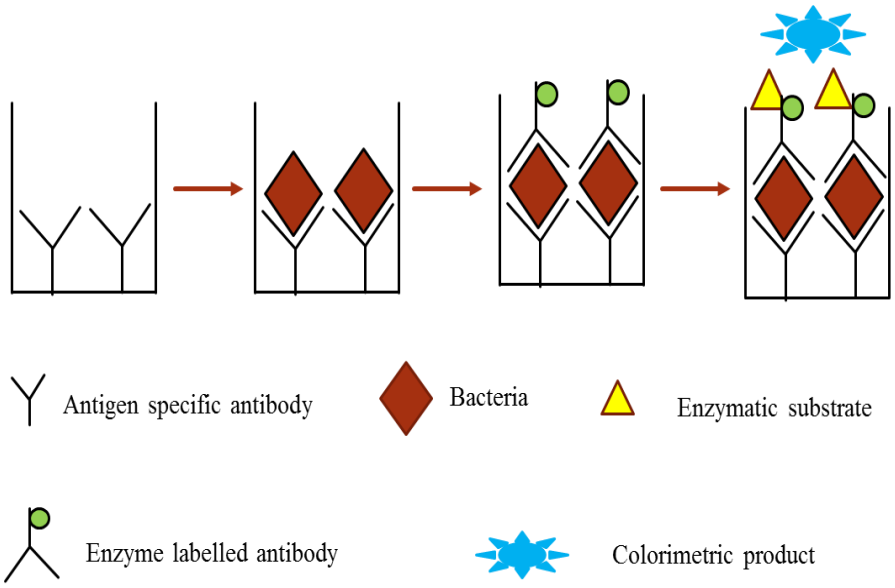

Figure 2.2 Schematic representation of the sandwich-ELISA protocol.

2.3.2.2 Immunomagnetic separation (IMS)

IMS as a useful laboratory method that can efficiently separate targets from sample suspension, is commonly used to pre-enrich or pre-concentrate samples before final test. In brief, a sample matrix containing target bacteria is mixed and incubated with antibody coated paramagnetic beads for a certain period of time. As the antibody on beads can bind to the antigens displayed on bacteria surface, target bacterial cells are isolated and captured when an appropriate magnetic separator is placed nearby the test tube. Then the bead-cell complex is

washed several times to remove unbound cells as well as other contaminants. Finally, the relatively purified target cells on the magnetic particles can be used in following experiments. Nowadays, polystyrene beads coated with iron oxide and antibodies are the most common type used for microorganism separation. For example, Dynabeads® is one of the commercially available beads for various pathogen detection, including *Salmonella*, *E. coli* O157:H7 and *Listeria*. Different bead sizes ranging from nanometers up to microns can be chosen depending on the specific application. For example, to isolate whole bacteria cells, bead size in the range of 1-2 micrometer may offer a good balance between sensitivity and time. (56) Furthermore, when combined with other detection methods (e.g., PCR, optical, fluorescence or electrochemical assay), IMS can minimize the total analysis time significantly. Bushon et al. found that the immunomagnetic separation coupled adenosine triphosphate (IMS-ATP) method by measuring bioluminescence induced by release of ATP from the captured bacterial cells could be performed within 1 h which was obviously much faster than other traditional methods. (80)

2.3.3 Polymerase chain reaction (PCR)

PCR is a nucleic acid amplification technology that developed in the 1980s and now widely applied in the field of bacterial detection.(81) It enables the synthesis of thousands to millions of copies of target DNA fragment from a single or a few copies. This technique is especially useful in identification of slow-growing or non-cultivatable microorganisms (e.g. anaerobic bacteria) as well as detection of infectious antigens. It also enables the discrimination of pathogenic from non-pathogenic strains through specific genes (e.g. toxin gene).

Besides conventional PCR, the emergence of multiplex PCR, reverse transcriptase PCR (RT-PCR) and real-time PCR (qPCR) also provide a rapid and sensitive detection and more advantages. (82) For instance, multiplex PCR is sufficient to detect more than one target

organism at the same time by adding different sets of primers corresponding to the specific genes of each target strain. Reverse transcriptase PCR (RT-PCR) is able to amplify only expressed genes by using the reverse transcriptase to reverse transcribe RNA into single-stranded DNA first. Then the single-stranded DNA (complementary DNA) served as a template can be exponentially amplified by conventional PCR. (13)

On the other hand, real-time PCR permits sensitive quantification of nucleic acids that can be used to diagnose pathogens for individual species. It is based on the detection of fluorescent signal generated by the fluorescent dye which increases in proportion with the amount of the amplicons. Fluorescence intensity is monitored during reaction cycle, thus allowing the analysis in real time without laborious post-amplification analysis (e.g. gel electrophoresis). (83) SYBR® Green and TaqMan® are the two different formats that specific to double-stranded DNA and sequence specific oligonucleotide, respectively. (81) Although there are many advantages of qPCR method, limitations still present as listed in Table 2.3.

Table 2.3 Advantages and limitations of real-time PCR

Advantage[a]	Limitation
Range of quantification (7–8 log decades)	PCR product increases exponentially
High technical sensitivity (<5 copies)	Variation increases with cycle number
High precision (<2% CV of CT values)	Increased variation after transformation to linear values
No post-PCR steps	Overlap of emission spectra[b]
Minimized risk of cross contamination	Maximal four simultaneous reaction[b]
High throughput	Increased risk of false negative result[c]
Multiplex approach possible	

a Adapted from Dieter Klein.(82)
b maybe new technology could improve this.
c particularly for pathogen detection.

PCR in combination with other detection techniques have also been well established. For example, a PCR and microarray-based assay offered a specificity of 98% and sensitivity of 96%

(initial sensitivity of 82%) in bacterial pathogen detection within three hours including the time required for sample preparation, DNA extraction, PCR and microarray steps.(84) Compared with culturing and plating, PCR based assay can rapidly provide reliable result in a less time-consuming manner.

2.4 Biotinylation

In biochemistry, the process of covalently attaching biotin to a protein, nucleic acid or other molecule is called biotinylation. Due to the small size of biotin, it is unlikely to disturb the original function of the attached molecule. Generally, there are chemical biotinylation and enzymatic biotinylation. Chemical biotinylation means the reaction is through conjugation of chemistries, such as conjugation to primary amines, carboxyls or carbohydrates. These often result in nonspecific biotinylation of the groups on the target protein. For example, N-hydroxysuccinimide coupling results in biotinylation of any primary amines in the protein. On the other hand, enzymatic biotinylation gives binding to a specific lysine within a certain sequence. The detailed reaction process is indicated in Figure 2.3.

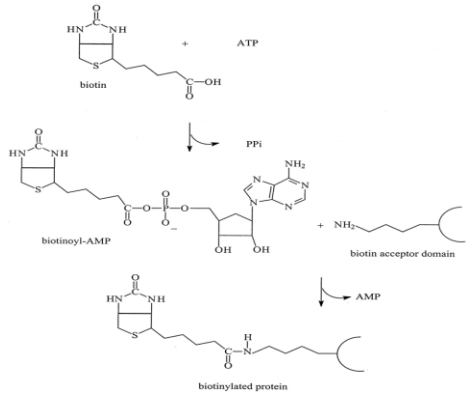

Figure 2.3 In vivo biotinylation: condensation reaction between biotin and a lysine residue to form a biotinylated protein that is catalyzed by biotin ligase protein.(85)

Table 2.4 List of some biotinylated molecules and their possible applications (abbreviations: ALP= alkaline phosphatase, HRP= horseradish peroxidase)

Biotinylated moiety[a]	Possible applications
Anti-immunoglobulins, Protein A, Protein G	Immunological assays, flow cytometry, cell sorting, immunohistochemistry, Western blots
Lectins	Glycoconjugate studies, mitogenic stimulation studies
Anti-lectins	Localization of lectin receptors
Enzymes (ALP, beta-galactosldase, glucose oxidase, HRP)	Immunological assays, nucleic acid hybridization
Ferritin, hemocyanin	Electron microscopy
Agarose, cellulose	Affinity chromatography
Anti-avidin, anti-streptavidin	Amplification assays
Nucleotides	Nucleic acid hybridization
DNA	Nucleic acid hybridization, molecular mass markers, DNA sequencing
Hormones	Affinity chromatograp, receptor-ligand interaction
Cells	Hybridoma production

a Adapted from Eleftherios P. Diamand and Theodore K. Christopoulos.(86)

2.4.1 Biotin protein ligase

Biotin-protein ligase (BirA, EC 6.3.4.15) can add biotin covalently to biotin-acceptor peptides/proteins via an ATP intermediate reaction which is highly efficient. The biotinylation process can be carried out by the cell's endogenous BirA or through the co-expression of an exogenous BirA.(87) Recently, AviTag or biotin acceptor peptide (BAP) has been fused to proteins for biotinylation as BirA recognizes the N-terminus or C-terminus of this 15 amino acid peptide. For in vivo enzymatic biotinylation, the BAP tagged protein and BirA are usually co-expressed. BirA also reacts with proteins fused to biotin carboxyl carrier protein (BCCP) as it can be recognized by biotin molecules as well and attached to it.(88) Table 2.4 shows some biotinylated molecules and their possible applications.

2.4.2 Streptavidin-biotin

Streptavidin is a 52.8 kDa protein found from the bacterium *Streptomyces* avidinii which has high affinity to biotin. The binding between biotin and streptavidin is extremely strong, highly specific and resistant to organic solvents, denaturants, detergents, proteolytic enzymes as well as extremes of temperature and pH.(86) Thus streptavidin-biotin interaction system is used extensively in molecular biology and biotechnology. Due to the small molecule size (244.31 Da) of biotin, the biological activity of the macromolecules after biotin binding will not be affected, e.g., enzymatic catalysis and antibody binding. In other words, biotinylation usually will not change the properties of these bio-molecules. (89) As one of the strongest noncovalent binding between a ligand and a protein and as per streptavidin molecule possess four binding sites, they can be applied for multiply biotinylated molecules for biotin. These features make the isolation of biotinylated molecules convenient and allow its application in various areas of modern biological techniques (e.g. orient immobilization for bacterial detection). For example, in the field of protein purification, biotinylation allows the purification step more easily by reducing background binding which often occurred within other affinity tags. In addition, the purified streptavidin is readily available and their derivatives e.g., with fluorophores, enzymes, antibodies and solid phases are also commercially available as listed in Table 2.5.

Table 2.5 List of streptavidin conjugates and their possible applications

Conjugate of avidin or streptavidin with[a]	Possible applications
Enzymes (ALP, HRP, p-galactosidase, glucose oxidase, etc), fluorophores (fluorescein, rhodamines, phycoerythrin, Texas Red)	Immunological assays, flow cytometry, cell sorting, Immunohistochemistry, Western blots, nucleic acid hybridization
Eu^{3+}-chelates	Immunological assays, Western blots, nucleic acid hybridization
Ferritin, gold	Electron microscopy
Chemiluminescent labels	Immunological assays, Western blots, nucleic acid hybridization
Agarose	Affinity chromatography
Magnetic particles	Nucleic acid hybridization, affinity chromatography, DNA sequencing
Polystyrene	Immunological assays

a Adapted from Eleftherios P. Diamand and Theodore K. Christopoulos.(86)

2.5 Bacteriophage and application

2.5.1 Phage structure

Typical bacteriophage is composed of a protein head that encapsulate its genetic material (DNA or RNA) inside and tail for specific binding to the receptor molecules on its host bacteria (90). As a protective shell for the genome, phage head is made up of many copies of one or more kinds of capsid proteins which connect with a hollow tube tail. Phage tail is helpful for the attachment to its host cell when phage infection occurs as tail fibers would specifically attach to the receptor on bacterial cell wall and tightly bound to it. It is also useful for phage genetic material passing into the host cell. However, there also exist some exceptions, a small portion of phages don't have a tail structure (found in dsDNA phages). Although different phages have different sizes and shapes, most of them are in a range from 24nm to 200 nm in length while the length of the tail can be very different (91). Miller and et al. showed a typical phage T4 structure containing a head and tail in Figure 2.4.

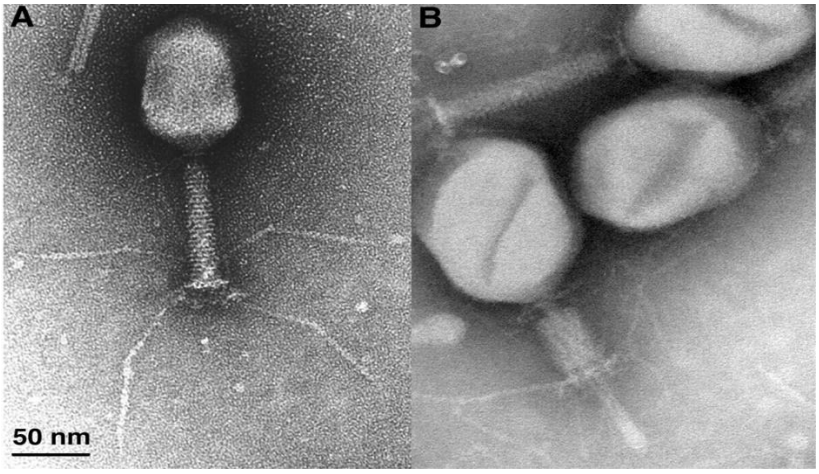

Figure 2.4 Electron micrographs of bacteriophage T4: (A) tail fibers and icosahedral head of phage T4; (B) DNA genome of phage T4 delivered into host cell through its hollow tail tube.(92)

2.5.2 The mechanism of phage adsorption to host

The mechanism of how phage adsorbs to its host as the first step of infection and parasitic metabolic life cycle is essential for better understanding phage based biosensors. Especially, as a key step in phage recognition to its specific host cell, both phage and bacterial structure as well as their interaction (eg. phage attachment, penetration to host cell) is worthy of attention and study.

2.5.2.1 Phage receptors on cell surface

Different phage is known to infect its corresponding bacterial strain or species, usually in a narrow host range and this specificity between phage-bacterial interaction relies on the specific attachment which is determined by the recognition of receptors on bacterial surface.(93) Moreover, receptor density, amount and site on bacterial cell wall play an important role in this process. For gram-negative bacteria, (such as *E.coli*) the external layer of

lipopolysaccharide (LPS) and proteins localized in the outer membrane usually serve as potential receptors during phage infection.

Lipopolysaccharide as an outer membrane component offers receptor function for phage conjugation. As a large molecule, its contains of lipid A and a polysaccharide which is consist of O-antigen (also named O side-chain), outer core and inner core in the outer membrane of gram-negative bacteria. It has been reported that the adsorption of *Salmonella flexneri* serotype 3a was related with the hydrolysis of a bond in the O-chain and absence of this O-antigen resulted in resistance to its corresponding phage. (94) In brief, phages recognize the receptor component in LPS through certain enzyme at tail fiber, followed by adsorption to it and hydrolysis of the bonds in O-chain. In the case of phage T4, only LPS serves as the receptor in *E.coli B* strain. For *E.oli* K12 strain, this residue is masked by an additional sugar chain which prevent its interaction with the tail fiber of T4. As a result, it need the OmpC protein to overcome the prevention and function as a receptor to interact with T4.(95) While to T-even phages, long tail fibers seem to play an important role during the specific attachment process. And for phage TuIb, it's possible that LPS plays two possible roles in the interaction with long tail fibers. In summary, polysaccharide part plays an important role for adsorption either by directly interacting with phage fibers for both short and long tail or by interacting with OmpC.

Based on different function, proteins can be classified into different types, including structural proteins, porins, enzymes, transport proteins and etc. As membrane proteins, OmpA, OmpC and OmpF from *E.coli* are known to serve as receptors during phage infection for phage K3, phage PA2 and phage T2, respectively.(95) As a major outer membrane protein, OmpC serves as a receptor which is needed along with cell wall LPS for the recognition of phage T4 to *E. coli* K12 strain. Mutants with an absence of either receptor could result in a reduced infection efficiency. Without both receptors, *E.coli* cells displayed resistance to T4 phage.(96) So for an

effective infection by phage T4, both OmpC and LPS are required for *E. coli* K12 while only LPS is required for *E.coli* B strain. Unlike phage T4, the receptor protein for phage T2 is OmpF and different phage tail fiber protein governs the recognition.

As another outer membrane protein of *E.coli* K12, OmpA has also been reported to serve as a receptor for T-even phage conjugation. (97) Studies indicated that phage Tulb attachment occurred when OmpA-LPS complex was present while missing of the protein resulted in resistance of phage infection. Similar result was found in phage K3 when bacterial mutants lacking the protein receptor were performed. (98) Moreover, for phage T6, researchers found tsx gene product (tsx-protein) served as phage receptors in *Shigella* and *E.coli.* Mutants with deficiency of this gene were fully resistant to some T6 like phage. (99) Other enzymes found at outer membrane, such as proteases OmpT, OmpX, protein TonA and TonB were receptors for T-like phage, phage T7 and T5, respectively.

Other components of bacterial cell including capsular polysaccharides, oligosaccharides, peptidoglycan or flagellum/pilus have also been reported to display receptor activities during phage adsorption process.

2.5.2.2 Phage adsorption under simple conditions

The initial stage of phage infection is an attachment onto the susceptible host cell surface. As there is no special structure for phage moving, the adsorption is dependent on random collision of phage and bacteria. So the first step of host recognition is regarded as a searching process. This is supposed to follow the mass-action kinetics which means higher reactant density resulting in higher number of random collisions and an increased adsorption rate. Moreover, the rate of adsorption is influenced by the physiological state of the bacteria. For *Ecoli*. B strains which are motile bacteria, the adsorption rate should be higher at optimal

condition as the bacteria can move faster than under adverse condition as their motility will be limited. Thus, the time for phage attachment relies on the concentrations of two particles and cell's physiological status.

Under optimal condition, the rate is directly proportional to both bacteria and phage concentrations. For most viruses, the maximum collision to achieve virus-host binding in nutrient broth takes place at 37°C. The attachment rate of phage T1 to *E. coli* B decreases when temperature is above or below 37°C. Usually, phage adsorption process contains two steps: reversible and irreversible binding. In a reversible one, phages can desorb from cells by dilution (e.g. by distilled water), while in irreversible one, phages are not reserved to liberate as their binding are with specific receptors. The irreversible adsorption of virus to receptor will induce an rearrangement of phage tail structure and following delivery of phage nucleic acid into host.(100) However, the molecular mechanism for the interaction between different phage-bacteria pair may vary according to specific receptor groups on surface. After the irreversible attachment, phage lysozyme and some specific enzymes will be synthesized and released to degrade peptidoglycan or exopolysaccharide (EPS) structure, resulting in the forming of pores in cell wall. ATP molecules and electrochemical membrane potential may also help the penetration.

2.5.2.3 Influence of ions in phage adsorption

In addition to physical factors (pH, temperature), phage adsorption rate is also influenced by chemical compositions, such as ions or organic substances in the surrounding environment. For example, in a plain medium, phages T1 and T3 display a rapid reaction rate to *E.coli* B at 37°C while phages T2 and T4 display no adsorption. But, when certain salts were added to the medium reaching an appropriate concentration, phage T2 and T4 exhibit the same

high adsorption velocity as phage T1 and T3. Similar result was found when phage T1 and *E. coli* B were suspended in distilled water as no measurable adsorption took place in this reaction system. These results suggest that the initial attachment of virus to cellular surface should follow an electrostatic pattern which is influenced by the ionic charges on the two surfaces.

For phage T1, when approximately 5 $X10^{-3}$ M NaCl is attained in buffered solution, virus adsorption rate can reach to nearly half that detected in nutrient medium. But when higher NaCl concentrations are applied (≥ 5 $X10^{-2}$ M), the reaction becomes inhibited and adsorption rates decrease to relatively low values. The same decreasing of adsorption rate at an excess of NaCl is also obtained in cultural medium. When NaCl is replaced by other univalent ions, such as KCl, LiCl and NH_4Cl, similar results are obtained. For example, when the concentration of NaCl reached 10^{-1} M, the adsorption rate diminished to less than half of the value obtained at the concentration level of 10^{-2} M. This means addition of ions at low concentration can increase the reaction velocity while decrease the velocity at high concentration.(101)

The effect of divalent ions on phage T1 adsorption rate to host cells also exhibit a similar pattern to that of monovalent ions. For example, when pure salt of $CaCl_2$ in buffered solutions is applied, only ≈5 $X10^{-4}$ M is needed to reach the maximal adsorption rate, while higher concentrations depress this reaction (eg. at ≈5 $X10^{-3}$ M, the velocity rate is < half of that at 5 $X10^{-4}$ M). The maximal reaction velocity at optimum Ca^{2+} concentration is also similar with that observed in nutrient broth. As their anion is the same, the concentration difference of NaCl and $CaCl_2$ is apparently caused by the cations. In addition, when anion is replaced by nitrate or sulfate, the result is similar with that of chloride. And when $CaCl_2$ was replaced by other divalent salts, such as $MgCl_2$ or $MnCl_2$, almost the same results were found.

Trivalent ions, such as Fe^{3+}, Al^{3+} and Cr^{3+} even at low concentration have inhibiting effect on phage and host bacteria as they can inactivate them quickly and irreversibly. Compared with

ionic substances, non-ionized substances in a reaction system show no effect on phage attachment velocity. So, the adsorption rate of phage to host cells is probably controlled by ionic components. In addition, the difference of total charge of these ions, instead of their chemical species influence most on phage adsorption rate. These experimental findings illustrate that electrostatic interaction maybe a key factor that influence phage attachment.

By adding $MgCl_2$ to a non-reacting mixture of phage and bacteria in distilled water, a rapid adsorption could be achieved which indicates the metallic cations have activation influence. Specifically, when *E. coli* B and phage T1 were mixed in distilled water at 37°C, no adsorption was detected until Mg^{2+} was added to the concentration of 10^{-3} M. The percentage of phage adsorption to host cells increased from 0 to 93% after the addition of Mg^{2+}. However, higher concentration of Mg^{2+} ultimately decreased the adsorption rate of phage to bacteria.

One proposed theory indicates that interaction between virus and host cell is a two-step process. The first step is a reversible one which may be similar to the attachment on an ion-exchange surface. The second step is an irreversible one which means infectious phage cannot be liberated from cell by dilution or agitation. An explanation of the irreversible binding between phage tail and the receptor is the key-lock interaction model.(102) Although most phages were found to display an irreversible adsorption to their host cells, PL-1 phages were reported to adsorb to host bacteria reversibly only.(103) But one thing in common is that the groups or structures of bacterial receptors are highly specific and differentiate widely according to different phage species.

Phage-host adsorption reaction would be influenced by the energy needed to different sites on both surfaces as well as the resulted number of ions that bind to each surface. In brief, the environment for effective phage-cell reaction needs appropriate charge distribution instead of special types of ions.

2.5.3 Phage life cycle

Although all phages need host cells to replicate and assemble progenies by using their metabolism and machinery, different phage species have different characteristic of life cycle. Generally, there are two different types of phages, one has lytic life cycle while the other has lysogenic life cycle. And there are basically five steps for a typical phage infection: 1) virus attachment; 2) DNA injection; 3) synthesis of virus components (phage nucleic acid and proteins); 4) assemble of phage particles to burst size; 5) release of new phages after cell lysis.

2.5.3.1 Lysogenic cycle

In a lysogenic cycle, phage's genome is integrated into the host chromosome instead of causing transcription in the bacteria. In this case, phage will not kill the host bacterial after the infection but replicate its DNA with the replication of bacterial DNA. Thus, phage genome will not be expressed except the gene coding for a repressor which can prevent the synthesis of viral lysozyme that is needed for a lytic cycle. Phage this unexpressed state is called prophage as it is not a phage but still keeps the ability to produce phage particles. Bacteria cells carry prophages are termed lysogen. Lysogen may turn into a lytic cycle stay until exposed to adverse conditions. In this case, repressor synthesis becomes inactivated or stopped while enzymes that can excise phage DNA from the host genome will be expressed. This process is named induction which means prophage DNA can be cut off from bacterial genome and following phage structural proteins will be synthesized and assembled for a lytic cycle.

2.5.3.2 Lytic cycle

Phage lytic cycle will cause the lysis of infected bacteria which is different from lysogenic cycle. In this case, phage DNA is separate from host bacterial genome and replicates separately. But both lysogenic and lytic life cycle need host cell's machinery for phage replication. Briefly, in a lytic cycle, after virus inject its nucleic acids (DNA or RNA) into the host cell, phage stars using cell's replication and translation mechanisms to produce their components immediately. Once the viral DNA and proteins are synthesized and assembled within the cell, new phage progeny particles are created and accumulated. Then some of phage specialized proteins, such as lysozyme (required for lytic cycle) are produced to dissolve the bacterial cell wall. This will result in the release of new phages into outside environment and keeping the infection to other cells. These two phage life cycles are shown in Figure 2.5.

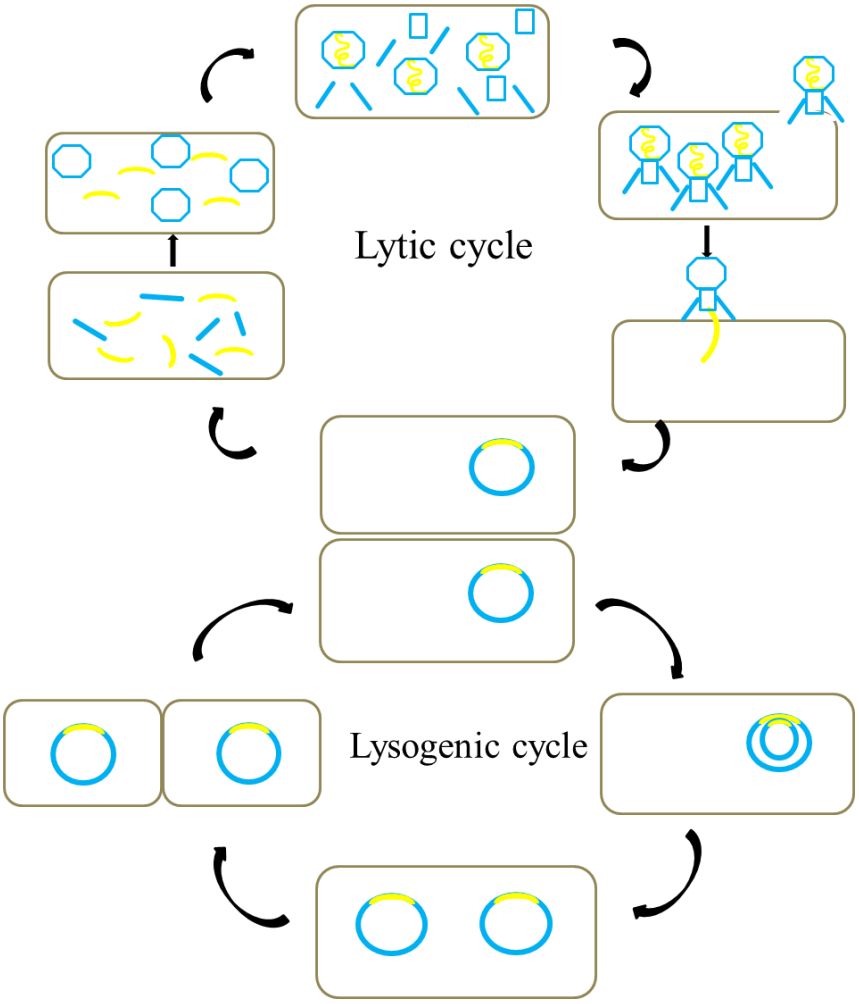

Figure 2.5 Overview of the general bacteriophage replication cycles. Phages can be either lytic or temperate.

2.5.4 Phage therapy

Phage therapy is a method by using lytic phage to treat pathogenic bacterial infections. As phages have the ability to infect their host bacteria, resulting in cell destruction, phage therapy is a potential alternative to antibodies and could be applied in many fields like human medicine, veterinary science, dentistry, and food industry. An advantage of phage therapy is its high specificity compared with antibiotics that used in clinic. Additionally, phage therapy is harmless to patient and other normal flora in environment. On the contrary, conventional antibiotics with wider effect range will kill not only harmful bacteria but also non-harmful one. Phage can also be used to treat bacterial infections that can't be treated by traditional antibiotic therapy. Typically, antibiotics cannot penetrate bacterial biofilm (composed of a polysaccharide matrix) and its concentration will decrease fast after permeating the infectious surface. On the contrary, due to the self-replicating and self-assembling characteristics of phage, effective treatment can be realized by applying phage based therapy theoretically.

2.5.5 Phage display technique

Phage display is a laboratory technique applied to express various peptides for different protein interactions. The goal is to select the one that can interact with a target of interest, such as peptide, protein or DNA. The first described case of phage display was on filamentous phage. Except this most commonly used type, other display systems by using T7, T4 and λ phage had also been applied. In this technique, the gene encoding target protein is inserted into phage coat protein gene, resulting in the expression of target protein fused with either minor or major phage coat protein. The general procedure of phage display is illustrated in Figure 2.6.

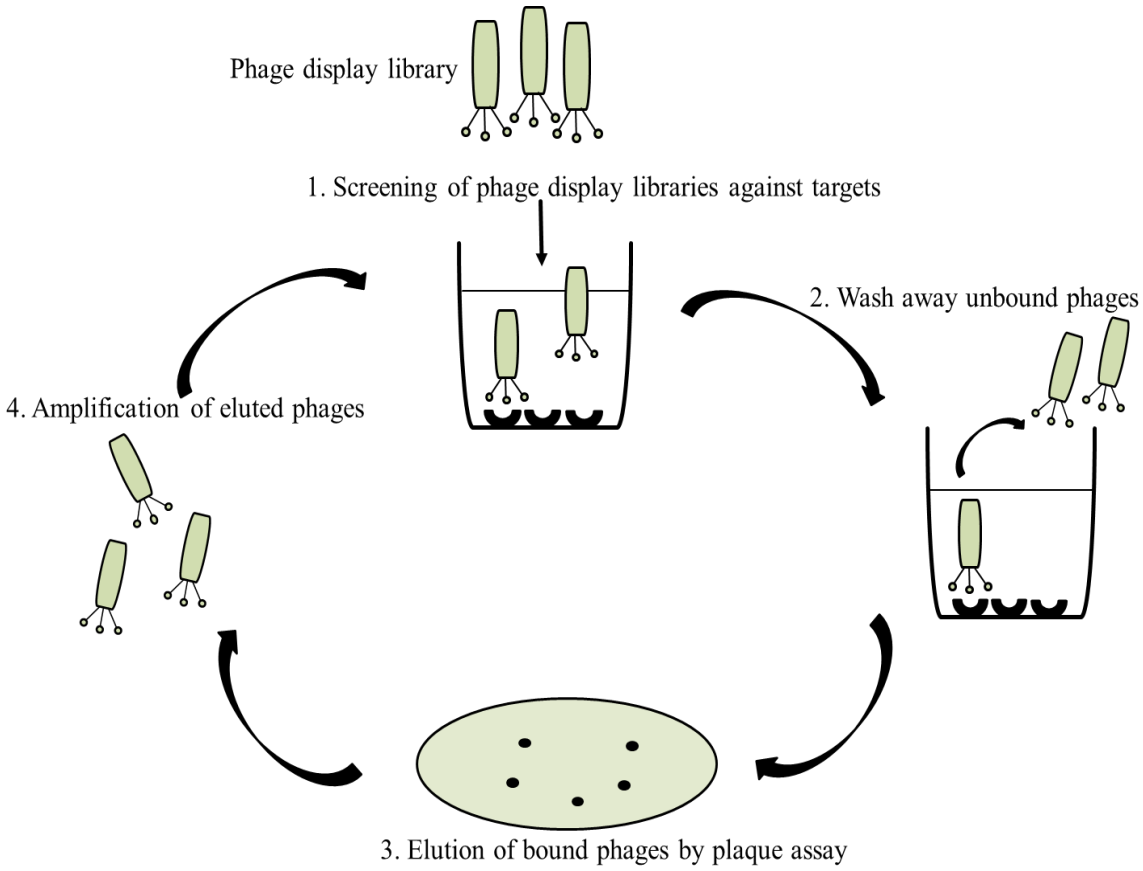

Figure 2.6 Schematic illustration of the general phage display protocol: 1) phage display libraries with potential ligand proteins bound to immobilized targets, 2) remove unbound phages by washing, 3) elution of bound phages by bacterial infection and plating on LB-agar, 4) amplification of the binding phage. Repeating of these steps (phage panning) is performed to enrich target phage and purified by infecting suitable host bacteria. The correct insertion of target peptides or protein fragments is verified by gene sequencing. (104)

CHAPTER 3

DEVELOPMENT OF A NOVEL BACTERIOPHAGE BASED BIOMAGNETIC SEPARATION METHOD AS AN AID FOR SENSITIVE DETECTION OF VIABLE *ESCHERICHIA COLI*

3.1 Introduction

The ability to sensitively and efficiently detect specific bacteria is paramount in many fields of study. In general, standard microbiological methods are based on traditional culture assays such as pre-enrichment, selective and differential culture medium, and serological tests to confirm the presence of targeted bacterial species. The total assay time can require 4 to 6 days to obtain results, which may hinder the timely response or prevention of outbreaks by virulent pathogens. (105-107) In these cases, improved analytical techniques that facilitate efficient bacterial separation and concentration, as well as reduce the assay time have become increasingly important.

Immunomagnetic separation (IMS) offers a simple but effective method to extract target organisms from heterogeneous suspensions, such as food matrices, agricultural water and environmental specimens. (108-110) Harmful foodborne pathogens, including *Escherichia coli* O157:H7, *Staphylococcus aureus*, *Salmonella spp*., and *Listeria monocytogenes* have been separated and detected using IMS combined with methods such as polymerase chain reaction, electrochemiluminescence detection, colorimetric detection, enzyme-linked immunosorbent assay or microfluidic biosensors. (111-116) Safarik and Safarikova found IMS to be superior in performance when compared with conventional culture methods for *Escherichia coli* O157:H7 detection in raw food products. (117) Lynch reported that the use of anti-*Salmonella* beads significantly improved the detection and separation rates of *Salmonella* in environmental samples. (118)

Immunomagnetic separation relies on the use of monoclonal or polyclonal antibodies as biorecognition elements. (119) Generally, the target organism in a mixed suspension can be captured and concentrated by antibodies coated on the surface of magnetic beads. (120) The retained target cells can then be eluted and analyzed after an external magnet is removed. In spite of the satisfactory results provided by this assay, false-positive results can still occur by cross-reactions between non-target components and magnetic beads. (121) Additionally, antibodies typically represent the most expensive part of biological assays. The immobilization of antibodies to magnetic beads is occasionally performed using covalent binding which suggests minor factors, such as pH value, cross-linking time or other changes in solution may easily affect this binding. (122) Alternatively, biotin-tagged antibodies can be used for immobilization onto streptavidin-coated surfaces. This method allows a more targeted approach if the biotin tag can be strategically placed. (123) However, the biotin-tagging can result in a full or partial loss of performance depending on the location of the tag. Thus, more research has focused on utilizing the affinity interaction between bacterial cells and functionalized magnetic particles for separation in complex samples. (121)

Bacteriophages are a group of viruses which target specific bacteria and have therefore been widely used for bacteria typing. (124) The specific recognition of their host bacteria by bacteriophages makes them a potential alternative to antibodies that are frequently used in biosensors. (125) It has been reported previously that by immobilizing *Salmonella*-specific phage on a polystyrene surface, *Salmonella* could be specifically from foods. (126) As the infected target bacteria is used to mass-produce (in a range of 10-1000) progeny of the introduced phage after cell lysis, detection of the target bacteria could base on assaying the phage titre in the solution which only happens at the presence of viable target bacteria. (127, 128)

Phages can be uses to increase the capture efficiency of a specific host bacterium. (129) This could allow higher specificity and accuracy of the downstream detection assay which then decreases the possibility of false positive results. (122) In order to facilitate the detection of target bacteria, many assays have been reported to introduce either affinity tags (biotin tag, Strep-tag and etc.) on phage capsid protein or separate reporter genes followed by monitoring of the gene expression products (green fluorescent protein, luciferase or yellow fluorescent protein). (41, 87, 130, 131) Among the affinity tags, the biotin acceptor protein containing a specific 15 amino acid peptide can be biotinylated through enzyme-mediated coupling of biotin to the acceptor domain. (88) Tolba had published an oriented immobilized bacteriophage T4 capable of capture and infecting *Escherichia coli* B specifically on the solid support. (42) Favrin also reported that biotin-modified bacteriophages could be used for *Salmonella* detection in broth. (132)

The aim of our study was to genetically modify an *E. coli* specific phage to introduce biotin tags on phage capsids that would be applied in bacterial separation and detection. In order to increase the bioinylation efficiency, the gene for the biotin ligase enzyme (*birA*) was incorporated into the phage genome for transcription during infection of a host *E. coli*. Bacteriophage T7 and *E. coli* B were selected to form a model system due to the importance of *E. coli* detection as an indicator of general coliform contamination. (126) We have previously demonstrated the use of T7 to detect *E. coli* with high specificity and sensitivity. (133)

Engineered phage particles were biotinylated *in vivo* during the infection cycle followed by coating on magnetic beads decorated with streptavidin. The phage-bead complexes were used for bacterial separation and the resulting capture efficiencies were investigated. Results from this research could be extended to other species for biosensor applications with an efficient, inexpensive, and technically simple manner.

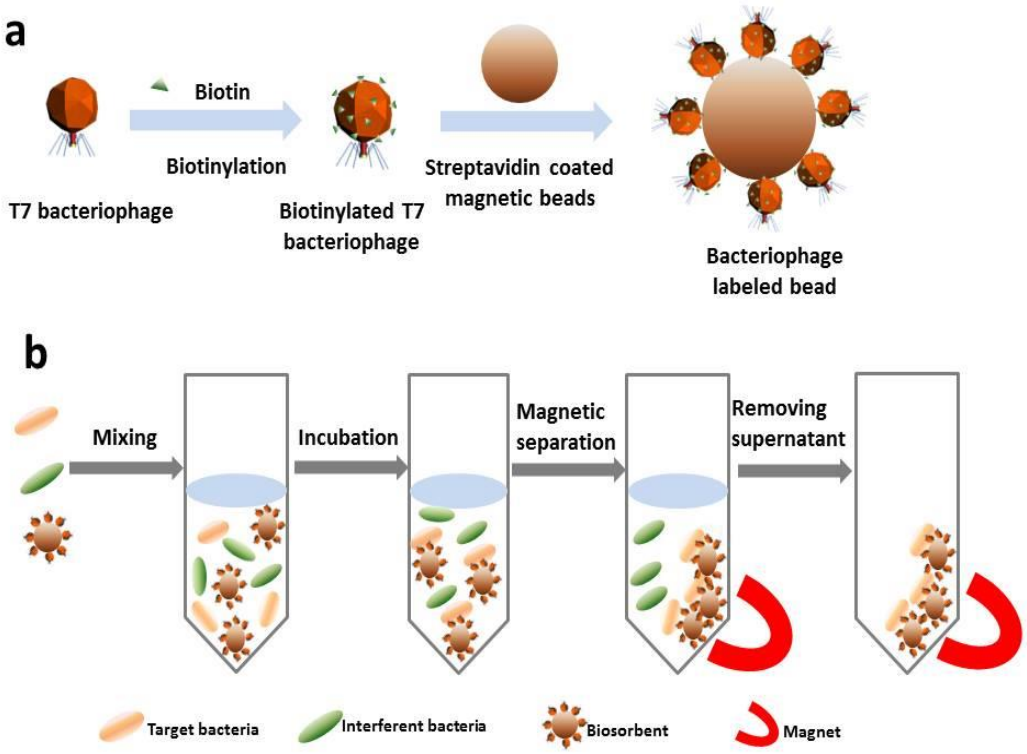

Figure 3.1 (a) Synthesis of phage bead biosorbent by streptavidin biotin interaction; (b) Schematic representation of the strategy for bacterial detection by the biosorbent.

3.2 Materials and methods

3.2.1 Chemicals, reagents and materials

Molecular biology grade water was purchased from Fisher Scientific (Fair Lawn, NJ). DNA molecular standards were purchased from Invitrogen (Carlsbad, CA). QIAquick PCR Purification Kits were obtained from Qiagen (Hilden, Germany). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). iProof™ High-Fidelity PCR kit and PCR reagents were purchased from Bio-Rad laboratories (Hercules, CA). Dynabeads® M-270 Streptavidin was purchased from Invitrogen (Oslo, Norway). Novagen® DNA ligase kit was obtained from EMD Millipore (San Diego, CA). All other chemicals of analytical grade were purchased from Fisher Scientific (Fair Lawn, NJ).

3.2.2 Bacterial strains and culture conditions

Escherichia coli BL21 (ATCC 25922) was used throughout development of the assay in this study. *E. coli* strain was cultured in Luria-Bertani broth (10 g of tryptone, 5 g of yeast extract, 10 g of sodium chloride per liter sterilized water; pH 7.4) by shaking at 150 rpm and incubating at 37°C overnight before use. A working stock of the strains was kept on LB plate (addition of 15 g agar to LB broth per liter) and stored at 4°C. Tenfold serial dilutions of *E. coli* cultures with phosphate buffered saline buffer (PBS: 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 1.8 mmol/L KH2PO4; pH 7.4) were plated on LB agar and incubated at 37°C overnight for viable cell counting. Enumeration of bacteria was expressed in CFU/mL.

3.2.3 Phage engineering

As a model system, Novagen®T7Select System (Madison, WI) was used for phage engineering and packaging. The target gene containing biotin ligase sequence (*birA*) and biotin acceptor peptide sequence (*bap*) was synthesized commercially (GenScript USA Inc., Piscataway, NJ) with designed overhang sequences for ligation with restriction endonuclease (HindIII and EcoRI) digested phage arms. Instead of cloning *birA* onto the phage capsid surface, we designed the T7Select phage to express biotin ligase intracellularly during infection under the control of the strong T7 Φ10 promoter downstream of the T7Select 415 capsid gene *10B*. The DNA fragment was digested with EcoRI and HindIII and purified with Qiaquick columns (Hilden, Germany). Then target DNA sequence was ligated to the arms of T7Select as recommended by the manufacturer. T7Select packaging extracts (Novagen, Madison, WI) was used to package the modified genome $T7_{birA\text{-}bap}$ into the phage head *in vitro* following the manufacturer's instruction. The packaged phage was amplified in liquid media of host *E. coli* BL21 by incubating at 37°C with shaking.

Single phage plaques on the soft agar plate were isolated and selected to verify mutant colonies. (107) The phage colonies were picked by a sterile loop and resuspended into nucleic acid free water. This served as template for the PCR amplification. To confirm the insertion of target gene in the genome of recombinant phage, T7Select primers (forward: 5'-GGAGCTGTCGTATTCCAGTC-3'; reverse: 5'-AACCCCTCAAGACCCGTTTA-3') were used. A PCR reaction was carried out in 20 µL reaction system including 1 µL phage DNA sample, 4 µL iProof HF buffer, 2 µL primer solution (1 µM of each primer), 0.4 µL dNTP mix, 0.2 µL DNA polymerase and nucleic acid free water. PCR amplification settings were as following: 95 °C, 10 min; 35 cycles of 95 °C, 20 s; 60 °C, 30 s; 72 °C, 45 s; then 72 °C for 10 min. Agarose (1.2 %) gel

electrophoresis was performed to separate PCR products in TAE buffer. SYBR® Safe DNA Gel Stain (Invitrogen, Eugene, OR) was used to stain the gel and UV illumination was applied for photograph.

3.2.4 Bacteriophage biotinylation level

The biotinylation efficiency was calculated using a Pierce® Biotin quantitation kit (Thermo Scientific, Rockford, IL) following the manufacturer's instruction. In brief, the purified biotinylated phage (10^{12} PFU/mL) was prepared and added to a mixture of HABA (4'-hydroxyazobenzene-2-carboxylic acid) and avidin in a 96-well plate followed by measuring at 500nm with a spectrophotometer from BioTek (Synergy™ 2, Winooski, VT). The change in absorbance of the HABA-avidin solution before and after addition of the biotinylated sample was measured and the ratio of biotin to modified phage head protein was calculated via the analytical formula provided by the manufacturer.

3.2.5 Bacteriophage propagation and purification

The engineered bacteriophage T7 was used in this study for bacteria separation. Amplification and purification of the phage were performed as following: an overnight *E. coli* culture (1 mL) was inoculated into 100 mL fresh LB broth followed by incubating at 37 °C with shaking (200 rpm) to reach an optical density of 0.6. After inoculating phage stock solution (100 µL) to the culture and incubating for 1.5 hour, the culture was centrifuged at 8000 x g for 10 min to pelletize the bacterial debris. In order to purify the phage, the supernatant was filtered using a 0.22 µm pore size filter (Corning Life Science, Corning, NY) followed by ultracentrifuging (35, 000 rpm, 2 hours) at 4°C. The phage pellet was resuspended in PBS buffer (1 mL) and stored at

4°C for future use. The phage titer was determined by double layer agar method as described by Adams. (107) Briefly, the phage solution (100 µL) and overnight *E. coli* culture (250 µL, 10^8 CFU/mL) were mixed with 3 mL top agar (10 g tryptone, 5 g yeast extract, 10 g sodium chloride and 7.5 g agar per liter; cooled to 40-50°C) and poured over the LB agar plates following incubation at 37°C for 3 hour. Enumeration of phage was expressed in PFU/mL.

3.2.6 Growth curve of the bacteriophage

To determine the burst sizes and latent period of recombinant phage T7, one-step growth curve experiment was conducted as described by Park and Leuschner with minor modifications. (134, 135) Firstly, log-phase *E. coli* B culture in fresh LB broth (100 mL) was prepared and incubated at 37°C. Then a phage suspension (100 µL, 10^8 PFU/mL) was added into 2.0-ml Eppendorf tube that contained 1 mL of the cell culture to give a multiplicity of infection of 0.1. The phage-*E. coli* solution was diluted at a ratio of 1:10^4 in PBS followed by incubation at 37°C with shaking (200 rpm). Starting at 0 min and with 10 min intervals for 1 h, the diluted suspension was enumerated through plaque assays as described previously. Latent time and burst size were calculated from this growth curve.

3.2.7 Preparation of bacteriophage based biosorbent

The magenetic beads were washed three times with PBS (pH 7.4) before use. To construct phage based biosorbent beads, biotinylated phage $T7_{birA\text{-}bap}$ (1 mL, 10^9 PFU/mL) was incubated with streptavidin coated magnetic beads (100 µL, 10^8 beads/mL) with mild agitation (10 rpm) at room temperature. After the phage-bead conjugation reaction, the bound phages were able to be removed using magnetic separation. The biosorbents were washed three times

and resuspended in 100 µL PBS (pH7.4) until further use. Wild type T7 bacteriophage ($T7_{WT}$) which served as a control was conjugated similarly. To calculate the immobilization efficiency, the titers of phage in the initial solution and that of supernatant solution (after capture by magnetic beads) were enumerated. The immobilized phage was assessed by subtraction of the two titers. In order to optimize the immobilization efficiency, different phage-beads incubation times (1 h, 6 h and 12 h) were investigated.

Additionally, the infectivity of the biosorbent beads was verified by examining the presence of plaques around plated beads. Briefly, the biosorbents were mixed with overnight *E. coli* culture (250 µL) and top agar (3 mL) followed by placing on LB agar plate and incubating overnight (at room temperature). All experiments were performed in triplicate.

3.2.8 SEM imaging

Scanning electron microscopy (SEM) was performed by using a FEI SEM Magellan (Hillsboro, OR) at 5 KV. Beads and bacterial cells samples were prepared in 2.5% glutaraldehyde at 4°C overnight. After fixation, these samples were rinsed twice with distilled water and dried, followed by dehydrating in gradient concentrations of ethanol. Then samples were coated with gold for 30 second with sputter coater Cressington 108 auto (TED PELLA, INC., Watford, UK).

3.2.9 Bacterial capture efficiency of the biosorbent

Manual magnetic separation was performed using 1 mL samples in micro centrifuge tubes. A scheme of the bacteriophage-MS assay was illustrated in Fig. 1. In brief, overnight *E. coli* culture with tenfold serial dilutions were prepared to make 10^2 to 10^6 CFU/mL cell suspension, as confirmed through viable plate counting. Then an aliquot (20 µL) of the

constructed biomagnetic beads was added to bacterial samples (10^2 to 10^6 CFU/mL) and incubated (room temperature) with agitation (10 rpm) for 15 min. The bead-bacteria complexes were then separated and concentrated using a magnetic separator rack from BD IMag™ (Sparks, MD) for 3 min. The supernatant (100 µL) was collected and spread on LB agar plates for quantification and the sample was aspirated and discarded. Bead-bacteria complexes were washed three times with PBS (1 mL) and resuspended in the same buffer in 100 µL for further analysis. *E. coli* cells in the initial dilutions and the supernatant were enumerated respectively to calculate the number of captured cells. Capture efficiency (CE) was defined as the percentage of the total bacterial cells captured by the biomagnetic beads and calculated using the following equation:

$$CE\ (\%) = 100\times(1-C_s/C_I);$$

Where C_I is the initial number (CFU/ mL) of cells present in the sample and C_s is the number (CFU/mL) of cells in the supernatant which is unbound to biomagnetic beads. (136)

3.2.10 PCR assays

To verify the ability of biosorbent beads to capture and infect viable bacteria, PCR assay was carried out after magnetic separation of *E. coli* cells. The bead-bacterial complex was resuspended in sterile water and incubated (20 min) for lysis which allowed release of the bacterial DNA. The magnetic beads were removed and one microliter of the supernatant was used as DNA template for PCR reaction. As a control, heat treated (65 °C, 10 min) bacteria samples (dead cells) were performed in the same way as non-heat treated bacteria (live cells). (137) Universal primer 27F and 1492R (1 µM) in a total volume of 2 µL were used for PCR. The PCR amplification was performed under the following conditions: 98 °C, 10 minute, 35 cycles of

98 °C, 10 sec, 55 °C, 30 sec, 72 °C, 30 sec followed by a final extension at 72 °C for 10 minutes. Gels were run at 120 V in TAE buffer for 30 minutes. PCR products were separated by agarose gel electrophoresis and visualized under UV illumination as previously described.

3.3 Results and discussion

3.3.1 Engineering the reagent phage

As depicted in Figure 3.2 a, biotin ligase in $T7_{415}$ was encoded as a non-fusion protein downstream of gene *10B* and *bap*. To construct the mutant, we designed a stop codon at the end of *bap* and a ribosome binding sequence (RBS) upstream of the biotin ligase gene. The T7 promoter in front of gene *10* drives transcription of the biotin acceptor peptide and biotin ligase in front of gene 10. The construct was packaged and amplified in *E. coli* culture. The resulting engineered phage ($T7_{birA\text{-}bap}$) was confirmed by PCR and sequencing as containing the appropriate gene (*birA*-*bap*) fragment. When the DNA sequence of *birA*-*bap* was incorporated into the genome of phage $T7_{415}$, we could identify the corresponding band at the size of ~1.3 kb. The control $T7_{WT}$ genome did not yield a detectable PCR amplicon (Fig. 3.2b). This cloning strategy resulted in the display of the BAP peptide on the phage capsid protein and intracellular expression of biotin ligase (BirA). Capsid proteins could then be biotinylated either *in vivo* or *in vitro* following lysis.

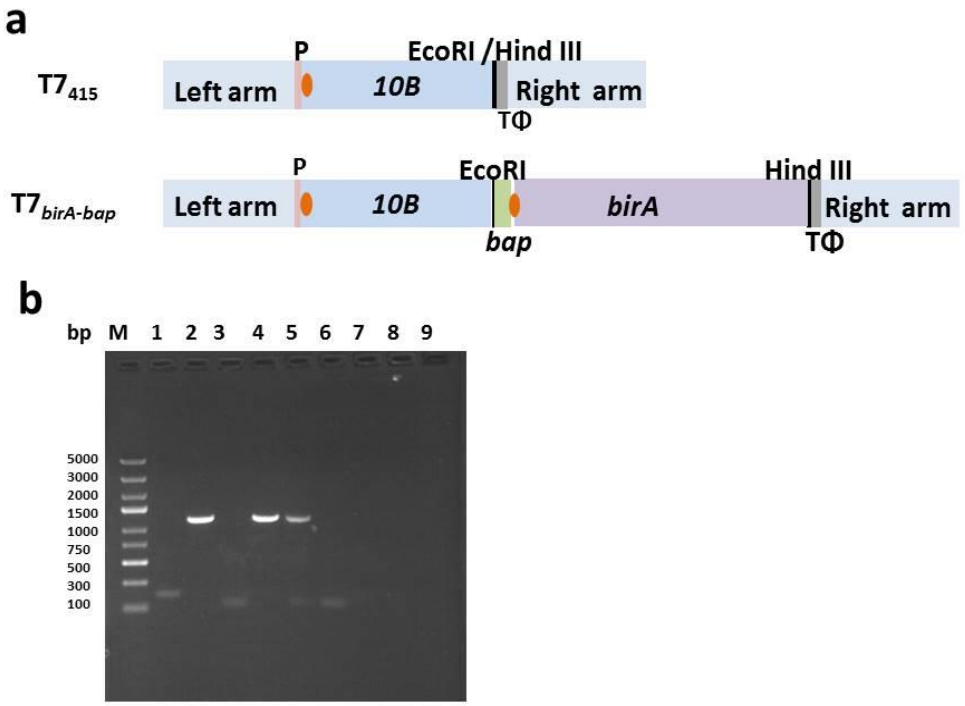

Figure 3.2 (a) Schematic representation of the recombinant construct. Cloning vector (T7415) included a T7 promoter (P) in front of gene 10B, a ribosome binding sequence (orange dot), T7 terminator Tϕ (gray) and restriction sites Eco RI & Hind III (black and vertical lines). Construct T7birA-bap containing the target gene between Eco RI and Hind III included a stop codon (white line) terminating the translation of 10B and BAP (fused with 10B), T7 promoter with a ribosome binding sequence and T7 terminator downstream of the cloning site. (b) Agarose gel electrophoresis showing the insertion of birA-bap into phage genome. Lanes 1 to 5, PCR products of engineered phage with a size of ~ 1300 bp, indicating the insertion of birA-bap; lanes 6 to 9, fragments obtained from T7WT phage as a negative control for the PCR; lane M, GeneRuler express DNA ladder.

3.3.2 Characterization of the recombinant phage

3.3.2.1 Biotinylation level

We used the commercially available kit containing HABA reagent for the colorimetric determination of biotinylation levels in labelled phage capsid proteins. The average number of biotins per modified phage ($T7_{birA\text{-}bap}$) was estimated to be 136.4±7.3, which was 32.9±1.8% of

the total peptides (415) displayed, while the control phage ($T7_{bap}$) had only 8.8±4.4% of the total peptides biotinylated. The result was in agreement with a previous report that recombinant proteins with the expression of biotin acceptor peptide in high and low levels were typically biotinylated at ~6% and 30% efficiency, respectively (41).

3.3.2.2 One-step growth curve

One-step growth curve experiment was determined to compare different characteristics of the recombinant phage with the wild type one. Generally, burst size and latent period had a progressive correlation: optimal latent period could induce high fitness of phage that would contribute to burst size or plaque size (107, 138). Burst size was calculated as the ratio of the final titer of released progeny phage to the original count of host cells that infected during the latent period (138). These parameters may be affected by many factors, including the host species, incubation temperature, medium components and bacterial physiology (129). As Fig. 3. 3 indicated, the latent period for the wild type phage was ~20 min and the burst time was 40 min with a burst size of 167±33 PFU; the recombinant phage latent period was ~30 min, with a burst time and size of 30 min and 218±80 PFU, respectively. The burst size of the wild type phage was similar to previously reported values. Nguyen and Kang had reported a latent period of 16.8 min and a burst size of 179 PFU when BL21 was infected by T7 phage at 37 °C (139). Thus, insertion of *birA-bap* into the T7 genome led to a prolonged latent period and enlarged bursts, which was in accordance with the normal relationship between burst size and latent time to maximize phage adaptation to environments: small burst size was compensated for by short latent time, while prolonged latent period usually yielded larger burst size (42).

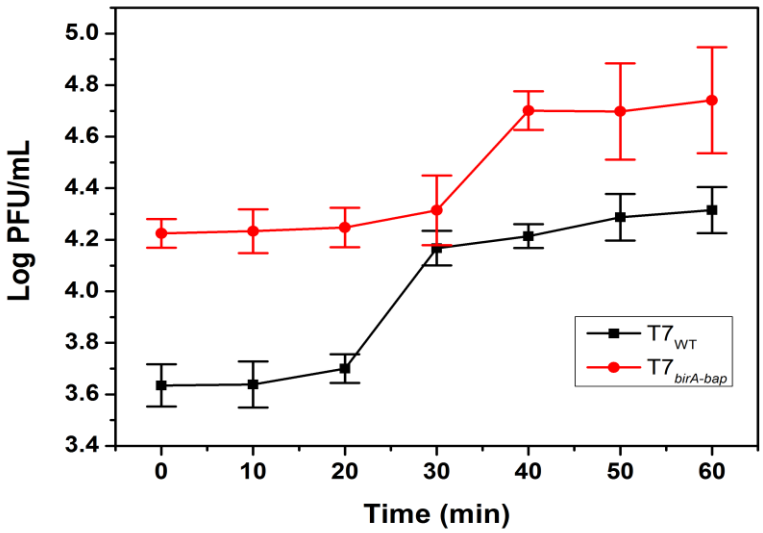

Figure 3. 3 One-step growth curves of the T7WT and T7birA-bap. Vertical error bars represented standard deviation.

3.3.3 Characteristics of the phage based biosorbent

3.3.3.1 Infectivity

The infectivity of bacteriophage based biosorbent was investigated by plaque assay to verify progeny phage released from *E. coli* cells that infected and lysed by the immobilized phage particles (140). After 3 h incubation, the plates were inspected to verify the presence of clear plaques produced by phage-coated beads when applied to *E. coli* lawn. The zone of lysis indicated that the immobilized recombinant phage maintained its infectivity to lyse *E. coli* in the lawn and not inactivated by the sorption on the beads.

3.3.3.2 Immobilization density

As the results shown in Figure 3.4, the immobilization density of the biotin labelled phage (10^9 PFU/mL) on magnetic beads after different incubation time (1 h, 6h and 12 h) was 29.8±9.2%, 43.9±12.2% and 82.8±5.1%, respectively. This was significantly different from the wild type phage which had no significant difference with respect to incubation time (from 4.3±1.6% to 5.1±2.4%). These results indicated that biotinylated phage particles were immobilized on the streptavidin coated magnetic beads due to the strong bond between biotin and streptavidin rather than physical absorption. To estimate the value of phage per bead, the number of immobilized phage particles was divided by the number of magnetic beads applied in the reaction. The result was summarized and compared in Table 3.1. The phage density on the surface of the beads was 89.3±27.7 PFU/bead, 131.5±36.7 PFU/bead, and 248.3±15.3 PFU/bead in 1, 6, and 12 hours, respectively.

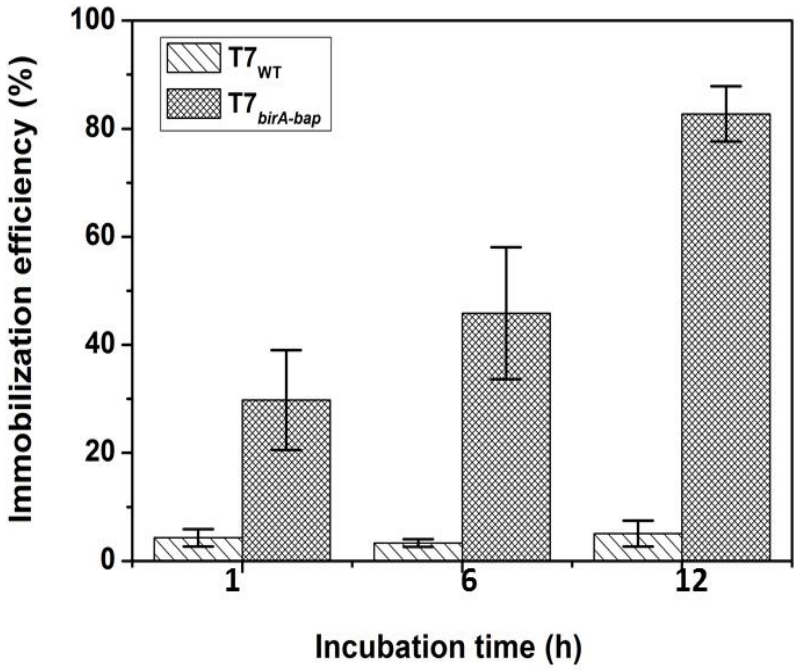

Figure 3.4 Immobilization efficiency of biotinylated phage on streptavidin coated magnetic beads after different incubation time (1h, 6h and 12h). T7WT phage was served as control. Error bars represented the standard deviations of six measurements.

Table 3.1 Summary and comparison of phage per bead after different immobilization times. Values represent the average of three independent tests ± the standard deviation

Incubation time	Immobilization efficiency	Phages per bead
1 h	29.8±9.2%	89.3±27.7 PFU
6 h	43.9±12.2%	131.5±36.7 PFU
12 h	82.8±5.1%	248.3±15.3 PFU

3.3.4 SEM image

As shown in Fig. 3.5, *E. coli* cells captured by phage immobilized magnetic bead were observed with a scanning electron microscope (SEM). The binding between *E. coli* cells and the biosorbent also indicated the specificity and high affinity of the biotin labelled phage to streptavidin coated magnetic bead mediated by biotin-streptavidin interactions.

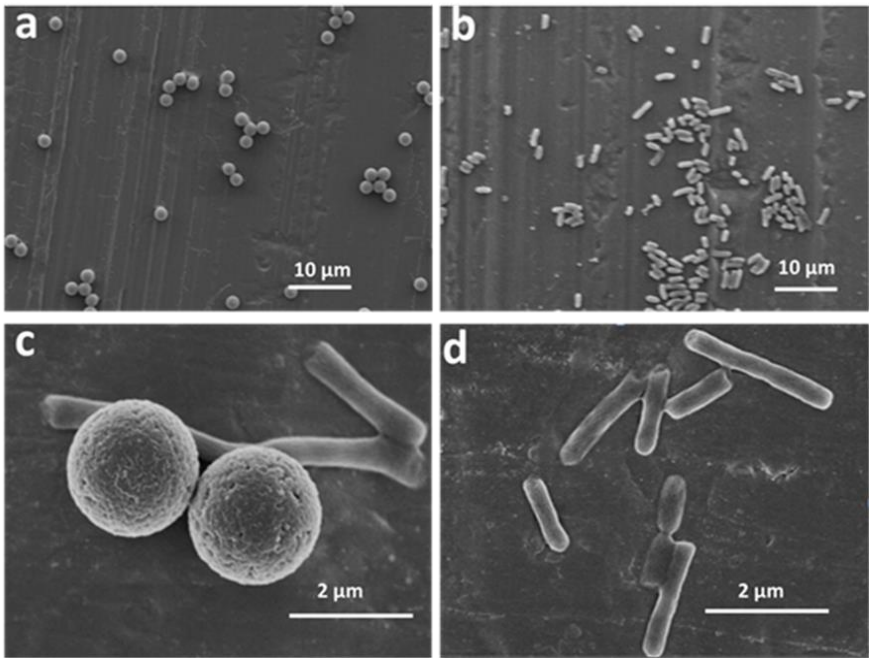

Figure 3.5 Scanning electron microscopic images of beads with *E.coli* cells: (a) Phage based magnetic beads (biosorbents); (b) *E. coli* cells (appear as rods); (c) High magnification of *E. coli* cells captured by the biosorbents; (d) High magnification of *E. coli* cells.

3.3.5 Capture efficiency of the phage-bead biosorbent

To estimate the ability of immobilized phage particles for bacterial capture and infection, phage-based biosorbents constructed using different immobilized phage densities (89.3±27.7 PFU/bead, 131.5±36.7 PFU/bead, and 248.3±15.3 PFU/bead) were investigated. As longer incubation time (exceed 30 minutes) with bead-phage complex could result in lysis of

bacteria, a shortened capture time (about 15 minutes) was performed prior to magnetic separation.

To assess the capture efficiency of phage bead biosorbent for *E. coli* cells (10^2-10^6 CFU/mL) in broth, the depletion of the number of *E. coli* from initial solution was determined. Significantly lower CEs were obtained with the use of $T7_{WT}$ conjugated magnetic beads than with $T7_{birA\text{-}bap}$ conjugated magnetic beads (Fig. 3.6). For the biosorbent coated with 89.3±27.7 PFU/bead, 50.7±9.2% of *E. coli* (10^2CFU/mL) was captured compared with to 17.1±7.2% captured by the control (Fig. 3.6a). The CEs of 131.5±36.7 PFU/bead increased to 69.1±9.4% accordingly (Fig. 3.6b). For the biosorbent conjugated with 248.3±15.3 PFU/bead, 86.2±4.7% of *E. coli* cells (10^2CFU/mL) were captured (Fig.3. 6c). Under the same conditions, capture efficiencies for the control remained ～10% to 20% (Fig. 3.6b-c). For all phage densities, an increasing bacterial load resulted in decreased capture efficiencies. This is typically due to a decreased number of particles per bacterial cell resulting in lower separation velocities. The results demonstrated the relationship between the density of the immobilized phages and the capture efficiencies at different *E. coli* concentrations.

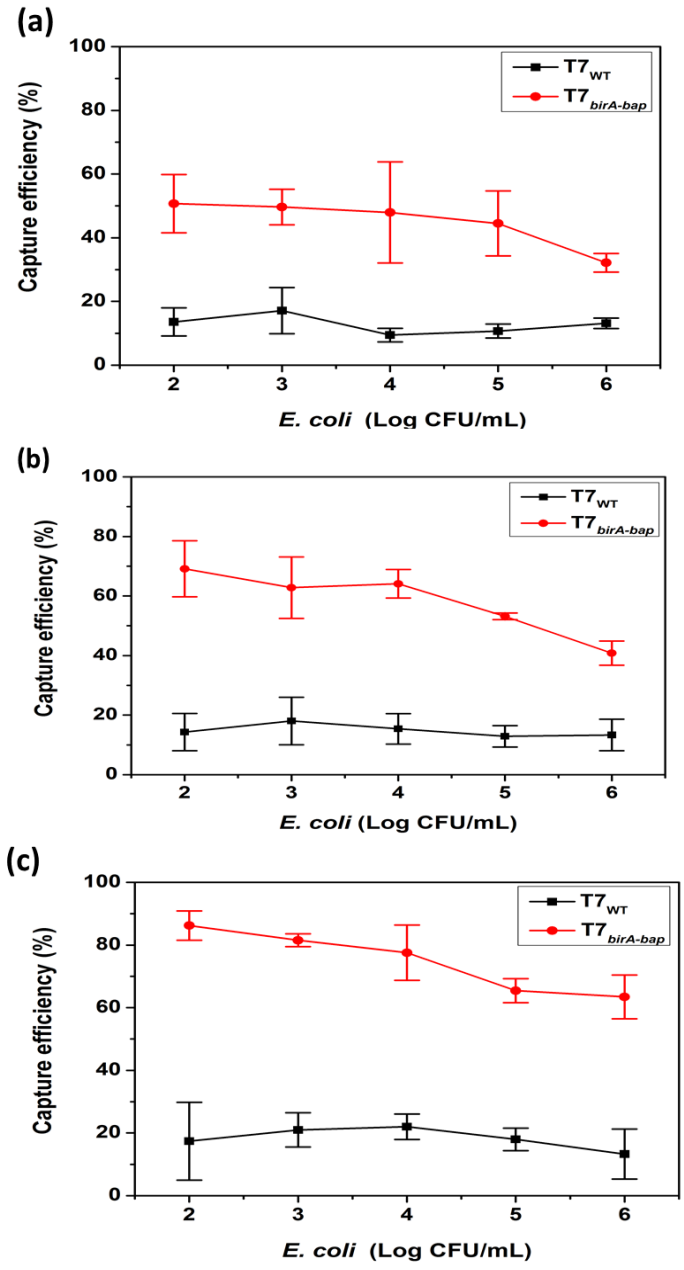

Figure 3.6 Evaluation of the constructed biosorbents: (a-c) Capture efficiency of the biosorbent immobilized with 89.3±27.7 PFU, 131.5±36.7 PFU and 248.3±15.3 PFU phage particles per bead, respectively. The time for *E. coli* BL21 cells attachment was 15 min. Wild-type T7 phage was served as a control. The error bars represented the standard deviations of six measurements.

3.3.6 PCR detection of viable *E. coli* captured by the biosorbent

The capture ability of viable cells was tested by PCR amplification after biosorbent separation of *E. coli* cells (heat treated and non-treated). Bacterial samples were performed in the same biomagnetic separation and PCR amplification operation. As indicated in Fig. 3.7, all of the non-heat treated samples (10^2 CFU/mL) yielded a PCR product band. While the heat treated did not contain an observable PCR amplicon.

Although non-viable *E. coli* may still be separated by the phage-bead complex, the adsorption would not result in an infection. Without a phage replication cycle, no lysis step would occur and the bacterial nucleic acids would not be deposited in the sample. In this case, the non-viable bacteria would be removed with the beads and the nucleic acids from viable bacteria would remain in the sample for PCR.

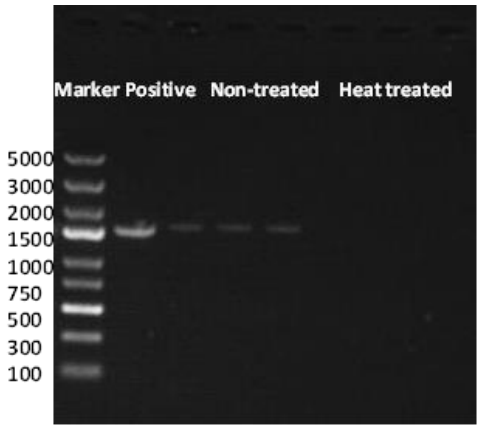

Figure 3.7 PCR detection of heat treated and non-treated *E. coli* cells captured by the biosorbent.

3.4 Conclusions

In our study, the genetically engineered phage was successfully biotinylated *in vivo* which offered a useful label for rapid and specific detection of bacteria. The efficiencies of bacterial capture by the immobilized phage particles were significantly different between the recombinant phage based biosorbents and the control. Approximately 86% of bacteria were captured by the constructed phage bead complexes which resulted in a limit of detection 10^2 CFU/mL. In conclusion, combined with PCR, the captured bacteria could be detected in less than 3 h. Compared with other published detection techniques, the proposed assay offers superiority in both sensitivity and assay time (Table 3.2). In addition, this developed novel separation method could also be applied to other bacterial specific phage which would open new avenues for the development of biosensors to detect bacteria efficiently and in a user friendly way.

Table 3.2 Comparison and summary of detection techniques for *E. coli*

Detection assay	Detection time (h)	Sensitivity (CFU/mL)
IMS-enzyme immunoassay	<48	10^4-10^6
IMS-microarray	6	10^3
IMS-PCR	3-4	4.52×10^2
IMS	<24	40-10^2
IMS (Immunomagnetic nanobeads based)	1	10^4
IMS-phage based	4-5	10^4
IMS-ELISA	3-4	2.6×10^5
Our study	<3	10^2

CHAPTER 4

APPLICATION OF BACTERIOPHAGE-CONJUGATED MAGNETIC PROBE FOR *ESCHERICHIA COLI* O157:H7 DETECTION IN WATER SAMPLES

4.1 Introduction

Serotype O157:H7 *Escherichia coli* is an important food-borne and water-borne pathogen that can cause outbreaks of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome. (141) It belongs to the broader class of Shiga-toxin producing *E. coli* (STEC) within which, *E. coli* O157:H7 has been the primary serotype that can cause human illnesses and deaths. (142) In addition, infection of this harmful pathogen is of particular concern in elderly people and young children as it is associated with hemolytic uremic syndrome which may permanently damage the kidneys. (143, 144) Outbreaks and sporadic cases of STEC infections have been reported worldwide and estimated to cause about 73,000 foodborne illnesses and approximately 61 deaths in the United States per year. (145, 146) Since 1983, more than 100 outbreaks of *E. coli* O157:H7 have been reported, with a substantial economic impact of the contamination of foods. (147) It has been estimated that E. coli O157:H7 infections cost around $ 405 million each year in the USA, including the costs of medical care, premature deaths and lost productivity.(146, 148) Thus, development of new techniques for rapid and effective detection of *E. coli* O157:H7 is crucial for food safety and public health.

The sources of *E. coli* O157:H7 infections include raw or undercooked meat products, unpasteurized milk, apple cider, fermented sausage, cheese, sprouts, spinach and ready-to-eat salad.(145, 149, 150) The other vehicle for illness associated with this pathogen includes contaminated drinking water, surface waters and water used in the irrigation of vegetables and fruits that contaminated with manure or cattle feces.(151, 152) In the recent decades, a number of *E. coli* O157:H7 outbreaks have been associated with contaminated water.(153, 154) In

addition, the surviving ability of this pathogen in water has been indicated for extended periods which emphasizes the importance of separating and detecting *E. coli* O157:H7 in water samples.(155) It has been reported that the infectious dose of *E coli* O157:H7 is between 10 to 100 colony-forming units (cfu) while detection at this low concentration is always challenging and needs long time pre-enrichment before analysis.(156) Traditional methods used to detect *E. coli* O157:H7 with selective media followed by biochemical identification and serotype confirmation are time-consuming and take up to 5–6 days.(157, 158) Thus, alternative ways that can bypass or reduce the time required in cultural enrichment is of great importance.(159, 160)

Nowadays, many advanced methods including enzyme linked immunosorbent assays (ELISA),(150) polymerase chain reaction (PCR) amplification,(161) DNA microarrays (162) and etc. have provided a useful tool for the sensitive detection of *E. coli* O157:H7 from various samples.(151) Although some of these methods allow a rapid detection, there still exist inadequateness of distinguishing viable cell from inactivated cell or avoiding false positive results that caused by the presence of DNA from non-viable bacteria.(18) In this case, multiple assays are in great need to ensure the sensitivity and specificity for accurate detection and quantification. Bacteriophages (phages) as are viruses that require viable bacteria to produce progeny particles offer a valuable way to circumvent this issue and provide a platform for viable bacteria detection.(163) Compared with antibodies which are expensive and inconsistent, phages are inexpensive, stable and essay to produce with robustness.(164) They target host bacteria with high specificity without affecting other bacteria.(165) The application of phage mediated assay for identification of specific bacteria has recently gained increased interest due to these features. Goodridge had reported using a fluorescent bacteriophage based assay for the detection of *E. coli* O157:H7 in broth with a detection limit of 10^2-10^3 cfu/mL.(166) Hoang

published a phage based colorimetric method that could detect E. coli O157:H7 in apple juice with a low concentration of 1 cfu/mL in 15 h.(167) The characteristics of specific and rapid interaction with target cells had made phages a potent alternative to antibodies served as a promising recognition element for bacterial capture and detection.

Currently, *E. coli* O157:H7 capture and concentration from sample matrix accompanied with high levels of natural microbial population, can become very difficult.(168) The efficiency of the following detection techniques such as quantitative real-time PCR (qPCR) can be seriously affected by the inhibitors present within the complex sample matrix.(54) Thus, magnetic separation (MS) technology combined with qPCR assay has been found effective to detect and quantify small numbers of water-borne *E. coli* O157:H7 which also improve the detection efficiency.(169)

The purpose of this study was to develop a biomagnetic separation method by using magnetic bead-bound phage for target capture (*E. coli*) in combination with qPCR for detection. The magnetic beads were conjugated with *E. coli* O157:H7 phages via the carbodiimide method (by amide bond). The captured bacteria were allowed for lysis as part of the natural phage infection cycle. DNA released from the capture cells were used as the template for qPCR reaction to provide quantitative analysis (Figure 4.1). This proposed detection principle has proven to be rapid and selective for viable bacteria identification as well as cost effective due to the advantageous self-replicating and self-assembling characteristics of phage particles.

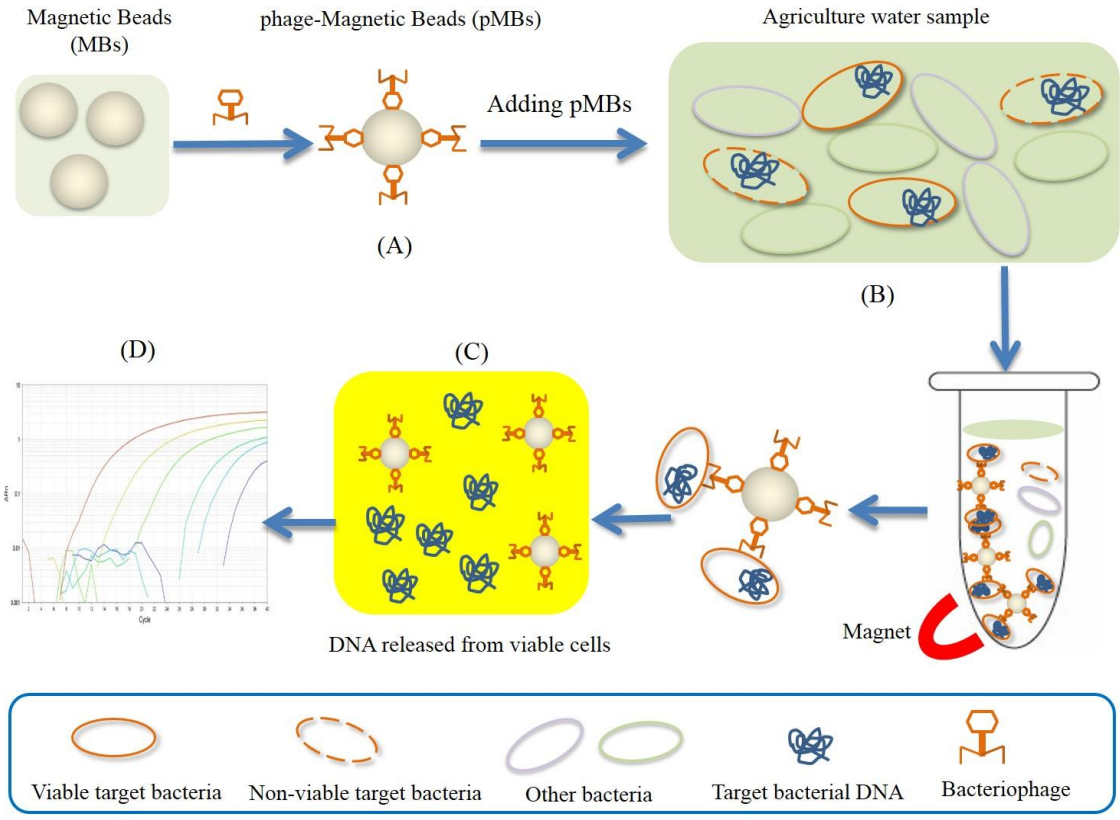

Figure 4.1 Schematic representation of pathogen detection of *Escherichia coli* O157:H7 in water samples by bacteriophage mediated magnetic separation combined with real time PCR.

4.2 Materials and method

4.2.1 Chemicals and Materials

N-hydroxysuccinimide (NHS), 2-(NMorpholino) ethanesulfonic acid (MES) hydrate and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) were obtained from Acros Organics (Morris Plains, NJ). Glutaraldehyde, bovine serum albumin (BSA), yeast extract, tryptone and agar were purchased from Fisher Scientific (Fair Lawn, NJ). Dynabeads® M270 carboxylic acid were obtained from Invitrogen (Oslo, Norway). MS washing steps were carried out with phosphate buffered saline buffer (PBS, 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, 0.24 g of KH_2PO_4; pH 7.4, in 1 L distilled water). A Millipore Mill-Q System (Thermo Scientific,

Asheville, NC) was used to prepare purified water with resistivity of 18.2 MΩ cm at 25 °C. Buffers and medium were sterilized by autoclaving at 121 °C before use. Other chemicals were reagent grade and used as received.

4.2.2 Bacterial strains and culture conditions

Escherichia coli O157:H7 (*E. coli* O157:H7; ATCC 35150) from frozen culture stock was streaked onto a Luria Bertani (LB) plate and incubated at 37 °C for 24 h. The specificity of the qPCR protocol was assessed by using other foodborne pathogens, including *Salmonella enterica* (*S. enterica*; ATCC 14028), *Staphylococcus aureus* (*S. aureus*; CD 489), and *Pseudomonas aeruginosa* (*P. aeruginosa*; CD 1006). The bacteria were stored at −80 °C in LB broth containing 30% glycerol. Prior to each experiment, a single colony was picked from the streaked LB plate and inoculated into LB broth for overnight growing at 37 °C with agitation (200 rpm). To collect the cell, fresh overnight bacterial culture was centrifuged (8000 g, 10 min) and washed with PBS buffer followed by tenfold serial dilution in the same buffer. The bacterial concentration (colony forming units (cfu)/mL) was enumerated from culture dilutions grown on LB plates. Diluted bacteria in PBS buffer at desired concentrations were prepared for further experiments.

4.2.3 Bacteriophage preparation

E. coli O157:H7 phage ECML-134 (ATCC PTA-7949) and ECML-134(ATCC#PTA-7949) were used in this study. The phage titer was enumerated by the double layer agar method.(107) In brief, the phage solution (100 µL) and overnight *E. coli* culture (250 µL, 10^8 cfu/mL) were mixed with 3 mL top agar (10 g tryptone, 5 g yeast extract, 10 g sodium chloride and 7.5 g agar per liter; cooled to 40-50°C) and poured over the LB agar plates following

incubation at 37°C for 3 hour. Titer of phage was expressed in plaque forming units (pfu)/mL. The phage was propagated and purified prior to conjugation with magnetic beads. Briefly, 100 µL of phage suspension (containing 10^8 pfu/mL) was added into 35 mL of a midexponential phase *E. coli* O157:H7 culture (OD600=0.6-0.8) followed by incubation at 37 °C for 1.5 hour to allow lysis. Lysate was centrifuged (7000g, 10 min) and the supernatant was collected following filtration through a 0.22-µm pore-size syringe filter (Corning Life Science, Corning, NY). Then the phage solution was concentrated by ultracentrifuge (35,000 rpm, 2 hour) and resuspended in PBS buffer (pH 7.4, 1mL) and stored at 4 °C until use.

4.2.4 Preparation of phage-conjugated magnetic beads (MBs)

The phage-MBs were prepared according to the manufacturer's instruction with slight modifications.(170) The amino groups on phage proteins allowed for covalent binding with the carboxylic acid group on the activated magnetic beads via an amide linkage. Initially, magnetic beads (100 µL, 10^8 beads) were washed twice with 25 mM MES (pH 5) and resuspended in the same buffer (1 mL). Immediately before use, EDC (200 µL, 20 mg/mL) and NHS (100 µL, 20 mg/mL) solutions were added to the washed beads following incubation with slow rotation at room temperature for 30 min. The supernatant was removed and magnetic beads were washed three times with ice-cold PBS (pH 7.4). After activation, phage stock (100 µL, 10^{10} PFU/mL, in PBS) was added and agitated slowly overnight at 4 °C. The phage-MBs were separated, washed and resuspended in PBS-BSA (PBS containing 0.1% (w/v) BSA (1 mL, 1 mg/mL)) for 2 hour to block any uncoated surface on the beads. After separation and washing, the blocked beads were stored in PBS (pH 7.4, 1mL) at 4 °C for further use.

4.2.5 Water qualities

Three types of water samples were used in this study. The agricultural water was collected from a local farm land. The drinking water and tap water were collected from water fountains and lab taps at University of Massachusetts, Amherst, respectively. These water samples were characterized by using water quality parameters including temperature, pH, turbidity and *E. coli*. Sample pH was measured using a Fisher Scientific Accumet Research AR25 pH/mV/°C/ion selective electrode meter. Turbidity was measured with a Hach model 2100Q portable turbidimeter (Hach Company, Loveland, CO). Temperature was measured with a Fisher Scientific thermometer. *E. coli* O157:H7 was tested by selective MacConkey agar plating method.(162) An aliquot of this water was served as negative control and others were inoculated with target bacteria.

4.2.6 Artificially contaminated water sample

Water samples (900 µL) were spiked with appropriate dilutions of overnight cultured *E. coli* O157:H7 (100 µL) to achieve the final concentrations ranging from 10^2 to 10^7 cfu/mL. Select solution (900 µL, containing 10^3 cfu *E. coli* O157:H7/mL) were mixed with a suspension (100 µL) of three other common water borne pathogens (*S. enterica*, *S. aureus* and *P. aeruginosa*) which were prepared at concentration of 10^4 cfu/mL. Artificially contaminated samples were used immediately and each sample (1 mL) was exposed to the desired amount of phage conjugated MBs. All water samples were tested as negative for *E. coli* O157:H7 by both selective plating and PCR methods before use.(120)

4.2.7 Bacteria cells capture and separation

The phage conjugated MBs (100 µL of 10^6 to 10^8 beads) were exposed to artificially contaminated water samples (1 mL) at desired contamination rate (10^3-10^6 cfu/mL) and incubated at room temperature for 15 min with gentle rotation. Following incubation, the microcentrifuge tube containing bacteria-beads complexes was inserted into a magnetic apparatus for 5 min and the supernatant was pipetted out carefully. The *E. coli* bound beads were washed twice to remove unbound cells with PBS, resuspended in PBS (30 µL) and subjected to qPCR. Bacterial capture efficiency (CE) was assessed with the following equation:

$CE(\%)=100\times(C_0/C_I)$;

where C_I is the initial number of cells in the sample and C0 is the number of captured target cells quantified by qPCR assay.(171)

In addition, Dynabeads anti-*E. coli* O157 was used following the manufacturer's instruction for the *E. coli* capture in parallel with the phage-based separation. All the experiment was performed in triplicate.

4.2.8 Imaging captured bacteria

For SEM images, the samples were fixed within 2.5 % glutaraldehyde solution overnight at 4 °C followed by dehydrating with serially diluted ethanol solutions. Scanning electron microscopy (SEM) images were obtained using FEI SEM Magellan (Hillsboro, OR) at voltage of 5 kV and current of 13 pA.

4.2.9 Real time PCR reactions

The real time PCR reaction was performed according to the manufacturer's protocol for TaqMan assay using MicroSEQ® *E. coli* O157:H7 detection kit (Life Technologies, Warrington, UK). The thermal cycling conditions were 95 °C with one cycle of initial denaturation of template DNA and activation of Taq DNA polymerase for 3 min, 40 cycles of 95 °C of denaturation for 3 s, and primer annealing at 60 °C for 20 s. A standard curve was constructed using ten-fold serial dilutions of *E. coli* O157:H7 suspensions (enumerated culturally) that exposed to phage-MBs and incubated for DNA release from the captured bacteria following subjected to qPCR. The data were plotted into Ct value (Y axis) as a function of the logarithm of *E. coli* concentration (X axis). (172) In all runs, a negative control of amplification was performed using 30 µL of water instead of bacterial template. Applied Biosystems® 7500 fast Real-Time PCR System (Thermo Fisher Scientific) was used for the assay. For unknown sample, the bacterial number recovered after phage MBs separation was estimated from the standard curve based on Ct values obtained from qPCR after capture. The capture efficiency (CE) was calculated as the percentage of the estimated cell number (after capture and PCR detection) to the initial spiked cell number.

4.2.10 Data analysis

Bacterial numbers were in terms of log values. A regression line was fitted to the plot of log numbers of *E. coli* captured by the beads probe against Ct values. A linear relationship and value for the regression coefficient (R^2) was obtained by calculating in Microsoft Excel 2010 (Microsoft Corp., Redmond, WA).

4.3 Results and discussion

The proposed method for *E. coli* O157 screening was dependent upon the detection of DNA released from viable *E. coli* cells following phage-mediated lysis. Although numerous PCR based assays for *E. coli* O157 detection have been reported, these typically depended on the presence of DNA with no indication of viability. On the other hand, accurate analysis by qPCR relies on efficient removal of PCR inhibitors and extraction of DNA. In this case, phage based magnetic separation not only assures signals (DNA) from viable bacteria but also avoids the use of organic or toxic reagents which simplifies the procedure. As the bio-capture efficiency is influenced by its microenvironments, reaction temperature, pH conditions, ionic strength, reaction time and the volume of pMBs are optimized in this study for a maximum bacterial capture. Our study applied two *E. coli* O157 specific lytic phages (Table 4.1and Table 4.2) for targeted bacterial DNA release and indicated that the phage-based magnetic separation provided specificity for target bacteria capture even under the interference of high numbers of background micro-flora.

Table 4.1 Bacteriophage lytic specificity for target bacteria, *E. coli* O157:H7 strains

E. coli O157:H7 strain number	Phage susceptibility	
	ECML-117	ECML-134
ATCC 700728	+	-
ATCC 35150	+	+
ATCC 43894	+	+
ATCC 43895	+	+
ATCC 43889	+	+

"ATCC" means the American Type Culture Collection;
"+" means lytic activity was detected
"-" means lytic activity was not detected.

Table 4.2 Bacteriophage lytic specificity for non-target bacteria, non-O157:H7 strains

Bacterial strain	Phage susceptibility	
	ECML-117	ECML-134
E. coli BL21	-	-
E. coli K12	-	+
S. aureus	-	-
Salmonella	-	-
P. aeruginosa	-	-

"+" means lytic activity was detected
"-" means lytic activity was not detected.

4.3.1 Optimizing bio-magnetic separation using pMBs

Different volume of phage-MBs (pMBs) was tested for the capture efficiency (CE) of *E. coli* O157 in the range of 10^2-10^5 CFU/mL. A significantly lower CE was obtained when 25 µL of pMBs was applied than with using 50 µL or 100 µL of pMBs (Table 4.1). Coupled with qPCR, detection limit by using 25 µL of pMBs was one log higher than that by using 50 µL or 100 µL of pMBs). As there was no significant difference between the CEs of higher volumes, volume of 50 µL was chosen for the separation experiments to reduce the use of pMBs.

Table 4.3 Effect of the volume of phage conjugated beads (pMBs) on bacteria capture efficiency and limit of detection (LOD) by qPCR

Volume of pMBs (µl)	Capture efficiency %				LOD by MS plus qPCR
	10^{2a}	10^3	10^4	10^5	
25	62.9±5.2 C[c]	50.5±4.1B	41.2±3.7 C	29.4±2.6 B	10^{3b}
50	80.2±3.4 AB	75.1±2.8 A	66.4±3.1 AB	52.1±2.2 A	10^2
100	86.5±4.1A	78.4±2.3 A	70.7±2.4 A	56.8±1.4 A	10^2

a *E. coli* O157 concentrations in PBS (CFU/ mL);
b Limit of detections (CFU/ mL);
c Mean±standard deviation (n=3);
The same capital letter within the same column are not significantly different (P>0.05), different letters are significantly different (P<0.05).

Effect of different incubation times (10, 20 and 30 min) on the CE of *E. coli* O157 (10^3 CFU/mL) were investigated at room temperature (Fig. 4.2). Compared with bared beads (CE < 10%), Dynabeads and pMBs reached high CE of 57.7±5.52 % and 64.2±4.83 % after incubating 10 min, respectively. After incubating 20 min, CEs of Dynabeads and pMBs increased to 72.3±3.45% and 74.8±3.22 %, respectively. However, a reaction time of 30 min did not increase the CE significantly compared with that of 20 min. In contrast, longer incubation time of pMBs would result in phage mediated lysis, thus 20 min were used as the reaction time.

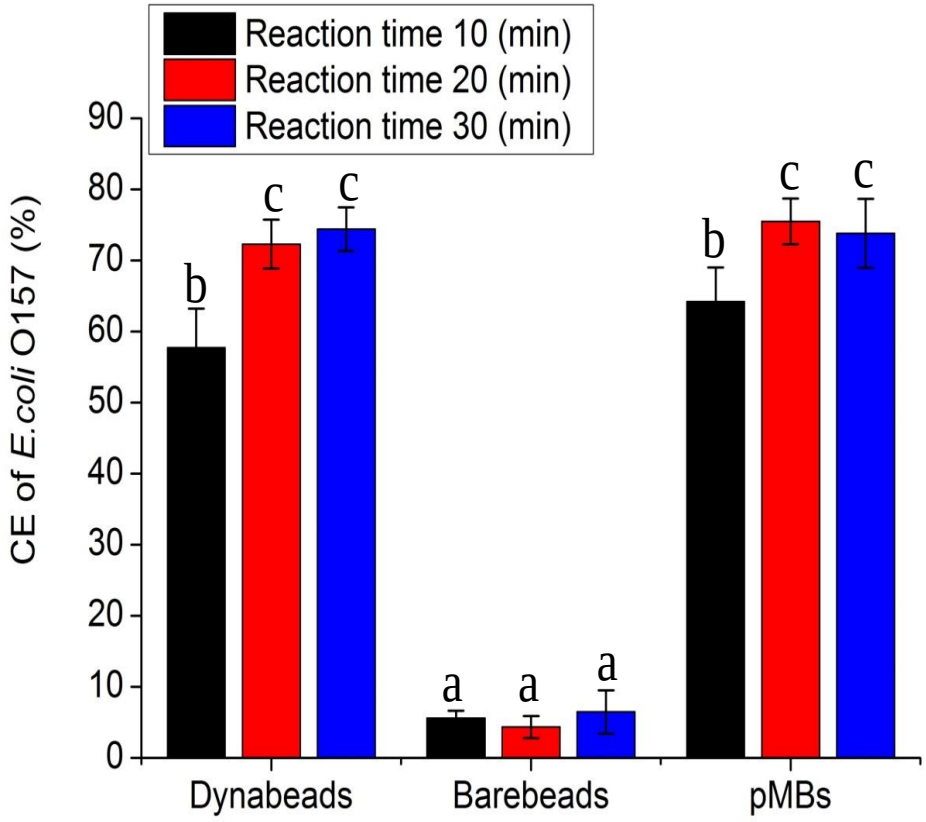

Figure 4.2 Effect of reaction time on the capture of *E. coli* O157:H7 at 10^3 CFU/mL in PBS (pH 7.0) at 37. Bars with same letter (a, b, c) are not significantly different (P>0.05), bars with different letter are significantly different (P<0.05).

Reaction temperatures from 4 °C-55 °C were used for the magnetic separation of 10^3 CFU/mL *E. coli* O157:H7 in 1.0×10^{-2} M PBS (pH 7.0). The capture efficiency increased first with the increase of temperature from 4°C to 37°C, and reached the maximum CE of 74.67±2.07% and 72.48±2.17% for pMBs and Dynabeads, respectively (Fig. 4.3). When reaction temperature increased to 42 °C and 55 °C, phage based beads still retained the ability of capturing bacteria after incubating 20 min while antibody based beads lost the ability significantly at 55 °C (39.6±7.33%). This may due to the activity of antibody receded after reaching maximum at the physiological temperature. The results showed that phage particles the on the beads surface was able to better resist changes in incubation conditions which was in accordance with other reported studies. For example, a small fraction of inovirus PH75 was reported to survive heating at 75 °C for many hours (173). The cubic phage PRD1 could survive when stored at −80 °C and T4-like Shigella phage C16 remained detectable after 32 years of storage at 4 °C (165). An immobilized phage P22 (50%) was also reported to remain the ability to recognize *Salmonella.* typhimurium after left in a dry state for as long as a week (174).

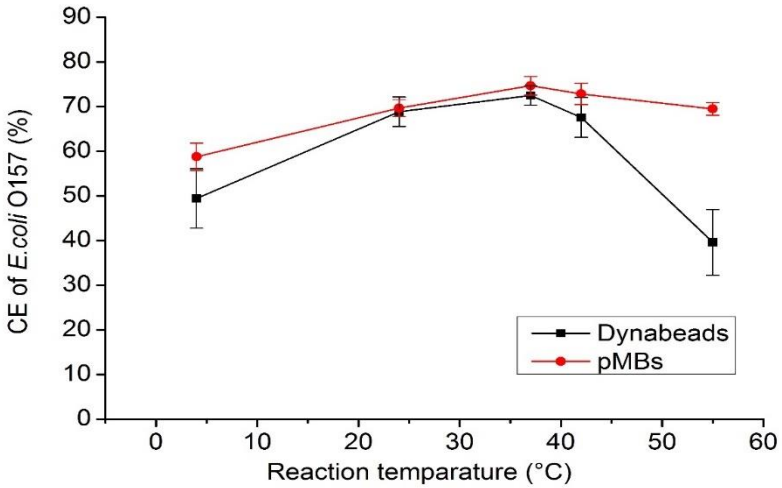

Figure 4.3 Effect of temperature on the capture of *E. coli* O157:H7 from 10^3 CFU/mL in PBS (pH 7.0) for 20 min.

In addition to the high tolerance with temperature fluctuation and drying condition, phages were less susceptible to pH stress as well. In this study, the influence of pH on the magnetic separation of *E. coli* O157 was evaluated in a range between pH 4.0 and pH 10. As Fig.4.4 indicated, the interaction between phage/antibody and *E. coli* was dependent on the pH of the reaction system. A maximum CE from 10^3 CFU/mL *E. coli* cells in $1.0x10^{-2}$ M PBS was observed at pH 7.0 for both types of beads. Specifically, the CE of pMBs still maintained comparatively high level at acidic and basic conditions, CE of 66.18±3.64 % at pH 4.0 and 71.37±1.99 % at pH 10. Although the CEs of antibody based MBs kept stable at pH 6.0-8.0, it was notable that it dropped significantly when the pH deceased below or increased above this stable range. In general, most phages are stable at the range of pH 4-10. Specifically, myovirus P78-77 was reported to survive at pH 2-13 for 24 h and acid-resistant tectivirus P78-76 could survive at pH 2 for 24 h (173).

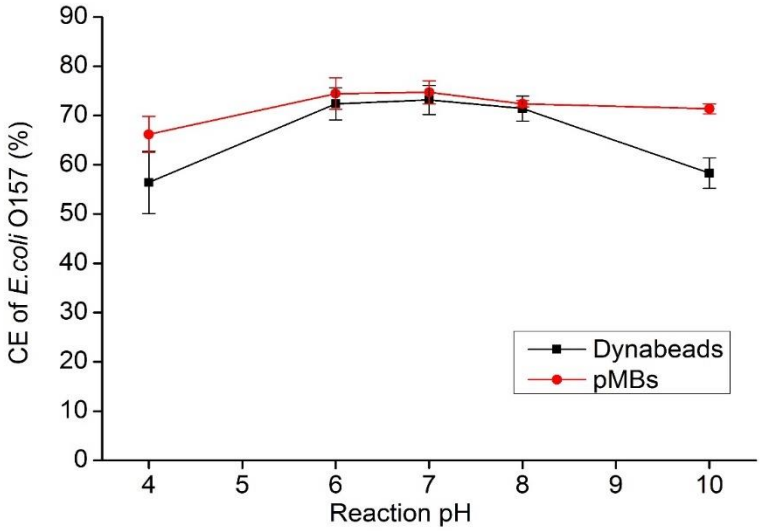

Figure 4.4 Effect of pH on the capture of *E. coli* at 37 °C for 20 min.

The effect of phosphate buffer concentration in the range of 10^{-2}-10^{-1} M PBS on the bio-magnetic separation was tested (Fig. 4.5). CEs of both beads kept stable at first when the buffer concentration increased up to 2.0×10^{-2} M PBS, and then decreased with the increase of PBS concentration. Compared with antibody based beads, the CE of pMBs was above 50 % at higher ionic concentration (>2.0×10^{-2} M). When the PBS concentration reached 5.0×10^{-2} M, the CEs of antibody based beads dropped to a significant lower level (25.27±3.65 %) than that of the phage based one (70.54±1.64 %). And at the concentration of 10^{-1} M PBS, the activity of Dynabeads for bacterial capture left only about 14 %. It was clear that antibody based magnetic separation was sensitive to extreme conditions, such as pH < 4 or pH > 10, temperature > 50 °C or salt concentration > 0.5 M (35). On the other hand, the phage particles were much more resistant to the environmental stress which still retained the capture efficiency of 54.4±4.58 % at 1.0×10^{-1} M PBS.

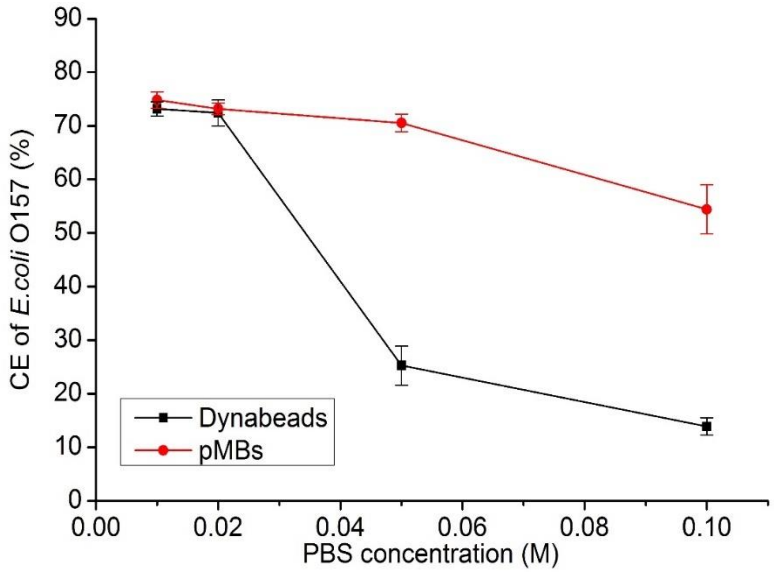

Figure 4.5 Effect of ionic strength on the capture of *E. coli* at 37 °C for 20 min.

The higher capture efficiency displayed by pMBs over Dynabeads under optimal conditions was shown in Fig.4.6. At the range of 10^2-10^6 CFU/mL bacterial culture, both the capture efficiency of pMBs and Dynabeads decreased with the increase of cell concentration (from 80.24±5.44% and 74.95±2.74 to 45.4±2.06 and 38.47±1.99, respectively). The specific isolation of target cells by Dynabeads beads was based on the coated antibody to recognize flagella antigens on the cell while the production of flagella was dependent on the incubation condition. Another factor resulting in the lower CE of Dynabeads was possibly due to the fragileness of flagella which could be disrupted during magnetic separation (175). Compared with antibodies, the unique property of high specificity and fast interaction between phage with protein/lipopolysaccharide receptors on cell surface which was a well-accepted mechanism contributed to a relatively higher capture efficiency at low concentrations of cell numbers (176).

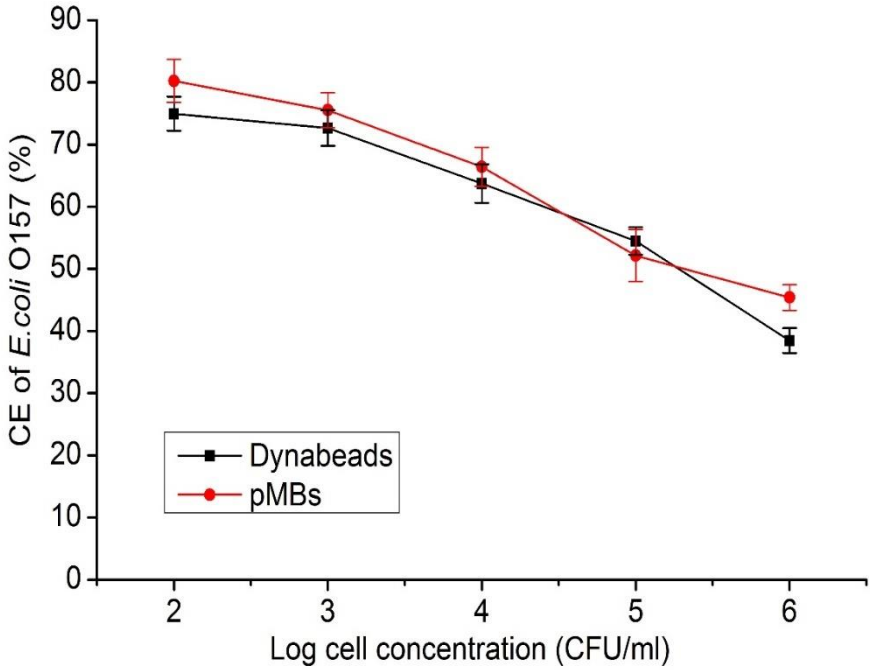

Figure 4.6 Capture efficiency of *E. coli* O157 obtained by using pMBs under optimal reaction conditions compared with Dynabeads.

4.3.1 SEM image

Magnetic beads immobilized with *E. coli* phage via the reaction between amine groups on phage coat protein and the functionalized groups on beads surface were prepared for bacterial capture (Fig. 4.7a). After exposing to *E. coli* cells (Fig.4. 7b), the cell was captured by the phage-beads as confirmed by the image (Fig. 4.7c). No detectable non-targeted bacteria were captured on phage-beads. The results indicated that phage based magnetic particles could be used as a reliable pre-concentration method for specific and selective capture of *E. coli* from sample matrices.

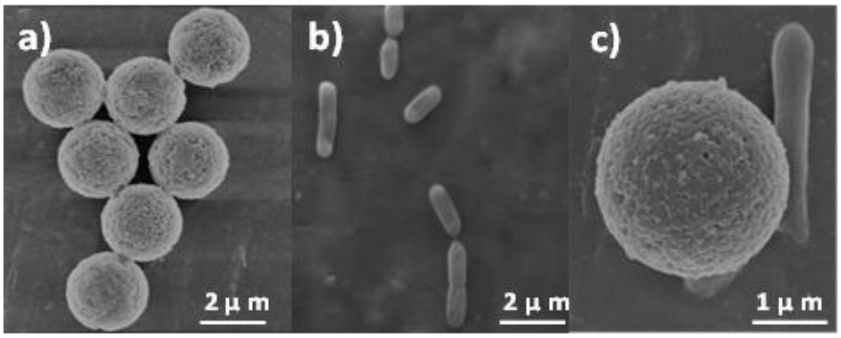

Figure 4.7 Scanning electron microscope images of bacterial capture by phage-beads. (a) SEM image of phage covalently immobilized on beads surface by the EDC-NHS coupling chemistry; (b) *E. coli* cells; (c) capture of *E. coli* on phage-beads.

4.3.2 Water sample quality

The water quality tests were included for characterizing the chemical quality of the water samples used in this study. As shown in Table 4.3, the average pH of the drinking water was similar with the tap water (pH 7.18 and 7.17, respectively) while the agricultural water showed acidic (pH 6.77). Turbidity of the water samples varied no widely, ranging from 0.19

nephelometric turbidity units (NTU; tap water) to 4.23 NTU (agricultural water). All samples showed negative results for the target bacteria.

Table 4.4 Water quality date

Water quality	Water sources (n)[a]		
	Agricultural water (2)	City water A (4)	City water B (4)
Turbidity (NTU)	4.23±0.37[b]	0.34±0.09	0.19±0.03
pH	6.77±0.08	7.18±0.17	7.17±0.05
Target *E. coli*	_	_	_

a n, number of samples;
b Mean ± standard deviation of three triplicates;
--, not detected.

4.3.3 *E. coli* capture assay of phage-bead probe

E. coli capture performance by the phage-beads probe was measured as well. As a negative control, bare beads were applied in the same immunomagnetic separation operation. At four concentrations of 10^3 cfu/mL, 10^4 cfu/mL, 10^5 cfu/mL, 10^6 cfu/mL, the capture efficiency of phage-beads reached 76.97±3.48%, 65.60±3.98%, 52.59±1.72% and 43.62±1.33%, respectively. When applied to the same amount of target bacteria (10^4 cfu/mL), much higher capture efficiency (55.41±6.05% and 65.60±3.98%) was obtained using high bead concentrations (10^7 and 10^8). The capture efficiency of lower bead concentration (10^6) was only 36.96±4.97%. It was believed that higher bacterial capture efficiency was induced by the introduction of more phages that could infect target bacteria. With the increasing number of beads, surface area for the binding with target bacteria also improved which contributed to higher capture efficiency.

4.3.4 Specificity of phage-beads probe

Generally, *E. coli* O157:H7was found to present along with other bacteria in environment, such as water isolates. (177) For accurate detection, it was necessary to test the

specificity of the detection assay. In this study, the specificity was tested by both conventional plate counting method and qPCR method (Fig. 4.8). The specificity and sensitivity of phage-beads probe was confirmed by detecting *E. coli* O157:H7 combined with other pathogenic bacteria strains including *S. enterica, P. aeruginosa* and *S. aureus*. As Fig. 4.8 showed, phage-beads probe had much higher capture efficiency (76.97±3.48%) for *E. coli* O157:H7 and lower cross reactivity (10.04±3.07%, 8.74±2.27% and 11.13±2.02%, respectively) against other competitor bacteria. For bare beads, higher nonspecific capture efficiency (about 12-16%) was obtained under the same condition. The results indicated that phage-beads could selectively capture *E. coli* O157:H7 with good specificity even in the presence of large number of competitor bacteria.

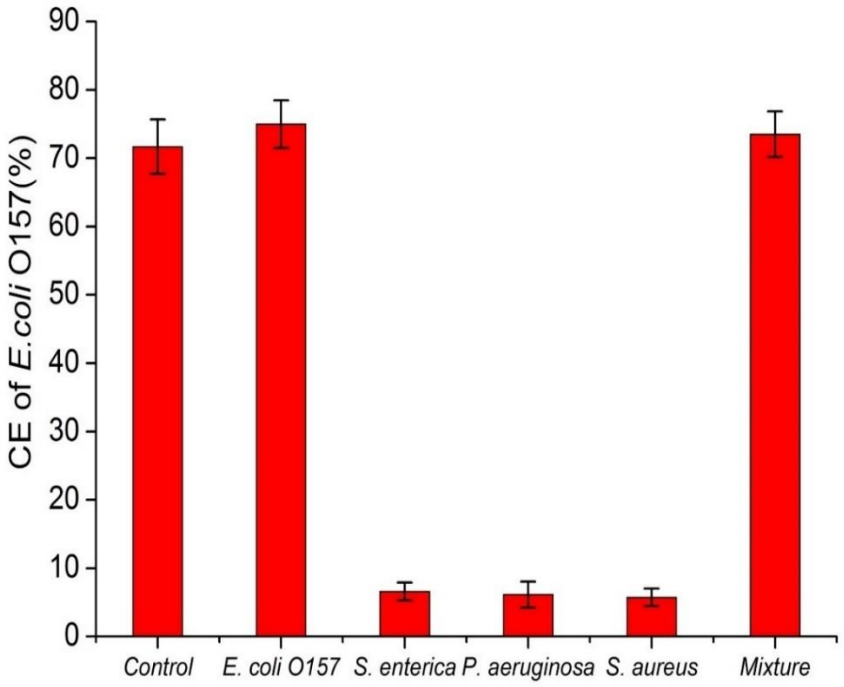

Figure 4.8 Specificity test of the phage-MBs for *E. coli* cells and other bacterium. The standard deviations derived from the mean value of capture efficiency of triplicate experiments.

4.3.5 Real time PCR analysis

Quantitative real-time PCR was performed to calculate the capture efficiency for *E. coli* O157:H7 cells by the phage-conjugated beads. Genomic DNA was released from captured cell through phage mediated lysis and served as templates for qPCR reaction. The sensitivity and dynamic range of the PCR assay was indicated in Figure 4.9. Serial dilutions of *E. coli* in different concentration ranges (10^8-10^1 cfu/mL) were tested in triplicates.

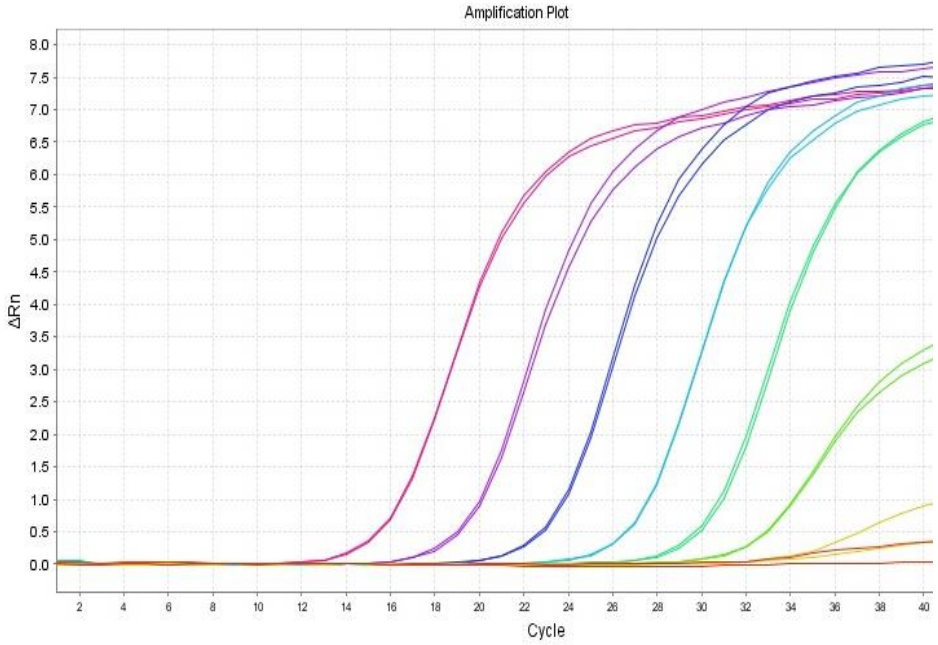

Figure 4.9 Amplification plot of ten-fold dilution series of *E. coli* O157:H7 performed in duplicate from 10^2 to 10^8 cfu/mL in PBS. Y-axis, the fluorescence signal (Delta Rn); X-axis, the cycle number.

Standard curve of phage-mediated bacterial capture and qPCR detection was shown in Figure 4.10. The standard curve created by correlating the Ct values to the logarithmic concentration of bacterial cells showed a good linear inverse relationship ($y=-3.379x + 40.661$, $R^2 = 0.997$) with a low limit of detection 10^3 cfu/mL. The efficiency value (E) of the qPCR assay was 97.67% by using the formula: $E = e^{-1/slope}-1$. The PCR reaction efficiency was comparable to

published reports when boiling was used to lyse bacterial cell and release DNA after magnetic beads separation.

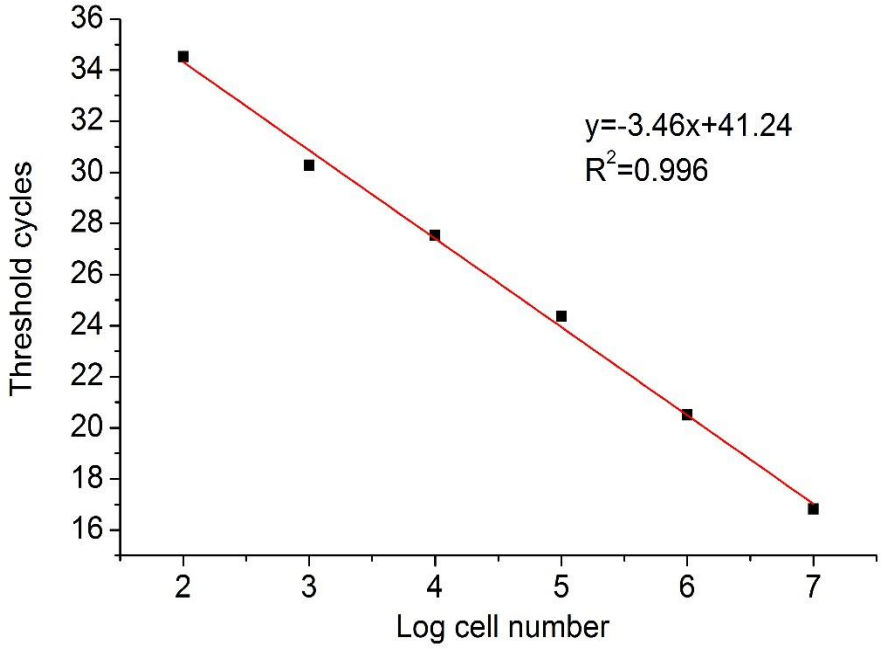

Figure 4.10 Standard curve derived from the correlation of threshold cycle (Ct) with the cell number (Log10) in various concentration. Slope, -3.379; correlation coefficient (R^2), 0.9967.

4.3.6 Detection of viable *E. coli* O157:H7 in spiked liquid samples

E. coli cells from inoculated water samples were detected by qPCR coupled with or without the phage functionalized beads separation. When DNA was extracted from the inoculated agricultural water at *E. coli* concentration below 10^4 CFU/mL without pMBs pre-concentration, fluorescence signals of qPCR above the threshold values were not detectable (corresponding to the absence of data points). When phage-based magnetic separation was applied prior to qPCR, the amplification of DNA from agriculture water samples in the range of 10^2-10^6 CFU/mL bacteria was consistently detectable (Fig. 4.11). However, the Ct values corresponding to the same range of *E. coli* concentrations derived from agricultural water were

higher than those from PBS buffer. These results might due to the PCR inhibitors present within the agricultural water. As the agricultural water was much more turbid (> 20 times) than the buffer and the pH condition was more acidic, these factors could affect the interaction between phage and bacteria resulting lower capture efficiency. But it was clear that pMBs pre-concentration resulted in an improvement in PCR sensitivity at levels of 10^2 CFU/mL.

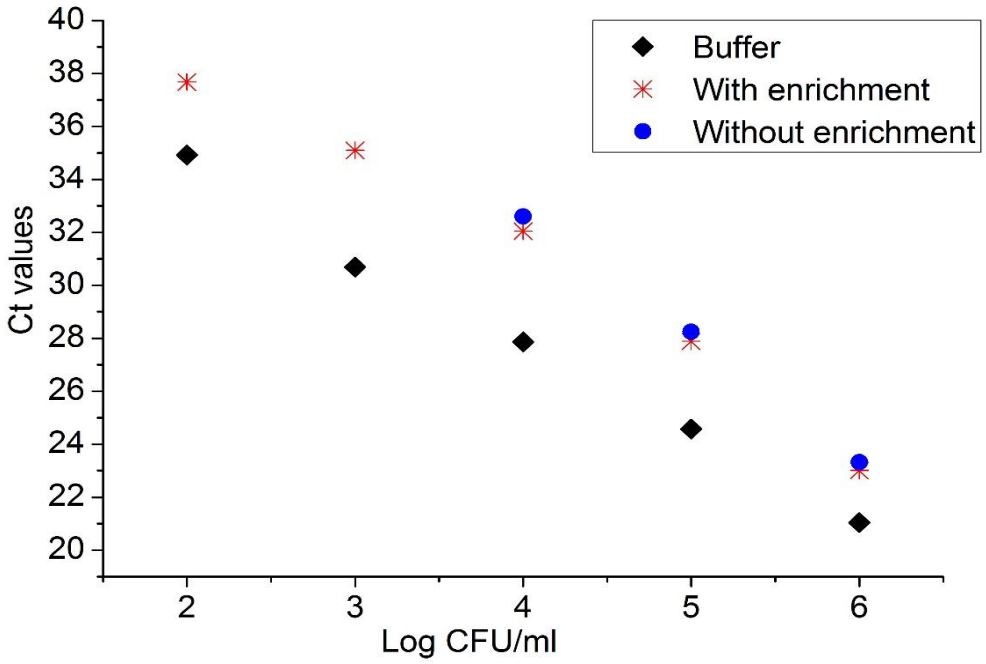

Figure 4.11 qPCR detection of artificially contaminated agricultural water (AW) samples with/without phage-based magnetic concentration.

The threshold cycle value (Ct) versus the logarithmic cell number of *E. coli* O157:H7 in spiked agricultural water sample was higher compared to reference sample (cells spiked in PBS buffer). Direct PCR without phage based isolation resulted in failure of amplification at lower concentrations, and pMBs pre-concentration contributed to improve the PCR efficiency. The feasibility of pMBs based magnetic separation plus qPCR detection was then tested in several additional samples (Fig. 4.12). The standard curves were obtained as described previously to

correlate the Ct values versus bacterial concentrations. Inclusion of the pMBs mediated pre-concentration allowed the standard curves created from city water, agricultural water and broth to be ($y=-3.39x + 41.04$, $R^2 = 0.99$), ($y=-3.65x + 45.76$, $R^2 = 0.98$) and ($y=-3.61x + 42.59$, $R^2 = 0.99$), respectively. The presence of positive PCR signals at all concentrations of inoculated bacterial cells after the pMBs pre-concentration showed that the phage-based magnetic separation offered an effective detection of low concentrations of bacteria, which was otherwise limited. Therefore, the ability of this separation technique to selectively isolate and purify *E. coli* O157 cells from sample suspensions provided a proof-of-concept of the potential combining of pMBs with qPCR to achieve an effective detection of waterborne pathogens. Thus, the novel phage based assay could be applied to rapidly screen waterborne pathogens associated with disease outbreaks or to control epidemic altogether.

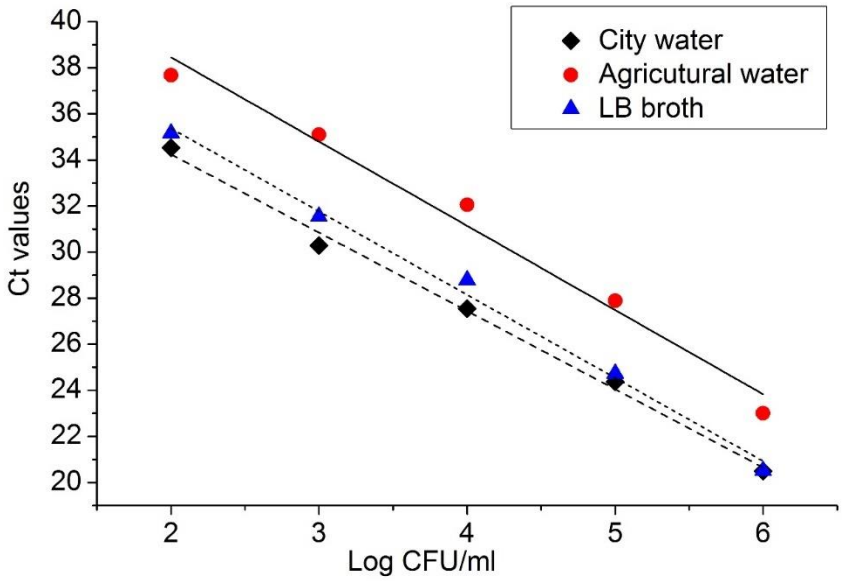

Figure 4.12 Standard curves of different samples including LB broth, AW and city water with pMBs magnetic separation coupled qPCR.

4.4 Conclusions

We have shown here the higher capture efficiency using phage based magnetic separation coupled qPCR compared with antibody based IMS assay. Due to the remarkable specificity and high affinity of phage particles, the developed combination of pMBs-qPCR assay quantified *E. coli* O157:H7 directly from water sample without additional labeling or time consuming pre-enrichment step. The assay was completed in 2 hours with a detection limit of 10^2 CFU/mL even in the presence of non-target bacteria. The proposed principle has proven to be rapid and selective for viable bacteria identification as well as cost effective due to the self-replicating and self-assembling characteristics of phage. Additionally, this method offers a 2-step specificity from both the phage and the primer selection which can be modified to other bacteria of interest and achieve the goal for multiple pathogen detection from environmental samples.

The robustness of the reported method could allow improved bacteria separation in food and environmental samples with extreme or unknown matrix compositions. In addition to the improved temperature tolerance, these characteristics could allow an assay to be better amenable to field testing where incubation conditions may be less controlled.

CHAPTER 5

DEVELOPMENT OF MEMBRANE FILTER BASED COLORIMETRIC DETECTION OF *ESCHERICHIA COLI* BY USING BACTERIOPHAGE FOR ON-SITE WATER QUALITY MONITORING

5.1 Introduction

Rapid detection of bacteria is essential to ensure the microbiological quality of waters, thus prevent illness or outbreaks. The coliform group has been used extensively as an indicator of water quality, and in particular *Escherichia coli* are generally deemed as the indicator of fecal contamination in water and food samples.(3) According to the Centers for Disease Control and Prevention (CDC) report, diarrhea cases linked with unsafe water, inadequate sanitation, or insufficient hygiene have resulted in 1.5 million deaths each year worldwide.(178) The guidelines for acceptable levels of *E. coli* cells in water vary slightly depend on the source and use of the water. Fecal coliform testing is one of the nine tests of water quality that form the overall water-quality rating in a process used by the EPA. This test requires a very careful set of sterile procedures, as well as expensive equipment and a five-day test. Field kits for fecal coliform are available but expensive.

Until now, the most sensitive and specific methods for bacterial detection are based on cultures. Traditional viable plating allows for target microorganisms growing to certain number where unique biochemical features can be detected distinctly. Although the gold standard method (culturing) provides powerful diagnostic tools, it usually requires at least several days to get results. As the need for more rapid tests is of essential for environmental and food analysis, numerous studies have focused on developing quicker assays with the same sensitivity and specificity as traditional methods. One of the techniques utilized to shorten the detection time for *E. coli* is polymerase chain reaction (PCR), based on the amplification of the *uidA* gene that code for beta-glucuronidase.(179) However, the PCR assay alone lack the ability to provide

live/dead cells differentiation, due to the persistence of DNA in the environmental sample even from the cells that without viability. Additionally, detection of coliforms by means of molecular methods requires sophisticated equipment, costly reagents and extensive laboratory work or highly trained personnel.

As a rapid and simple detection method, immunomagnetic separation (IMS) has been investigated widely which relies on specific antibodies coated magnetic beads to capture and concentrate the target bacterium via the binding with antigens on cell surface. The combination of IMS with PCR or electrochemical assay has also been reported to isolate and detect pathogenic bacteria, including *Escherichia coli*, *Salmonella spp*. and *Listeria monocytogenes*.(180, 181) Although, the rapidity and selectivity of these methods enable the separation of the target organism from the non-target micro flora, the requirement of expensive reagents or equipment renders the method costly.

As a routine and widely accepted technique, membrane filter (MF) method offers a simple and inexpensive way to enumerate coliforms from water samples. Compared with microfluidic and lab-on-a chip devices which only analyze microliter-volume samples, MF technique can be used directly to test liquid samples in larger volume. Recently, the detection of specific enzymatic activity from the localized coliforms following MF has been reported to detect *E. coli* successfully. For example, Bernal et al. employed enzyme-linked immuno-filter assay (ELIFA) to detect *E. coli* from aqueous samples and attained a limit of detection (LOD) of 10^4 per membrane in 2 h 15 min. (182) The *E. coli* cells were collected on cellulose ester membranes first and then treated with rabbit anti-*E. coli* IgG, phosphatase-conjugated anti-rabbit IgG and chromogenic enzyme substrate. After color development, the results were assessed visually. Another filtration coupled electrochemical method showed a LOD of 5×10^3 cfu/mL *E. coli* O157:H7 in a 25-min assay. The bacterium labelled with alkaline phosphatase

conjugated antibodies were captured on a membrane filtration. The filter was placed against a carbon electrode and incubated with enzyme substrate for electrochemical detection. (183) Although these techniques offered a rapid detection, they still relied on immuno-labelling which meant relatively high cost, inconsistent reagents which may impede their practical application in industry.

Utilizing bacteriophages as a capturing and sensing element is promising because they provide rapid interaction and high specificity in target recognition. Many bacteriophages mediated detection formats have been investigated, including phage mediated lysis coupled bioluminescence/electrochemical assay, phage biosorbents coupled PCR/immunoassay as well as enzyme labelled or recombinant phages based colorimetric/luminescence assay. (128, 131, 184) According to Blasco et al., the presence of *Escherichia coli* and *Salmonella newport* can be assayed by measuring adenosine triphosphate (ATP) bioluminescence after phage induced host lysis. (185) The sensitivity was improved by detecting released adenylate kinase (AK) instead of ATP as the cytoplasmic marker and the detection limit was fewer than 10^4 cells within 1 h and 2 h for *E. coli* and *Salmonella Newport*, respectively. β-D-galactosidase as another common cellular constituent can be released from intact cells upon phage infection and its enzymatic activity can be monitored by the amperometic measurement in real-time. Based on the electrochemical assay, Neufeld and co-workers indicated a detection of as low as 1 cfu/100 mL *E. coli* (K-12, MG1655) among mixed populations. The assay required a pre-incubation step for bacteria less than 2.5×10^3 cfu/ml, thus rendered the total assay time to 6−8 h. (186) The detection limit of this experimental format relies on the released intracellular enzymatic activity. Similarly, Yemini et al. indicated using phage induced release of α- and β-glucosidase as cell markers for the detection of *Bacillus cereus* and *Mycobacterium smegmatis*, respectively. The detection limit of the amperometric, phage-based biosensor was 10 cfu/ml within 8 h. (187)

However, the requirement for portable handheld devices used to measure bioluminescence or electrochemistry prevents the practical application of in-situ test.

On the contrary, visual colorimetric examinations are more convenient compared with other available methods. Simplicity of the detection by naked eyes without the need for specific apparatus makes it the most preferable assay to perform under field conditions. Additionally, quantifying the optical change by a spectrophotometer is relatively easier than by an epifluorescence microscope or a luminescence counter. Thus, remarkable progress has been made on the design of colorimetric sensing systems for bacteria detection over the years. Jokerst et al. developed a paper based analytical device for pathogen detection by measuring the color change associated with three enzyme−substrate pairs: β-galactosidase with chlorophenol red β-galactopyranoside (CPRG); esterase with 5-bromo-6-chloro-3- indolyl caprylate (magenta caprylate) and phosphatidylinositol-specific phospholipase C (PI-PLC) with 5-bromo-4-chloro-3-indolyl-myo-inositol phosphate (X-InP). The colorimetric method allowed a detection limit of 10 cfu/cm^2 in ready-to-eat meat sample but required an enrichment time of 8-12 h, also it was limited to micro spot test. (188) Wen et al. utilized the bacterial intrinsic peroxidase activity for *Shewanella oneidensis* detection. The optical signal was detectable in a dynamic range between 5.0×10^3 and 5.0×10^6 cfu/mL target cells. (189) Thus, convenience of the colorimetric assay together with the rapidity and selectivity of the phage-based detection may allow the development of a useful technique for a low-cost, disposable, and robust test for field applications.

In the present work, a phage based membrane filter technique coupled colorimetric assay will be developed. As a model system, the well characterized T7 bacteriophage and its host bacterium, *E. coli* BL21, are used. The choice of ALP as a reporter protein is based on the availability of a wide variety of methods for detection of the enzyme activity including

colorimetry, fluorescence and bioluminescence. (190, 191) According to our previous work, the ALP phage-based probe was able to detect 10^3 cfu/mL of *E. coli* in 6 hours by chemiluminescent assay and 10^4 cfu/mL in 7.5 hours by colorimetric assay. Compared with the previous methods, the adoption of membrane filter as an isolation and pre-concentration without the need of specific antibody coating in the current method is the novelty and advantage. Furthermore, both sample preparation and detection scheme are more simple and cost-friendly as no particular equipment are required. Thus, the developed assay offers a disposable test scheme which is practical and affordable for water quality detection and could be further applied in developing countries as a low cost, rapid diagnostic aid for on-site detection of viable bacteria, without the need of sophisticated equipment and highly trained personnel.

5.2 Material and methods

5.2.1 Materials

Nitro-blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate solution was purchased from Thermo Scientific Pierce (Rockford, lL, USA). Sodium chloride, potassium chloride, tripton, agar and yeast extract were obtained from Sigma (St.Louis, MO, USA). Nalgene™ sterile analytical filter units (0.45 µm/0.22 µm pore size) were ordered from Thermo Fisher Scientific. Disposable petri dishes were purchased from Fisher Scientific. Ampicillin sodium salt was purchased from Thermo Fisher Scientific and filter sterilized before use. All chemicals and solutions used throughout this study were dissolved or diluted with deionized water.

5.2.2 Apparatus

Ultrospec 10 cell density meter (Amersham Biosciences, Piscataway, NJ) was used to measure the bacterial culture optical density in this study. The photographs were taken with cell phone. The pH measurement was carried out on Fisher Scientific Accumet Research AR25 pH/mV/°C/ion selective electrode meter. Stainless steel membrane forcep was purchased from Thermo Scientific. JCM-6000PLUS NeoScope benchtop scanning electron microscope (JEOL USA, Inc., Peabody, MA) was used for SEM images analysis. Cressington sputter coater 108auto (Ted Pella, Inc., Redding, CA) was used for SEM sample preparation.

5.2.3 Bacterial strain and culturing

E. coli BL21 (ATCC700550) was grown from a glycerol stock in Luria-Bertani (LB) broth as described in a previous report. (180) For the bacteria, a single colony was inoculated in bacterial medium at 37 °C with gentle shaking at 200 rpm overnight. The *E. coli* cell culture (1 mL) was centrifuged at 8000 g for 10 min to collect cells and washed three times with phosphate buffer solution (PBS, 0.01 M PBS; 137 mM NaCl, 6.4 mM Na_2HPO_4, 2.7 mM KCl, 0.88 mM KH_2PO_4; pH 7.4). After removal of supernatant, the collected cell pellet was dispersed in autoclaved PBS (1 mL) and stored at 4°C for further use. Before each measurement, cell solution was serially diluted to the desired concentration with LB broth and determined by plating onto standard agar plates for cell count.

5.2.4 Phage preparation

Engineered phage T7 ALP that overexpress alkaline phosphatase was used in the study. The T7 phage was genetically engineered to carry a reporter gene (ALP) through standard

molecular cloning techniques. The designed ALP gene under control of the T7 promoter was synthesized by Genscript (Piscataway, NJ) in plasmid pUC57 firstly and then the reporter gene was PCR amplified and purified to ligate with T7Select 415-1 arms. The ligated DNA was packaged into mature phage capsids via the T7select packaging extracts to create T7 ALP and screened for the correct insert. The positive clones were propagated in BL21 for further use.

Overnight culture of *E. coli* (100 µL) were inoculated into fresh LB broth (35 mL) and grown at 37 °C with constant shaking (200 rpm) to reach an optical density (OD) at 600 nm of 0.6. Then 100 µL of phage stock solution was added to the cell culture for phage propagation by standard techniques. (28) Lysates were filter-sterilized using 0.22 µm pore size filters (Corning Life Science, Corning, NY). Purified phage stock solution was prepared by ultracentrifuge as described in previous paper. Double-layer LB plates with 0.75 % soft agar was used to enumerate phage titers.

5.2.5 Membrane filter capture of *E. coli*

Membrane filtration was performed according to US EPA standard protocol. Serial 10-fold dilutions of *E.coli* cells were prepared in the range of 10^2–10^5 cfu/ml in LB from a washed *E. coli* suspension. An aliquot of sterile LB broth was prepared as negative control. Then 1 mL of various cell dilutions were inoculated into 99 ml sterile water sample in the funnel followed by connecting the funnel unit to a tubing attached with a vacuum pump. Then the sample was filtered through 0.45 µm/ 0.22 µm cellulose nitrate membrane under partial vacuum. The membrane filter was removed from the funnel unit by sterile forceps and placed into a clean petri dish containing nutrient medium (2.0 mL of LB broth). The device was incubated at 37 °C for bacterial recovery before phage induced colorimetric reaction. The humidity in the incubator was kept in constant to avoid evaporation of the liquid medium.

5.2.6 Optimization of the reaction conditions

The membrane filter retaining cells was incubated for 0h, 2h, 4h, 6h and 8h respectively, followed by adding 500 µL purified phage stock solution (5×10^6 PFU/mL) over the top of each membrane and incubating for another 30 min with shaking for phage infection. After each incubation, the membrane filter was removed and placed into a fresh petri dish and 500 µL of BCIP/NBT reaction solution was added on the top of membrane immediately. The filter was incubated at 37 °C for various time, ranging from 1h to 3h to determine the optimal reaction time for color change. Experiments were carried out in triplicate. Negative control test was performed by filtering water sample without inoculating bacterial cells while positive control was performed by inoculating the same amount of cells followed by incubating at 37 °C overnight. Traditional plate counting assay was performed in parallel.

5.2.7 Colorimetric method

Colorimetric detection of living *E.coli* cell by naked eyes was conducted through the enzymatic reaction of alkaline phosphatase released from each captured bacteria and its chromogenic substrate (BCIP/NBT) under optimal condition as described above. Hydrolysis of this BCIP following oxidation by NBT can produce a blue-colored precipitate at the site of alkaline phosphatase presence. In our case, the localized precipitate resulting from alkaline phosphatase activity released from each colony would indicate the location of *E.coli* cell that effectively infected by the engineered T7 ALP phage. Image of the membrane with colored precipitate was taken using a smart phone. Chemiluminescent signal was recorded by using chemiluminescent substrate and the image was taken using a CCD imaging station (Kodak, Rochester, NY, USA).

5.2.8 Specificity test

To investigate the specificity of the assay, other non-targeted bacterial strains, including *Salmonella. Typhimurium* and *Staphylococcus. aureus* at the concentration of 100 cells/100 mL water sample were tested under the same conditions. Different bacteria cells were collected, prepared and serial diluted followed by colorimetric detection as described above.

5.2.9 SEM analysis of the membrane filter

Each of the membrane filter specimen was removed and fixed by immersing in 2.0% glutaraldehyde for 1 h. Then the membrane was washed twice with sterile water and air dried at room temperature. Segments of the prepared membrane filter was aseptically affixed on the top of a sample holder by using double adhesive cellulose tape followed by sputter coating with gold. Then the specimen was observed at an accelerating voltage of 10 kv by scanning electron microscopy and photographed.

5.2.10 Statistical analysis

All experiments were performed at least three times with a minimum of two replicates in each experiment. Data presented as the mean value and standard deviation was calculated from each experiment using Microsoft Excel 2016. Statistical analysis was carried out using Student's t test (Microsoft Excel Data Analysis) and the results were deemed to be statistical significance when $p<0.05$.

5.3 Results and discussion

E. coli as an indicator of fecal contamination of water has been used commonly since their presence indicates the possibility of other pathogenic organisms may also be present in the water. Thus alkaline phosphatase based chromogenic detection methods have been published to investigate the bacterial contamination of drinking or well water. However, false positive results still exist due to the presence of other bacteria in water which express alkaline phosphatase. In our study, the detection of viable *E. coli* based on the enzymatic reaction of alkaline phosphatase is relatively quantitative compared with common colorimetric assay as the bacterial colony can be counted through the dark blued/purple precipitates. And our goal is to apply this phage based assay which specifically infects its corresponding host bacteria to reduce the rate of false positives resulting from non-targeted bacteria that also produce alkaline phosphatase. The schematic representation of the assay is shown in Figure 5.1.

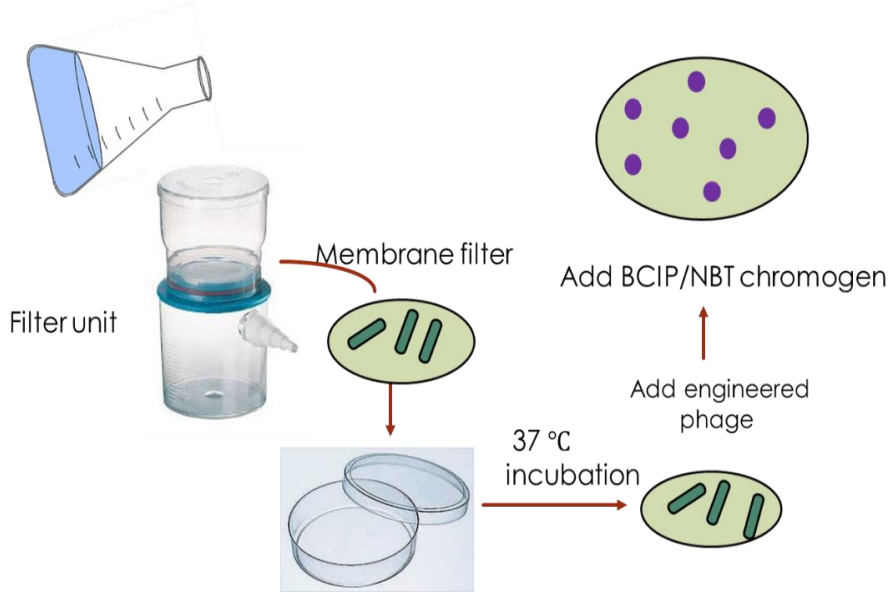

Figure 5.1 The schematic representation of *E. coli* detection approach contains the following steps: 1) artificially inoculated water sample (100 ml) was vacuum filtered to retain target bacteria, 2) membrane filter was incubated in a petri dish containing appropriate medium at 37 °C for various time period; 3) T7 APL phage was added to the membrane and incubated at 37 °C for 30 min 4) chromogenic alkaline phosphatase substrate was added onto the membrane and incubated for color development.

5.3.1 Proof-of-principle detection of *E.coli* by ALP based assay

As the amount of alkaline phosphatase released from the bacterium on the filter determines the detection sensitivity, both bacteria concentration and the phage infection during the incubation become the key factors for the proposed approach. Two assay patterns of alkaline phosphatase were explored and compared. One is based on the alkaline phosphatase detection from engineered *E. coli* cells that overexpress ALP (ALP *E.coli*) and released by wild type phage T7. The other method is based on infection of the wild type *E. coli* cell with our engineered T7 phage carrying the ALP gene (T7 ALP) which will induce ALP expression upon infection. Both patterns allow alkaline phosphatase releasing and the enzyme reaction with chromogenic substrate resulting precipitated enzyme products (purple dots) which are in proportion to cell number. In the first model, colored signal is visual detectable as presented in Figure 5.2. Compared with the wild type strain, the ALP *E.coli* strain yields significantly higher intensity of colored signal both in culture solution and on membrane filter which confirms the principle of the second detection pattern by using T7ALP phage to detect wild type *E.coli* is possible.

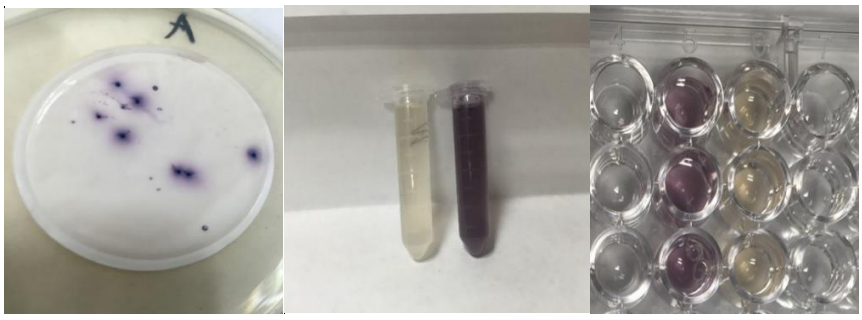

Figure 5.2 Colored signal from the reaction of *E.coli* strain that overexpressed alkaline phosphatase (ALP *E.coli*) with BCIP/NBT substrate on 0.22 µm membrane filter (left); comparison of enzymatic reaction of ALP *E.coli* with wild type *E.coli* at the presence of BCIP/NBT substrate in centrifuge tube and 96-well plate, respectively (middle and right).

5.3.2 Optimization of the membrane filtration system

In order to maximize the detection limit of the developed assay, combination of different incubation time for the phage based infection was investigated. The membrane filter containing bacterial cells incubated for 1h following addition of phage T7 and another 1h of enzyme incubation showed lowest signal intensity, while the combination of 4h incubation of bacterial cell with 4h incubation of enzyme reaction produced the highest signal intensity (Fig. 5.4 A and D). The intensity of colored signals increased along with the increasing of incubation time as indicated in Fig. 5.3. For example, the signal intensity of the combination of 2h incubation of cells plus 2h incubation of enzyme as well as 3h of each incubation were almost 4-fold of the signal from just 1h incubation. This may due to the longer incubation time allowed more phage induced infection and enzyme released from bacterial cells, as well as enough enzyme reaction time with the chromogenic substrate. Moreover, the colored signal from counting the precipitated dye was easy to quantify the number of bacteria in the test sample without using other equipment.

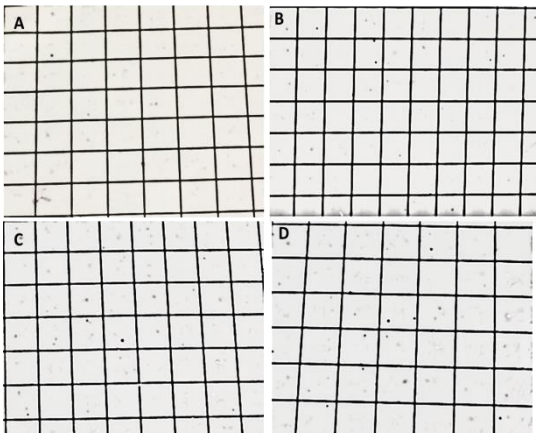

Figure 5.3 Images of signals on the membrane filter (pore size 0.45 µm) under different incubation time A) Cell incubation 1h + phage infection 45min + enzyme incubation 1h; B) Cell incubation 2h + phage infection 45min + enzyme incubation 2h; C) Cell incubation 3h + phage infection 45min + enzyme incubation 3h; D) Cell incubation 4h + phage infection 45min + enzyme incubation 4h.

After the proof-of-concept detection, phage T7 ALP was employed for real sample detection, the overexpression of alkaline phosphatase by the engineered phage was verified by comparing it with wild type T7 in normal plaque assay. Then Novex® AP chemiluminescent substrate (CDP-Star®, 100 µL) was added onto the plaques to verify the signal from infectious T7 ALP phage to *E.coli* strain. The result was acquired using the imaging device and showed in Fig. 5.4. Positive signal (plaques) was observed from the engineered phage T7 ALP while the wild *E.coli* strain yield no signal.

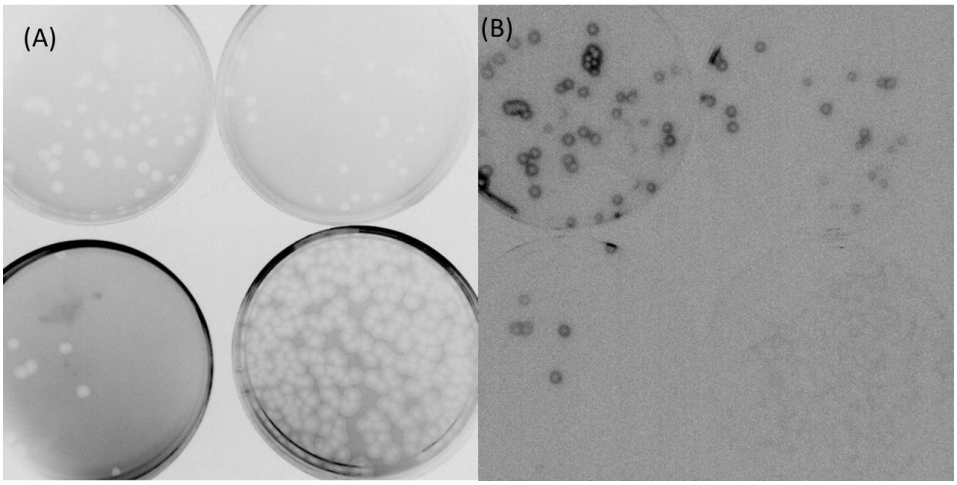

Fig. 5.4 (A) Optical images of engineered phage plaques (top left and right & bottom left) and wild phage plaque (bottom right) on *E.coli* BL21 wild type lawn using plaque assays. (B) chemiluminescent images from these plaques corresponding to the same plates in (A), the bottom right plate containing wild type T7 phage on BL21 lawn produce no signal.

Sterile water sample was inoculated with *E.coli* cells of around 10 CFU/100ml and filtered through membrane filter followed by incubation for various time: 0h, 2h, 4h, 6h, 8h. Then phage T7 ALP was added and incubated for 30 min at 37 ℃. Substrate BCIP/NBT was added on top of the membrane filter in a new petri dish and incubated for enzyme reaction: 8h, 6h, 4h, 2h,1h, respectively which made the total assay time for each set was almost the same, except the last one. The result from different combination times of incubation was summarized

in Fig 5.5 which indicated the optimal combination was 4h incubation of bacterial with 4h incubation of enzymatic reaction. Controls of only *E.coli* or *E.coli* with substrate without phage infection were also carried out in parallel and no signal was detected (Fig.5.6).

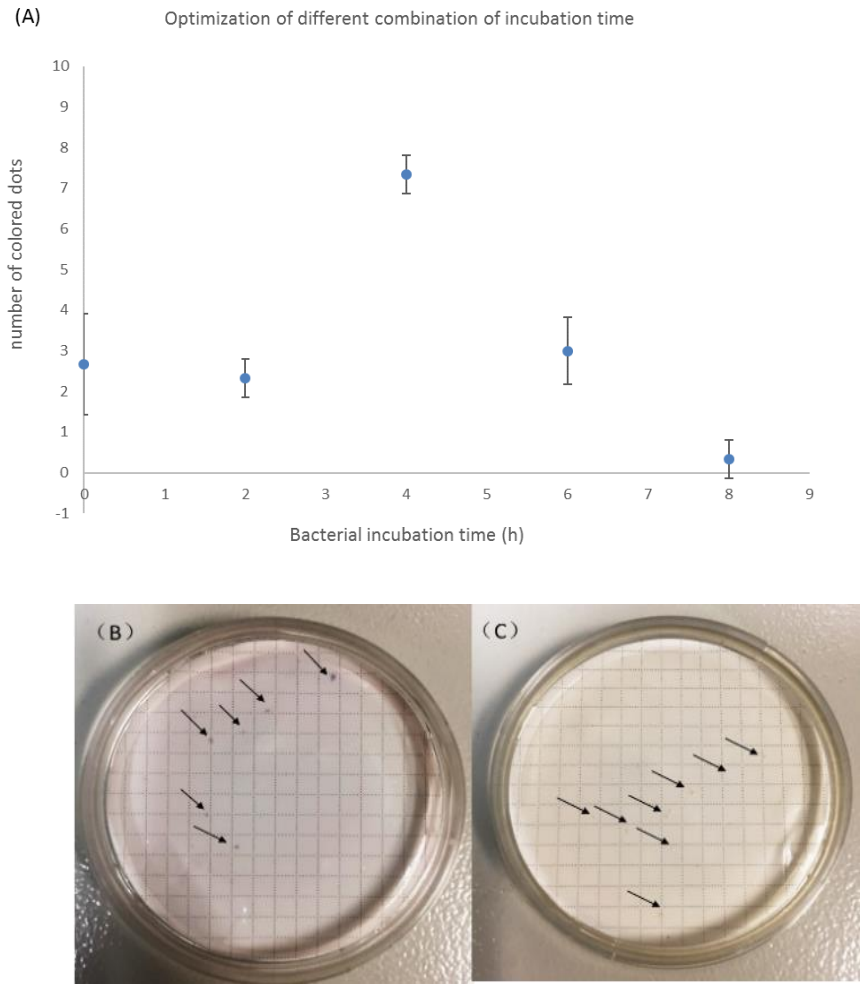

Fig. 5.5 Comparison of different incubation time for colored precipitates forming on the membrane filter. (A) Membrane filters incubated for different combinations of bacterial incubation and enzyme incubation, and the number of precipitate was counted according; (B) number of colored precipitates (indicated with arrow) from phage based assay: reaction condition of 4h incubation for bactria+30 min phage infection+ 4h enzyme reaction; (C) number of bacterial colony from traditional assay: incubation time 12h.

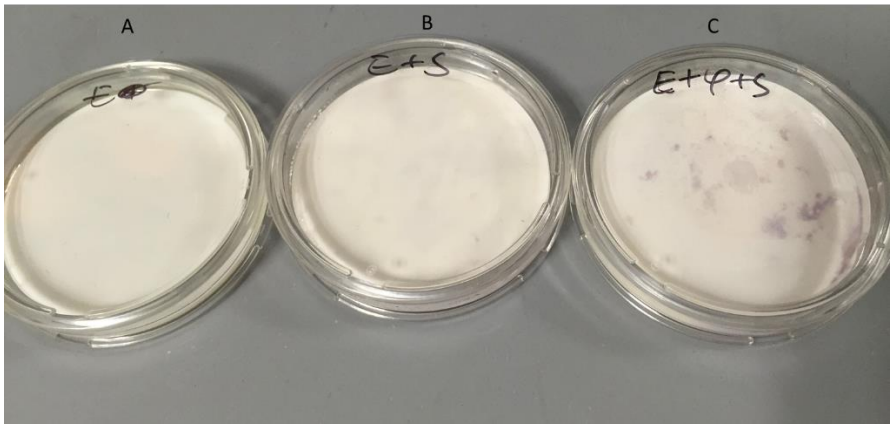

Fig. 5.6 Negative controls of the phage based detection assay. (A) only *E.coli* cells on the membrane after 6h incubation; (B) *E.coli* cells after incubation with substrate; (C) *E.coli* cells after incubation and phage infection with substrate.

These results indicated that without phage infection, colored signal was not detectable in a short period of time, while the T7 ALP could help to synthesize and release alkaline phosphatase into the surrounding area resulting the reaction of its chromogenic substrate and forming colored precipitate.

5.3.3 Specificity of *E. coli* detection

As the proposed detection assay is based on the detection of alkaline phosphatase released from phage mediated lysis, the specificity relies on the interaction of phage to the host cell. Non-targeted bacteria showed no detectable signal during the short incubation period which confirmed the sensitivity by using this testing format.

5.3.4 SEM analysis of the membrane filter

It is clear that the bacteria retained in the membrane filter determines parts of the detection limit, thus the structure of membrane filter was investigated. Theoretically, compared

with larger pore size, smaller pore size would more easily keep bacteria on it due to the size restriction. Different section of the membrane filter after incubation and phage infection was analyzed by the SEM as shown in Fig. 5.7. As these images showed, *E.coli* cell was retained by the filter on top/ inside with intact shape. While after phage infection, bacteria were lysed and cell debris were found inside the membrane indicating the liberation of cell enzymes for following colorimetric reaction.

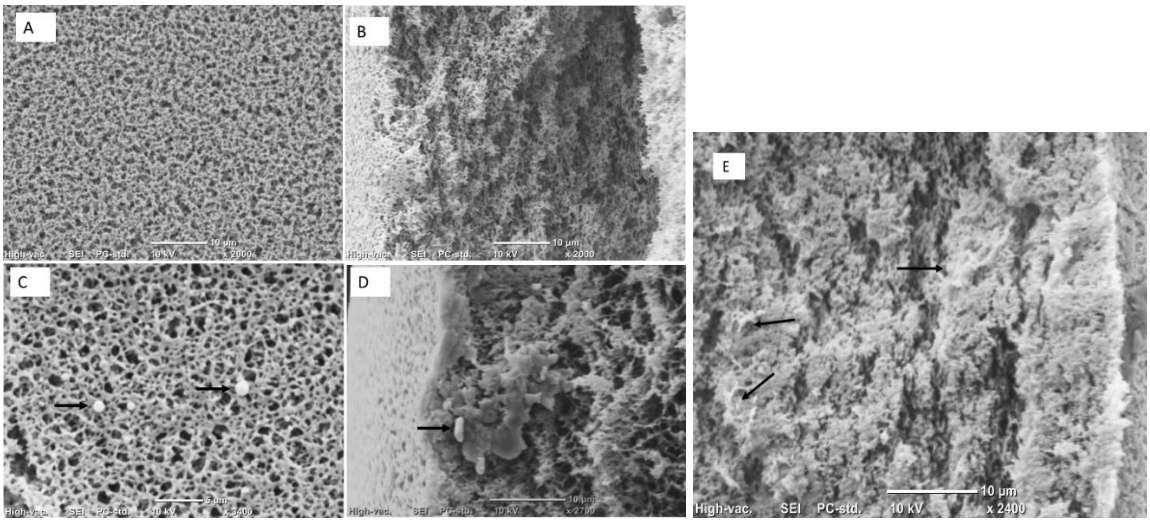

Figure 5.7 SEM images of different sections of the membrane filters. (A) Top view of the 0.22 µm pore size membrane without bacteria; (B) cross section of the same membrane; (C) Top view of the membrane with bacteria (indicated with arrow) on top after water sample filtered through; (D) cross section of the same membrane containing bacterial cells (indicated with arrow) inside; (E) cross section of the membrane after phage induced lysis (indicated with arrow).

In conclusion, we could achieve colorimetric detection of separate colony after a short period of pre-incubation followed by phage T7 ALP infection. The engineered phage that overexpress ALP should simplify the detection format and reduce the total assay time compared with conventional method. By using phages, the probability of false-positive signals originating from the ALP released during the growth of non-targeted bacteria could also be reduced. Thus, we expect the proposed assay to be helpful for real sample testing, especially in resource limited

regions. Future work will be carried out to quantitatively deal with the colored signal, such as using ImageJ to analyze the intensity of precipitate colony.

CHAPTER 6

CONCLUSION

Escherichia coli is frequently linked with foodborne disease and some serotype are even human pathogens that cause acute infections, thus a rapid and effective detection system for it is closely related to public safety. In addition, government and industry have pay more and more attention to food safety issue in order to minimize the possibility of exposing general public to foodborne illness. Although golden standard microbiological methods can offer the sensitivity for bacteria detection, the needs for long time incubation and specialized facilities restrict most practical applications. In recent decade, phage based biosensors for bacterial detection and pathogen treatment has been developed widely and obtained considerable attention. The natural features of phage, including highly specific, simple and rapid self-reproducing render them ideal tools to deal with pathogen infection issues. Their natural self-assembling allows synthesized nanoparticle almost identical, with exactly the same shape and nanostructured size which is difficult to achieve via laboratory synthesis. Furthermore, their resistance to extreme conditions, stability after long time storage as well as relatively large surface area of phage coat protein make them promising for many biochemical modification and reaction.

In the first part of the thesis, biotinylated phage by genetic modification to express biotin acceptor peptide on the capsid protein was achieved and immobilized on streptavidin-modified magnetic beads. The phage immobilization efficiency was indicated to be high enough for target bacterial capture from both broth and water samples. The bio- magnetic separation assay offered a rapid and simple way to pre-concentrate bacterial cells without pre-enrichment compared with traditional culture based method. This assay was also able to distinguish viable cell from non-viable one as phage infection only occurs in the presence of living cells thus allows

the detection of nucleic acid only from viable one which minimize the possibility of false positive signals from dead cells. The specific character of phage ensured the specificity of the assay and was verified by the fail from detection of non-targeted bacteria. These results provide further evidence that phage particles may open another avenue for accurate detection of bacteria and be more superior than antibody based immunoassays.

In the second part of this thesis, phage based detection assay was further compared with antibody based immunoassay. Unlike nonpathogenic *E.coli* strains (e.g. *E.coli* BL21 or *E.coli* K12) pathogenic *E.coli* O157:H7 was not sensitive phage T7, thus two of *E.coli* O157:H7 specific phages were selected for the new assay format. The chemically immobilized specific phages on magnetic beads showed a higher capture efficiency than antibody immobilized beads under extreme conditions, including high temperature, extreme pH range thus indicated phage-based biosorbent could be a useful tool for practical detection under extreme environments. Especially, when combined with PCR based assay, the total detection time can be shortened to three hours which is a great advantage compared with conventional one.

In the third part of the thesis, a membrane filter base assay for a colorimetric determination of *E.coli* in water sample has been developed. The enzymatic assay has been optimized for *E. coli* detection with reduced enrichment time relative to standard culture techniques. We have demonstrated a proof-of-concept for this assay using engineered an *E.coli* strain that overexpressed alkaline phosphatase following infection with phage T7 and the colorimetric assay was validated. Then engineered phage T7 overexpress alkaline phosphatase was constructed and applied in the detection of wild type *E.coli* cell with similar assay format. The assay could be performed within 9h to detect 100 cells in 100mL water sample. The colored signal was easy to count by naked eye and in proportion to the real cell number.

In the end, our goal is to develop phage based bacterial detection procedures that could turn complicated pathogen detection into a routine test and adopted by the food industry using relatively easy method without losing specificity and detection limit. And we have testified phage as a rapid and economical tool for identifying certain pathogen based on different detection formats. Since these approaches require no more than 4 h pre-enrichment, the test can be completed in about a working shift. Presumably, by replacing the phage species specific to different pathogenic bacteria, the developed assay model could be adopted as a universal tool for food quality and environmental safety applications.

BIBLIOGRAPHY

1. Hagens S, Loessner MJ. Application of bacteriophages for detection and control of foodborne pathogens. Appl Microbiol Biotechnol. 2007;76(3):513-9.

2. Gracias KS, McKillip JL. A review of conventional detection and enumeration methods for pathogenic bacteria in food. Canadian Journal of Microbiology. 2004;50(11):883-90.

3. Rompre A, Servais P, Baudart J, de-Roubin MR, Laurent P. Detection and enumeration of coliforms in drinking water: current methods and emerging approaches. J Microbiol Methods. 2002;49(1):31-54.

4. Byrd JJ, Xu H-S, Colwell RR. Viable but nonculturable bacteria in drinking water. Applied and environmental microbiology. 1991;57(3):875-8.

5. Lazcka O, Campo FJD, Muñoz FX. Pathogen detection: A perspective of traditional methods and biosensors. Biosensors and Bioelectronics. 2007;22(7):1205-17.

6. Meays CL, Broersma K, Nordin R, Mazumder A. Source tracking fecal bacteria in water: a critical review of current methods. Journal of Environmental Management. 2004;73(1):71-9.

7. Ivnitski D, Abdel-Hamid I, Atanasov P, Wilkins E. Biosensors for detection of pathogenic bacteria. Biosensors and Bioelectronics. 1999;14(7):599-624.

8. Chappelle EW, Levin GV. Use of the firefly bioluminescent reaction for rapid detection and counting of bacteria. Biochemical Medicine. 1968;2(1):41-52.

9. Gunasekera TS, Attfield PV, Veal DA. A Flow Cytometry Method for Rapid Detection and Enumeration of Total Bacteria in Milk. Applied and Environmental Microbiology. 2000;66(3):1228-32.

10. Yang L, Bashir R. Electrical/electrochemical impedance for rapid detection of foodborne pathogenic bacteria. Biotechnology Advances. 2008;26(2):135-50.

11. Floriano PN, Christodoulides N, Romanovicz D, Bernard B, Simmons GW, Cavell M, et al. Membrane-based on-line optical analysis system for rapid detection of bacteria and spores. Biosensors and Bioelectronics. 2005;20(10):2079-88.

12. McKillip JL, Drake M. Real-Time Nucleic Acid–Based Detection Methods for Pathogenic Bacteria in Food. Journal of food protection. 2004;67(4):823-32.

13. Noble RT, Weisberg SB. A review of technologies for rapid detection of bacteria in recreational waters. Journal of water and health. 2005;3(4):381-92.

14. Olsvik O, Popovic T, Skjerve E, Cudjoe KS, Hornes E, Ugelstad J, et al. Magnetic separation techniques in diagnostic microbiology. Clinical Microbiology Reviews. 1994;7(1):43-54.

15. Mandal P, Biswas A, Choi K, Pal U. Methods for rapid detection of foodborne pathogens: an overview. American Journal Of Food Technology. 2011;6(2):87-102.

16. Hudson J, Lake R, Savill M, Scholes P, McCormick R. Rapid detection of Listeria monocytogenes in ham samples using immunomagnetic separation followed by polymerase chain reaction. Journal of applied microbiology. 2001;90(4):614-21.

17. Skjerve E, Rørvik L, Olsvik O. Detection of Listeria monocytogenes in foods by immunomagnetic separation. Applied and environmental microbiology. 1990;56(11):3478-81.

18. Yang H, Qu L, Wimbrow AN, Jiang X, Sun Y. Rapid detection of Listeria monocytogenes by nanoparticle-based immunomagnetic separation and real-time PCR. International Journal of Food Microbiology. 2007;118(2):132-8.

19. Fratamico PM, Schultz FJ, Buchanan RL. Rapid isolation of Escherichia coli O157: H7 from enrichment cultures of foods using an immunomagnetic separation method. Food microbiology. 1992;9(2):105-13.

20. Wright D, Chapman P, Siddons C. Immunomagnetic separation as a sensitive method for isolating Escherichia coli O157 from food samples. Epidemiology and Infection. 1994;113(01):31-9.

21. Jeníková G, Pazlarová J, Demnerová K. Detection of Salmonella in food samples by the combination of immunomagnetic separation and PCR assay. International Microbiology. 2010;3(4):225-9.

22. Mansfield LP, Forsythe S. Immunomagnetic separation as an alternative to enrichment broths for Salmonella detection. Letters in applied microbiology. 1993;16(3):122-5.

23. Linda S, Uknalis J, Tu S-I. Immunomagnetic separation methods for the isolation of Campylobacter jejuni from ground poultry meats. Journal of immunological methods. 2001;256(1):11-8.

24. Lamoureux M, MacKay A, Messier S, Fliss I, Blais B, Holley R, et al. Detection of Campylobacter jejuni in food and poultry viscera using immunomagnetic separation and microtitre hybridization. Journal of applied microbiology. 1997;83(5):641-51.

25. Khare S, Ficht TA, Santos RL, Romano J, Ficht AR, Zhang S, et al. Rapid and Sensitive Detection of Mycobacterium avium subsp. paratuberculosis in Bovine Milk and Feces by a Combination of Immunomagnetic Bead Separation-Conventional PCR and Real-Time PCR. Journal of clinical microbiology. 2004;42(3):1075-81.

26. Rijpens N, Herman L, Vereecken F, Jannes G, De Smedt J, De Zutter L. Rapid detection of stressed Salmonella spp. in dairy and egg products using immunomagnetic separation and PCR. International Journal of Food Microbiology. 1999;46(1):37-44.

27. GOODING CM, CHOUDARY PV. Rapid and sensitive immunomagnetic separation–polymerase chain reaction method for the detection of Escherichia coli O157[ratio]H7 in raw milk and ice-cream. Journal of Dairy Research. 1997;64(01):87-93.

28. Adams MH. Bacteriophages. Bacteriophages. 1959.

29. Allwood PB, Malik YS, Maherchandani S, Vought K, Johnson LA, Braymen C, et al. Occurrence of Escherichia coli, noroviruses, and F-specific coliphages in fresh market-ready produce. Journal of food protection. 2004;67(11):2387-90.

30. Balasubramanian S, Sorokulova IB, Vodyanoy VJ, Simonian AL. Lytic phage as a specific and selective probe for detection of Staphylococcus aureus--A surface plasmon resonance spectroscopic study. Biosens Bioelectron. 2007;22(6):948-55.

31. Hagens S, Loessner MJ. Bacteriophage for Biocontrol of Foodborne Pathogens: Calculations and Considerations. Current Pharmaceutical Biotechnology. 2010;11(1):58-68.

32. Ackermann H-W. Phage classification and characterization. Bacteriophages: Springer; 2009. p. 127-40.

33. Minikh O, Tolba M, Brovko L, Griffiths M. Bacteriophage-based biosorbents coupled with bioluminescent ATP assay for rapid concentration and detection of Escherichia coli. J Microbiol Methods. 2010;82(2):177-83.

34. Favrin SJ, Jassim SA, Griffiths MW. Development and Optimization of a Novel Immunomagnetic Separation-Bacteriophage Assay for Detection ofSalmonella enterica Serovar Enteritidis in Broth. Applied and environmental microbiology. 2001;67(1):217-24.

35. Sorokulova IB, Olsen EV, Chen I-H, Fiebor B, Barbaree JM, Vodyanoy VJ, et al. Landscape phage probes for Salmonella typhimurium. J Microbiol Methods. 2005;63(1):55-72.

36. Sun W, Brovko L, Griffiths M. Use of bioluminescent Salmonella for assessing the efficiency of constructed phage-based biosorbent. Journal of Industrial Microbiology and Biotechnology. 2001;27(2):126-8.

37. Høyer-Hansen G, Hamers MJ, Pedersen AN, Nielsen HJ, Brünner N, Danø K, et al. Loss of ELISA specificity due to biotinylation of monoclonal antibodies. Journal of immunological methods. 2000;235(1):91-9.

38. Shenoy BC, Wood HG. Purification and properties of the synthetase catalyzing the biotination of the aposubunit of transcarboxylase from Propionibacterium shermanii. The FASEB journal. 1988;2(8):2396-401.

39. Tsao K-L, DeBarbieri B, Michel H, Waugh DS. A versatile plasmid expression vector for the production of biotinylated proteins by site-specific, enzymatic modification in Escherichia coli. Gene. 1996;169(1):59-64.

40. Schatz PJ. Use of peptide libraries to map the substrate specificity of a peptide-modifying enzyme: a 13 residue consensus peptide specifies biotinylation in Escherichia coli. Nature Biotechnology. 1993;11(10):1138-43.

41. Edgar R, McKinstry M, Hwang J, Oppenheim AB, Fekete RA, Giulian G, et al. High-sensitivity bacterial detection using biotin-tagged phage and quantum-dot nanocomplexes. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(13):4841-5.

42. Tolba M, Minikh O, Brovko LY, Evoy S, Griffiths MW. Oriented Immobilization of Bacteriophages for Biosensor Applications. Applied and Environmental Microbiology. 2010;76(2):528-35.

43. Bratkovič T. Progress in phage display: evolution of the technique and its applications. Cellular and molecular life sciences. 2010;67(5):749-67.

44. Favrin SJ, Jassim SA, Griffiths MW. Application of a novel immunomagnetic separation-bacteriophage assay for the detection of Salmonella enteritidis and Escherichia coli O157:H7 in food. Int J Food Microbiol. 2003;85(1-2):63-71.

45. Burnham S, Hu J, Anany H, Brovko L, Deiss F, Derda R, et al. Towards rapid on-site phage-mediated detection of generic Escherichia coli in water using luminescent and visual readout. Analytical and bioanalytical chemistry. 2014;406(23):5685-93.

46. Burnham S, Hu J, Anany H, Brovko L, Deiss F, Derda R, et al. Towards rapid on-site phage-mediated detection of generic Escherichia coli in water using luminescent and visual readout. Analytical and bioanalytical chemistry. 2014;406(23):5685-93.

47. Gebeyehu G, Rao PY, SooChan P, Simms D, Klevan L. Novel biotinylated nucleotide-analogs for labeling and colorimetric detection of DNA. Nucleic acids research. 1987;15(11):4513-34.

48. King W, Raposa S, Warshaw J, Johnson A, Halbert D, Klinger J. A new colorimetric nucleic acid hybridization assay for Listeria in foods. International journal of food microbiology. 1989;8(3):225-32.

49. Schmid-Hempel P, Frank SA. Pathogenesis, Virulence, and Infective Dose. PLoS Pathogens. 2007;3(10):e147.

50. Byrne B, Stack E, Gilmartin N, O'Kennedy R. Antibody-based sensors: Principles, problems and potential for detection of pathogens and associated toxins. Sensors. 2009;9(6):4407-45.

51. Samoylova TI, Ahmed BY, Vodyanoy V, Morrison NE, Samoylov AM, Globa LP, et al. Targeting peptides for microglia identified via phage display. Journal of neuroimmunology. 2002;127(1):13-21.

52. Weber PC, Ohlendorf D, Wendoloski J, Salemme F. Structural origins of high-affinity biotin binding to streptavidin. Science. 1989;243(4887):85-8.

53. Beckett D, Kovaleva E, Schatz PJ. A minimal peptide substrate in biotin holoenzyme synthetase-catalyzed biotinylation. Protein Science. 1999;8(04):921-9.

54. Flekna G, Schneeweiss W, Smulders FJM, Wagner M, Hein I. Real-time PCR method with statistical analysis to compare the potential of DNA isolation methods to remove PCR inhibitors from samples for diagnostic PCR. Molecular and Cellular Probes. 2007;21(4):282-7.

55. Donaldson K, Griffin DW, Paul J. Detection, quantitation and identification of enteroviruses from surface waters and sponge tissue from the Florida Keys using real-time RT–PCR. Water research. 2002;36(10):2505-14.

56. Lazcka O, Del Campo FJ, Munoz FX. Pathogen detection: a perspective of traditional methods and biosensors. Biosensors and bioelectronics. 2007;22(7):1205-17.

57. Caplice E, Fitzgerald GF. Food fermentations: role of microorganisms in food production and preservation. International Journal of Food Microbiology. 1999;50(1–2):131-49.

58. World Health Organization. Food safety. 2015 [cited 2016 21 Jan]. Available from: http://www.who.int/mediacentre/factsheets/fs399/en/.

59. Allos BM, Moore MR, Griffin PM, Tauxe RV. Surveillance for sporadic foodborne disease in the 21st century: the FoodNet perspective. Clinical Infectious Diseases. 2004;38(Supplement 3):S115-S20.

60. Centers for Disease Control and Prevention. CDC and Food Safety. 2015 [cited 2016 22 Jan]. Available from: http://www.cdc.gov/foodsafety/cdc-and-food-safety.html.

61. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson M-A, Roy SL, et al. Foodborne illness acquired in the United States—major pathogens. Emerg Infect Dis. 2011;17(1).

62. Tauxe RV. Emerging foodborne diseases: an evolving public health challenge. Emerging infectious diseases. 1997;3(4):425.

63. McCabe-Sellers BJ, Beattie SE. Food safety: emerging trends in foodborne illness surveillance and prevention. Journal of the American Dietetic Association. 2004;104(11):1708-17.

64. Mead PS, Griffin PM. Escherichia coli O157: H7. The Lancet. 1998;352(9135):1207-12.

65. Doyle MP. Escherichia coli O157: H7 and its significance in foods. International journal of food microbiology. 1991;12(4):289-301.

66. Besser RE, Lett SM, Weber JT, Doyle MP, Barrett TJ, Wells JG, et al. An outbreak of diarrhea and hemolytic uremic syndrome from Escherichia coli O157: H7 in fresh-pressed apple cider. Jama. 1993;269(17):2217-20.

67. Ackers M-L, Mahon BE, Leahy E, Goode B, Damrow T, Hayes PS, et al. An outbreak of Escherichia coli O157: H7 infections associated with leaf lettuce consumption. Journal of Infectious Diseases. 1998;177(6):1588-93.

68. World Health Organization. Salmonella (non-typhoidal) 2013 [cited 2016 23 Jan]. Available from: http://www.who.int/mediacentre/factsheets/fs139/en/.

69. Neidhart FC. Escherichia coli and Salmonella: Blackwell Science Ltd; 1996.

70. Tauxe RV. Salmonella: a postmodern pathogen. Journal of Food Protection®. 1991;54(7):563-8.

71. Poppe C, Smart N, Khakhria R, Johnson W, Spika J, Prescott J. Salmonella typhimurium DT104: a virulent and drug-resistant pathogen. The Canadian veterinary journal. 1998;39(9):559.

72. Centers for Disease Control and Prevention. Salmonella 2015 [cited 2016 23 Jan]. Available from: http://www.cdc.gov/salmonella/index.html.

73. Tompkin R. Control of Listeria monocytogenes in the food-processing environment. Journal of Food Protection®. 2002;65(4):709-25.

74. Cocolin L, Rantsiou K, Iacumin L, Cantoni C, Comi G. Direct identification in food samples of Listeria spp. and Listeria monocytogenes by molecular methods. Applied and Environmental Microbiology. 2002;68(12):6273-82.

75. Velusamy V, Arshak K, Korostynska O, Oliwa K, Adley C. An overview of foodborne pathogen detection: In the perspective of biosensors. Biotechnology Advances. 2010;28(2):232-54.

76. World Health Organization. Water-related diseases. 2014 [cited 2016 25 Jan]. Available from: http://www.who.int/water_sanitation_health/diseases/en/.

77. Straub TM, Chandler DP. Towards a unified system for detecting waterborne pathogens. J Microbiol Methods. 2003;53(2):185-97.

78. Aw TG, Rose JB. Detection of pathogens in water: from phylochips to qPCR to pyrosequencing. Current Opinion in Biotechnology. 2012;23(3):422-30.

79. Brooks B, Devenish J, Lutze-Wallace C, Milnes D, Robertson R, Berlie-Surujballi G. Evaluation of a monoclonal antibody-based enzyme-linked immunosorbent assay for detection of Campylobacter fetus in bovine preputial washing and vaginal mucus samples. Veterinary microbiology. 2004;103(1):77-84.

80. Bushon RN, Likirdopulos CA, Brady AMG. Comparison of immunomagnetic separation/adenosine triphosphate rapid method to traditional culture-based method for E. coli and enterococci enumeration in wastewater. Water Research. 2009;43(19):4940-6.

81. Valasek MA, Repa JJ. The power of real-time PCR. Advances in physiology education. 2005;29(3):151-9.

82. Klein D. Quantification using real-time PCR technology: applications and limitations. Trends in Molecular Medicine. 2002;8(6):257-60.

83. Yang S, Rothman RE. PCR-based diagnostics for infectious diseases: uses, limitations, and future applications in acute-care settings. The Lancet infectious diseases. 2004;4(6):337-48.

84. Järvinen A-K, Laakso S, Piiparinen P, Aittakorpi A, Lindfors M, Huopaniemi L, et al. Rapid identification of bacterial pathogens using a PCR- and microarray-based assay. BMC Microbiology. 2009;9(1):1-16.

85. van Geest M, Lolkema JS. Membrane Topology and Insertion of Membrane Proteins: Search for Topogenic Signals. Microbiology and Molecular Biology Reviews. 2000;64(1):13-33.

86. Diamandis EP, Christopoulos TK. The biotin-(strept)avidin system: principles and applications in biotechnology. Clinical Chemistry. 1991;37(5):625-36.

87. Cull MG, Schatz PJ. [26] Biotinylation of proteins in vivo and in vitro using small peptide tags. In: Jeremy Thorner SDEJNA, editor. Methods in enzymology. Volume 326: Academic Press; 2000. p. 430-40.

88. Duffy S, Tsao KL, Waugh DS. Site-specific, enzymatic biotinylation of recombinant proteins in Spodoptera frugiperda cells using biotin acceptor peptides. Analytical biochemistry. 1998;262(2):122-8.

89. Livnah O, Bayer EA, Wilchek M, Sussman JL. Three-dimensional structures of avidin and the avidin-biotin complex. Proceedings of the National Academy of Sciences. 1993;90(11):5076-80.

90. Tomat D, Quiberoni A, Casabonne C, Balagué C. Phage adsorption on Enteropathogenic and Shiga Toxin-Producing Escherichia coli strains: Influence of physicochemical and physiological factors. Food Research International. 2014;66:23-8.

91. Bebeacua C, Tremblay D, Farenc C, Chapot-Chartier M-P, Sadovskaya I, van Heel M, et al. Structure, Adsorption to Host, and Infection Mechanism of Virulent Lactococcal Phage p2. Journal of Virology. 2013;87(22):12302-12.

92. Miller ES, Kutter E, Mosig G, Arisaka F, Kunisawa T, Rüger W. Bacteriophage T4 Genome. Microbiology and Molecular Biology Reviews. 2003;67(1):86-156.

93. Braun V, Hantke K. Bacterial Receptors for Phages and Colicins as Constituents of Specific Transport Systems. In: Reissig JL, editor. Microbial Interactions. Boston, MA: Springer US; 1977. p. 99-137.

94. Lindberg AA, Wollin R, Gemski P, Wohlhieter JA. Interaction between bacteriophage Sf6 and Shigella flexner. J Virol. 1978;27(1):38-44.

95. Yu F, Yamada H, Mizushima S. Role of lipopolysaccharide in the receptor function for bacteriophage TuIb in Escherichia coli. Journal of bacteriology. 1981;148(2):712-5.

96. Yu F, Mizushima S. Roles of lipopolysaccharide and outer membrane protein OmpC of Escherichia coli K-12 in the receptor function for bacteriophage T4. Journal of bacteriology. 1982;151(2):718-22.

97. Morona R, Klose M, Henning U. Escherichia coli K-12 outer membrane protein (OmpA) as a bacteriophage receptor: analysis of mutant genes expressing altered proteins. Journal of bacteriology. 1984;159(2):570-8.

98. Alphen Lv, Havekes L, Lugtenberg B. Major outer membrane protein d of Escherichia coli K 12. FEBS Letters. 1977;75(1):285-90.

99. Manning PA, Reeves P. Outer membrane of Escherichia coli K-12: Tsx mutants (resistant to bacteriophage T6 and colicin K) lack an outer membrane protein. Biochemical and Biophysical Research Communications. 1976;71(2):466-71.

100. Baptista C, Santos MA, São-José C. Phage SPP1 Reversible Adsorption to Bacillus subtilis Cell Wall Teichoic Acids Accelerates Virus Recognition of Membrane Receptor YueB. Journal of bacteriology. 2008;190(14):4989-96.

101. Puck TT, Garen A, Cline J. THE MECHANISM OF VIRUS ATTACHMENT TO HOST CELLS : I. THE ROLE OF IONS IN THE PRIMARY REACTION. The Journal of Experimental Medicine. 1951;93(1):65-88.

102. Moldovan R, Chapman-McQuiston E, Wu XL. On Kinetics of Phage Adsorption. Biophysical Journal. 2007;93(1):303-15.

103. Watanabe K, Takesue S, Ishibashi K. Reversibility of the adsorption of bacteriophage PL-1 to the cell walls isolated from Lactobacillus casei. The Journal of general virology. 1977;34(1):189-94.

104. Tonelli RR, Colli W, Alves MJM. Selection of binding targets in parasites using phage-display and aptamer libraries in vivo and in vitro. Frontiers in Immunology. 2012;3:419.

105. Favrin SJ, Jassim SA, Griffiths MW. Application of a novel immunomagnetic separation–bacteriophage assay for the detection of Salmonella enteritidis and Escherichia coli O157:H7 in food. International Journal of Food Microbiology. 2003;85(1–2):63-71.

106. Tram LLT, Cao C, Høgberg J, Wolff A, Bang DD. Isolation and detection of Campylobacter jejuni from chicken fecal samples by immunomagnetic separation–PCR. Food Control. 2012;24(1–2):23-8.

107. Abedon ST, Culler RR. Optimizing bacteriophage plaque fecundity. Journal of theoretical biology. 2007;249(3):582-92.

108. Yoshitomi KJ, Zapata R, Jinneman KC, Weagant SD, Fedio W. Recovery of E. coli O157 strains after exposure to acidification at pH 2. Letters in Applied Microbiology. 2012;54(6):499-503.

109. Wadud S, Leon-Velarde CG, Larson N, Odumeru JA. Evaluation of immunomagnetic separation in combination with ALOA Listeria chromogenic agar for the isolation and identification of Listeria monocytogenes in ready-to-eat foods. J Microbiol Methods. 2010;81(2):153-9.

110. Hibi K, Abe A, Ohashi E, Mitsubayashi K, Ushio H, Hayashi T, et al. Combination of immunomagnetic separation with flow cytometry for detection of Listeria monocytogenes. Analytica Chimica Acta. 2006;573–574(0):158-63.

111. Fluit AC, Widjojoatmodjo MN, Box AT, Torensma R, Verhoef J. Rapid detection of salmonellae in poultry with the magnetic immuno-polymerase chain reaction assay. Applied and Environmental Microbiology. 1993;59(5):1342-6.

112. Xu J, Yin W, Zhang Y, Yi J, Meng M, Wang Y, et al. Establishment of magnetic beads-based enzyme immunoassay for detection of chloramphenicol in milk. Food Chemistry. 2012;134(4):2526-31.

113. Amagliani G, Omiccioli E, Campo A, Bruce IJ, Brandi G, Magnani M. Development of a magnetic capture hybridization-PCR assay for Listeria monocytogenes direct detection in milk samples. J Appl Microbiol. 2006;100(2):375-83.

114. Leon-Velarde CG, Zosherafatein L, Odumeru JA. Application of an automated immunomagnetic separation-enzyme immunoassay for the detection of Salmonella enterica subspecies enterica from poultry environmental swabs. J Microbiol Methods. 2009;79(1):13-7.

115. Sung YJ, Suk H-J, Sung HY, Li T, Poo H, Kim M-G. Novel antibody/gold nanoparticle/magnetic nanoparticle nanocomposites for immunomagnetic separation

and rapid colorimetric detection of Staphylococcus aureus in milk. Biosensors and Bioelectronics. 2013;43:432-9.

116. Wang D, Wang Z, Chen J, Kinchla AJ, Nugen SR. Rapid detection of Salmonella using a redox cycling-based electrochemical method. Food Control.

117. Safarik I, Safarikova M. Magnetic techniques for the isolation and purification of proteins and peptides. Biomagnetic Research and Technology. 2004;2:7-.

118. Lynch MJ, Leon-Velarde CG, McEwen S, Odumeru JA. Evaluation of an automated immunomagnetic separation method for the rapid detection of Salmonella species in poultry environmental samples. J Microbiol Methods. 2004;58(2):285-8.

119. Weagant SD, Bound AJ. Evaluation of techniques for enrichment and isolation of Escherichia coli O157:H7 from artificially contaminated sprouts. International Journal of Food Microbiology. 2001;71(1):87-92.

120. Bushon RN, Brady AM, Likirdopulos CA, Cireddu JV. Rapid detection of Escherichia coli and enterococci in recreational water using an immunomagnetic separation/adenosine triphosphate technique. Journal of Applied Microbiology. 2009;106(2):432-41.

121. Steingroewer J, Bley T, Bergemann C, Boschke E. Biomagnetic separation of Salmonella Typhimurium with high affine and specific ligand peptides isolated by phage display technique. Journal of Magnetism and Magnetic Materials. 2007;311(1):295-9.

122. Gervais L, Gel M, Allain B, Tolba M, Brovko L, Zourob M, et al. Immobilization of biotinylated bacteriophages on biosensor surfaces. Sensors and Actuators B: Chemical. 2007;125(2):615-21.

123. Wilchek M, Bayer EA. Introduction to avidin-biotin technology. Methods in enzymology. 1990;184:5-13.

124. Singh A, Arutyunov D, Szymanski CM, Evoy S. Bacteriophage based probes for pathogen detection. The Analyst. 2012;137(15):3405-21.

125. Sun W, Brovko L, Griffiths M. Use of bioluminescent Salmonella for assessing the efficiency of constructed phage-based biosorbent. Journal of industrial microbiology & biotechnology. 2001;27(2):126-8.

126. Bennett AR, Davids FG, Vlahodimou S, Banks JG, Betts RP. The use of bacteriophage-based systems for the separation and concentration of Salmonella. J Appl Microbiol. 1997;83(2):259-65.

127. Clark JR, March JB. Bacteriophages and biotechnology: vaccines, gene therapy and antibacterials. Trends in Biotechnology. 2006;24(5):212-8.

128. van der Merwe RG, van Helden PD, Warren RM, Sampson SL, Gey van Pittius NC. Phage-based detection of bacterial pathogens. The Analyst. 2014;139(11):2617-26.

129. Carey-Smith GV, Billington C, Cornelius AJ, Hudson JA, Heinemann JA. Isolation and characterization of bacteriophages infecting Salmonella spp. FEMS microbiology letters. 2006;258(2):182-6.

130. Piuri M, Rondón L, Urdániz E, Hatfull GF. Generation of Affinity-Tagged Fluoromycobacteriophages by Mixed Assembly of Phage Capsids. Applied and Environmental Microbiology. 2013;79(18):5608-15.

131. Tawil N, Sacher E, Mandeville R, Meunier M. Bacteriophages: biosensing tools for multi-drug resistant pathogens. The Analyst. 2014;139(6):1224-36.

132. Favrin SJ, Jassim SA, Griffiths MW. Development and optimization of a novel immunomagnetic separation- bacteriophage assay for detection of Salmonella enterica serovar enteritidis in broth. Appl Environ Microbiol. 2001;67(1):217-24.

133. Chen J, Duncan B, Wang Z, Wang L-S, Rotello VM, Nugen SR. Bacteriophage-based nanoprobes for rapid bacteria separation. Nanoscale. 2015;7(39):16230-6.

134. Park M, Lee J-H, Shin H, Kim M, Choi J, Kang D-H, et al. Characterization and Comparative Genomic Analysis of a Novel Bacteriophage, SFP10, Simultaneously Inhibiting both Salmonella enterica and Escherichia coli O157:H7. Applied and Environmental Microbiology. 2012;78(1):58-69.

135. Leuschner RK, Arendt E, Hammes W. Characterization of a virulent Lactobacillus sake phage PWH2. Appl Microbiol Biotechnol. 1993;39(4-5):617-21.

136. Xiong Q, Cui X, Saini JK, Liu D, Shan S, Jin Y, et al. Development of an immunomagnetic separation method for efficient enrichment of Escherichia coli O157:H7. Food Control. 2014;37(0):41-5.

137. Lee J, Kaletunç G. Evaluation of the Heat Inactivation of Escherichia coli and Lactobacillus plantarum by Differential Scanning Calorimetry. Applied and Environmental Microbiology. 2002;68(11):5379-86.

138. Wang I-N, Dykhuizen D, Slobodkin L. The evolution of phage lysis timing. Evol Ecol. 1996;10(5):545-58.

139. Nguyen HM, Kang C. Lysis Delay and Burst Shrinkage of Coliphage T7 by Deletion of Terminator Tϕ Reversed by Deletion of Early Genes. Journal of Virology. 2014;88(4):2107-15.

140. Mansfield LP, Forsythe SJ. The detection of Salmonella using a combined immunomagnetic separation and ELISA end-detection procedure. Lett Appl Microbiol. 2000;31(4):279-83.

141. Besser RE, Lett SM, Weber J, et al. An outbreak of diarrhea and hemolytic uremic syndrome from escherichia coli o157:h7 in fresh-pressed apple cider. JAMA. 1993;269(17):2217-20.

142. Tarr PI. Escherichia coli O157:H7: clinical, diagnostic, and epidemiological aspects of human infection. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1995;20(1):1-8; quiz 9-10.

143. Blackall DP, Marques MB. Hemolytic uremic syndrome revisited: Shiga toxin, factor H, and fibrin generation. American journal of clinical pathology. 2004;121 Suppl:S81-8.

144. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, et al. Food-related illness and death in the United States. Emerging Infectious Diseases. 1999;5(5):607-25.

145. Hilborn ED, Mermin JH, Mshar PA, et al. A multistate outbreak of escherichia coli o157:h7 infections associated with consumption of mesclun lettuce. Archives of Internal Medicine. 1999;159(15):1758-64.

146. Frenzen PD, Drake A, Angulo FJ. Economic cost of illness due to Escherichia coli O157 infections in the United States. Journal of food protection. 2005;68(12):2623-30.

147. Bell BP, Goldoft M, Griffin PM, Davis MA, Gordon DC, Tarr PI, et al. A multistate outbreak of Escherichia coli O157:H7-associated bloody diarrhea and hemolytic uremic syndrome from hamburgers. The Washington experience. Jama. 1994;272(17):1349-53.

148. Scallan E, Hoekstra RM, Angulo FJ, Tauxe RV, Widdowson MA, Roy SL, et al. Foodborne illness acquired in the United States--major pathogens. Emerg Infect Dis. 2011;17(1):7-15.

149. Mead PS, Griffin PM. Escherichia coli O157:H7. Lancet (London, England). 1998;352(9135):1207-12.

150. Johnson RP, Durham RJ, Johnson ST, MacDonald LA, Jeffrey SR, Butman BT. Detection of Escherichia coli O157:H7 in meat by an enzyme-linked immunosorbent assay, EHEC-Tek. Applied and Environmental Microbiology. 1995;61(1):386-8.

151. Zhu P, Shelton DR, Karns JS, Sundaram A, Li S, Amstutz P, et al. Detection of water-borne E. coli O157 using the integrating waveguide biosensor. Biosensors & bioelectronics. 2005;21(4):678-83.

152. Solomon EB, Yaron S, Matthews KR. Transmission of Escherichia coli O157:H7 from Contaminated Manure and Irrigation Water to Lettuce Plant Tissue and Its Subsequent Internalization. Applied and Environmental Microbiology. 2002;68(1):397-400.

153. Swerdlow DL, Woodruff BA, Brady RC, Griffin PM, Tippen S, Donnell JHD, et al. A Waterborne Outbreak in Missouri of Escherichia coli O157:H7 Associated with Bloody Diarrhea and Death. Annals of Internal Medicine. 1992;117(10):812-9.

154. Wachtel MR, Whitehand LC, Mandrell RE. Association of Escherichia coli O157:H7 with Preharvest Leaf Lettuce upon Exposure to Contaminated Irrigation Water. Journal of food protection. 2002;65(1):18-25.

155. Islam M, Doyle MP, Phatak SC, Millner P, Jiang X. Persistence of Enterohemorrhagic Escherichia coli O157:H7 in Soil and on Leaf Lettuce and Parsley Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water. Journal of food protection. 2004;67(7):1365-70.

156. Szalanski AL, Owens CB, McKay T, Steelman CD. Detection of Campylobacter and Escherichia coli O157:H7 from filth flies by polymerase chain reaction. Medical and Veterinary Entomology. 2004;18(3):241-6.

157. March SB, Ratnam S. Sorbitol-MacConkey medium for detection of Escherichia coli O157:H7 associated with hemorrhagic colitis. Journal of clinical microbiology. 1986;23(5):869-72.

158. Vuddhakul V, Patararungrong N, Pungrasamee P, Jitsurong S, Morigaki T, Asai N, et al. Isolation and characterization of Escherichia coli O157 from retail beef and bovine feces in Thailand2000 2000-01-01 00:00:00. 343-7 p.

159. Gordillo R, Rodríguez A, Werning ML, Bermúdez E, Rodríguez M. Quantification of viable Escherichia coli O157:H7 in meat products by duplex real-time PCR assays. Meat Science. 2014;96(2, Part A):964-70.

160. de Boer E, Beumer RR. Methodology for detection and typing of foodborne microorganisms. Int J Food Microbiol. 1999;50(1-2):119-30.

161. Higgins JA, Nasarabadi S, Karns JS, Shelton DR, Cooper M, Gbakima A, et al. A handheld real time thermal cycler for bacterial pathogen detection. Biosens Bioelectron. 2003;18(9):1115-23.

162. Bekal S, Brousseau R, Masson L, Prefontaine G, Fairbrother J, Harel J. Rapid Identification of Escherichia coli Pathotypes by Virulence Gene Detection with DNA Microarrays. Journal of clinical microbiology. 2003;41(5):2113-25.

163. Carter CD, Parks A, Abuladze T, Li M, Woolston J, Magnone J, et al. Bacteriophage cocktail significantly reduces Escherichia coli O157: H7 contamination of lettuce and beef, but does not protect against recontamination. Bacteriophage. 2012;2(3):178-85.

164. Brussow H, Hendrix RW. Phage genomics: small is beautiful. Cell. 2002;108(1):13-6.

165. Jończyk E, Kłak M, Międzybrodzki R, Górski A. The influence of external factors on bacteriophages—review. Folia Microbiologica. 2011;56(3):191-200.

166. Goodridge L, Chen J, Griffiths M. Development and Characterization of a Fluorescent-Bacteriophage Assay for Detection of Escherichia coli O157:H7. Applied and Environmental Microbiology. 1999;65(4):1397-404.

167. Hoang HA, Dien LT. Rapid and Simple Colorimetric Detection of Escherichia coli O157:H7 in Apple Juice Using a Novel Recombinant Bacteriophage-Based Method. Biocontrol science. 2015;20(2):99-103.

168. Johnson JL, Brooke CL, Fritschel SJ. Comparison of the BAX for Screening/E. coli O157:H7 Method with Conventional Methods for Detection of Extremely Low Levels of Escherichia coli O157:H7 in Ground Beef. Applied and Environmental Microbiology. 1998;64(11):4390-5.

169. Foddai A, Elliott CT, Grant IR. Maximizing Capture Efficiency and Specificity of Magnetic Separation for Mycobacterium avium subsp. paratuberculosis Cells. Applied and Environmental Microbiology. 2010;76(22):7550-8.

170. Chen J, Alcaine SD, Jiang Z, Rotello VM, Nugen SR. Detection of Escherichia coli in Drinking Water Using T7 Bacteriophage-conjugated Magnetic Probe. Analytical Chemistry. 2015.

171. Dai F, Zhang M, Hu B, Sun Y, Tang Q, Du M, et al. Immunomagnetic nanoparticles based on a hydrophilic polymer coating for sensitive detection of Salmonella in raw milk by polymerase chain reaction. RSC Advances. 2015;5(5):3574-80.

172. Shelton DR, Karns JS. Quantitative Detection of Escherichia coli O157 in Surface Waters by Using Immunomagnetic Electrochemiluminescence. Applied and Environmental Microbiology. 2001;67(7):2908-15.

173. Yu MX, Slater MR, Ackermann HW. Isolation and characterization of Thermus bacteriophages. Archives of virology. 2006;151(4):663-79.

174. Handa H, Gurczynski S, Jackson MP, Auner G, Mao G. Recognition of Salmonella Typhimurium by Immobilized Phage P22 Monolayers. Surface science. 2008;602(7):1392-400.

175. Fluit AC, Torensma R, Visser MJ, Aarsman CJ, Poppelier MJ, Keller BH, et al. Detection of Listeria monocytogenes in cheese with the magnetic immuno-polymerase chain reaction assay. Applied and Environmental Microbiology. 1993;59(5):1289-93.

176. Rakhuba DV, Kolomiets EI, Dey ES, Novik GI. Bacteriophage receptors, mechanisms of phage adsorption and penetration into host cell. Polish journal of microbiology / Polskie Towarzystwo Mikrobiologow = The Polish Society of Microbiologists. 2010;59(3):145-55.

177. Jokinen C, Edge TA, Ho S, Koning W, Laing C, Mauro W, et al. Molecular subtypes of Campylobacter spp., Salmonella enterica, and Escherichia coli O157:H7 isolated from

faecal and surface water samples in the Oldman River watershed, Alberta, Canada. Water Research. 2011;45(3):1247-57.

178. Centers for Disease Control and Prevention. Global WASH-related diseases and contaminants 2012 [cited 2015 Nov 12]. Available from: http://www.cdc.gov/healthywater/wash_diseases.html.

179. Bej AK, DiCesare JL, Haff L, Atlas RM. Detection of Escherichia coli and Shigella spp. in water by using the polymerase chain reaction and gene probes for uid. Applied and Environmental Microbiology. 1991;57(4):1013-7.

180. Wang Z, Wang D, Chen J, Sela DA, Nugen SR. Development of a novel bacteriophage based biomagnetic separation method as an aid for sensitive detection of viable Escherichia coli. The Analyst. 2015.

181. Wang D, Wang Z, Chen J, Kinchla AJ, Nugen SR. Rapid detection of Salmonella using a redox cycling-based electrochemical method. Food Control. 2016;62:81-8.

182. Bernal CS, Robinson G, Miles RJ, Price RG. Development of a sensitive and quantitative enzyme-linked immunofilter assay (ELIFA) for whole bacterial cells. J Microbiol Methods. 1994;19(2):135-43.

183. Mazenko RS, Rieders F, Brewster JD. Filtration capture immunoassay for bacteria: optimization and potential for urinalysis1. J Microbiol Methods. 1999;36(3):157-65.

184. Schmelcher M, Loessner MJ. Application of bacteriophages for detection of foodborne pathogens. Bacteriophage. 2014;4:e28137.

185. Blasco R, Murphy MJ, Sanders MF, Squirrell DJ. Specific assays for bacteria using phage mediated release of adenylate kinase. Journal of Applied Microbiology. 1998;84(4):661-6.

186. Neufeld T, Schwartz-Mittelmann A, Biran D, Ron EZ, Rishpon J. Combined Phage Typing and Amperometric Detection of Released Enzymatic Activity for the Specific Identification and Quantification of Bacteria. Analytical Chemistry. 2003;75(3):580-5.

187. Yemini M, Levi Y, Yagil E, Rishpon J. Specific electrochemical phage sensing for Bacillus cereus and Mycobacterium smegmatis. Bioelectrochemistry (Amsterdam, Netherlands). 2007;70(1):180-4.

188. Jokerst JC, Adkins JA, Bisha B, Mentele MM, Goodridge LD, Henry CS. Development of a Paper-Based Analytical Device for Colorimetric Detection of Select Foodborne Pathogens. Analytical Chemistry. 2012;84(6):2900-7.

189. Wen J, Zhou S, Chen J. Colorimetric detection of Shewanella oneidensis based on immunomagnetic capture and bacterial intrinsic peroxidase activity. Scientific Reports. 2014;4:5191.

190. Porstmann B, Porstmann T, Nugel E, Evers U. Which of the commonly used marker enzymes gives the best results in colorimetric and fluorimetric enzyme immunoassays: Horseradish peroxidase, alkaline phosphatase or β-galactosidase? Journal of Immunological Methods. 1985;79(1):27-37.

191. Bronstein I, Voyta JC, Thorpe GH, Kricka LJ, Armstrong G. Chemiluminescent assay of alkaline phosphatase applied in an ultrasensitive enzyme immunoassay of thyrotropin. Clinical Chemistry. 1989;35(7):1441-6.