Nanomagnetic Immunoassay for Rapid Detection of *Escherichia coli*

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ABSTRACT

This study identifies a simple process and a hand held device to quickly conduct an analysis, under field conditions, for detection of pathogens. Hand held devices for rapid detection of pathogens have been proposed to be used by first responders. A magnetic sandwich immunoassay is developed for rapid isolation and detection of targeted pathogen. *Escherichia coli* BL21 Star (DE3) pLysS is used as a model in this study. The magnetic immunoassay takes advantage of the multiple epitopes that are found on the target. On one epitope a magnetic particle is attached. On another epitope a glucose molecule is attached. Glucometers have been designed with suitable accuracy to detect small amounts of glucose through the oxidation of glucose to gluconic acid at the site of platinum electrode. The sandwich magnetic assay that contains the glucose label can be easily correlated to the concentration of the *Escherichia coli* BL21 Star (DE3) pLysS.

Keywords: Magnetic immunoassay, Glucose, E.coli, Nanoparticles

INTRODUCTION:

Rapid detection of biological warfare and biological terrorist threat agents and emerging diseases are significant military and civilian challenges. Biological agents when properly prepared are extremely potent and could be disseminated to anticipate or kill thousands of individuals. In addition, worldwide 22 million people die, every year, of infectious diseases. Bacterial infections are responsible for a high percentage of these infections [1]

Traditionally, detection of microorganisms is relying on the same biochemical basis developed by Pasteur and others in the last century and the disk diffusion method developed by Kirby-Bauer for antimicrobial susceptibility testing [2]. Last decade emphasis had been on automation of these basic biochemical tests. This has accelerated the identification but still takes, with the most sophisticated system, a minimum of 30 hrs under optimal conditions, for a precise diagnosis of bacterial infection.

Possibilities of low concentration of collectable bacteria at the site of investigation will cause a challenge if traditional culture methods are used. Studies suggest that only 1-10% of ambient bacteria are culturable [3]. According to the Center for biological Defense aerosol bacteria is of major concern when used in warfare or terrorist threats. A portable lightweight easy to use device that is able to detect with high sensitivity and very short time would provide a tremendous advantage when searching for pathogens.

Magnetic immunoassay for isolation *E.coli* was reported by Wright et al. [2] with an assay time of approximately 24 h. Detection methods for *E.coli* based on enzyme-linked immunosorbent assays (ELISA) [3] and polymerase chain reaction (PCR) [4] have been developed and improvements in their performance time and sensitivity are ongoing. PCR detection methods have the possibility of single cell detection with the potential of taking less than 8 h to perform [5]. However, some molecular methods of bacterial detection have not been fully accepted by routine microbiology testing laboratories. This is maybe due to the need for relatively expensive equipment and associated specialist skills to perform the analyses [6]. By comparison, immunological detection tests for bacterial pathogens (including latex agglutination, immunomagnetic separation, lateral flow

immunoassays and ELISA) are used frequently. Furthermore, ELISA detection methods have sensitivities of 10^5 – 10^7 Bacterial cells.ml^{-1}[7] and require overnight enrichment of the sample prior to analysis [8]. Some sensitive immunological methods have been developed using elector-chemiluminescence [9] and rapid flow through systems [10].

Current detection methods have led to the possible detection within a single working day. This study presents a significant bacterial detection technique that takes less than one hour to detect the exact amount of *E.coli* in the solution using glucose detection techniques. The basic assay uses the magnetic immunoassay technology to detect the minute concentration of *E.coli* in a sample. It utilizes a sandwich immunoassay formed by attaching two different antibodies to different epitopes on the same target antigen which is in this case, *E.coli* strain BL21 Star (DE3) pLysS. One antibody is attached to a solid surface of the magnetic particle, and the other is attached to a glucose molecule. The first antibody is used for the separation of the antigen (*E.coli*) from the sample, while the second antibody, attached to a glucose molecule(s), is used to measure the relative concentration of bacteria in the tested sample. Attaching glucose molecules at the end of the anti-Bacterial Endotoxin antibody facilitates the detection of the bacteria because it will be easily measured by a regular spectrophotometer at a wavelength of 500nm.

Traditionally, glucose is measured by one of three methods; Electrical, colorimetric, and acidity measurement. Due to sensitivity problems in both electrical and acidity approaches, colorimetric approach is used in this study. In colorimetric assay, Glucose was allowed to react with its enzyme Glucose Oxidase (GOX) to form Gluconic acid and hydrogen peroxide (H_2O_2).

$$\text{Glucose} + O_2 \xrightarrow{\text{GOX}} \text{Gluconic acid} + H_2O_2$$

$$H_2O_2 + \text{Reduced Chromogen} \longrightarrow H_2O + \text{Oxidized Chromgen}$$

The hydrogen peroxide then allowed to react with a reduced form of chromogen compound to form a colored oxidized chromogen compound, which was monitored by reflectance photometry [11].

MATERIALS AND METHODS

All magnetic microspheres (MMS) used in this study were produced using protocols that were established by [12, 13]. The MMS were coated with albumin and coupled with avidin to conjugate the anti-*E.coli* Rabbit antibody. Affinity purified antibodies both Rabbit antibody against *E.coli* and Monotype Antibody Endotoxin against *E.coli* BL21were obtained from ViroStat Company (Portland, ME, USA). The Rabbit Antibodies were already biotinylated while the Monotype Antibodies Endotoxin were biotinylated using EZ-link Sulfo-NHS-LC biotinylation kit obtained from Pierce chemicals (Rockford, IL, USA). The antigen *E.coli* strain BL21 Star (DE3) pLysS was obtained from Invitrogen Corporation (Carlsbad, CA, USA). Both Streptavidin (SA), Isolated from the microorganism *Streptomyces avidinii* and Concanavalin A (Con A) lectin were bought from Vector Laboratories (Burlingame, CA, USA). Glucose Oxidase Reagent was obtained from TECO Diagnostics (Anaheim, CA, USA).

Con-A is one of the most widely used and well characterized lectins. It has broad applicability primarily because it recognizes a commonly occurring sugar structure, α-D-Mannose and α-D-Glucose through its four saccharide binding sites. At neutral and alkaline pH, Con-A exists as a tetramer of four identical subunits of approximately 26,000 daltons each. Below pH 5.6, Con A dissociates into active dimers of 52,000 daltons. In this study, biotinylated Con-A lectins were prepared in a concentration of 1µL/ml by mixing 2µL Con-A with 9.998 µL HEPES buffer solution. The solution then kept refrigerated under -4^{o}C for further usage.

Decimal dilutions (10^1, 10^2, 10^3, 10^4, 10^5, and 10^6) of E.coli BL21 were prepared in Trypticase Soy Broth and cultured in Trypticase soy agar and incubated overnight under 37^{o}C to count the growth of bacteria. Bacterial colonies were then

counted. Each dilution was multiplied by its dilution factor and a final bacterial concentration of (3.4 x 10^9 cell/ml) was calculated.

The target conjugate of the experiment composed of two major parts; each was prepared separately as follows. The first part (Endotoxin-SA-Con A) was formed by adding an exact volume amount of 200 µl of Con-A and SA and was allowed to link each other for 30 minutes at 30°C while shaking, then the same volume amount of Biotinylated Endotoxin antibodies were added to the mixture and allowed again to react for 30 minutes at 30°C while shaking.

The second part of the ELISA conjugate (Rabbit Antibody against E.coli –microspheres (MMS)) was formed by adding 600 µl of biotinylated Rabbit antibodies to 7.6 ml of (0.01 mg/ml) of MMS and incubated together for 30 minutes at 30°C while shaking then an addition of 1ml of Human Serum Albumin protein (HSA) was performed to block any unreacted active sites on the microspheres to prevent any secondary signal that might result from the addition of the second part of the complex. Then, a 200 µl of each bacterial concentration was added to the conjugate and incubated under the same conditions.

To form the whole conjugate, the previous two major parts were added up to each other and incubated for 30 minutes at 30°C while shaking and a 300 µl of glucose was added to each tube and incubated under the same incubation conditions. After that, two 1.5 tesla magnets were applied to the side of each tube to isolate the reactants (SPM-Rabbit Antibody-E.coli-Endotoxin-SA-Con A-Glucose) from other excess solution.

The isolated reactants were then washed several times by Tris Buffered Saline (TBS) while the magnets applied, then a volume of 50 µl of each washed reactants was added to preheated tubes each contained 600 µl Glucose oxidase enzyme reagent and read spectrophotometrically at 500nm.

RESULTS

The determination of peripheral glucose molecules attached to the end of the whole conjugate is used to determine the concentration of *E.coli* that was taken up by the conjugate. The preheated Glucose oxidase enzyme reagent was incubated with a 50 µl samples for 10min in a water bath at 30°C. The chemical reaction that occurred between the Glucose oxidase enzyme and its substrate (Peripheral glucose) resulted in a pink-like color which can be detected spectrophotometrically at A_{500}. Since the serial dilutions of *E.coli* were used and inserted in the conjugate, different color intensities were resulted where each dilution correlated to the amount of *E.coli* that was linked on the conjugate.

Fig. 1 represents the relationship between various bacterial concentrations and their correlated glucose concentrations. The absorbencies of each bacterial dilution were matched with the standard absorbencies that were taken from the glucose standard curve, and the glucose concentration was calculated for each dilution using the curve standard equation $y = 41.046x^{-0.1945}$.

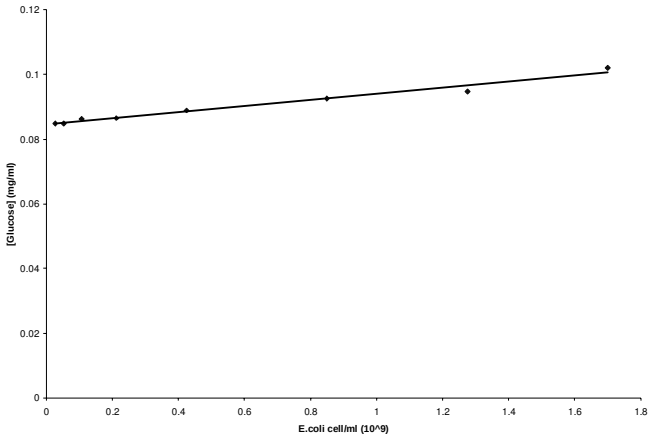

Fig.1. The relationship between various bacterial concentrations and their calculated glucose concentrations.

CONCLUSIONS

Magnetic ELISA that this study presents is an innovative bacterial detection method that involves using Streptavidin-biotin-antibody interaction. The literature shows that ELISA with SA-Biotin-Ab reaction has better assay sensitivity than the ELISA using the direct antibody

absorption format. Furthermore, glucose molecules which are attached to the end of the conjugate could be measured by multiple glucose measurement methods (electrical, colorimetric, and acidity measurement). Although, the electrical method, which can be easily carried out by using a regular glucometer, is rapid, convenient and affordable, it has some sensitivity problems. This disadvantage could be overcome by increasing the ability of glucometers to detect small glucose concentrations. Therefore, in this study colorimetric glucose assay was preferred over others for its rapidity and convenience, as well as its ability to detect minute terminal glucose concentrations.

Magnetic ELISA assay described can be applied to any sandwich immunoassay ELISA. It is predictable that this technique could be useful for the detection of a wide range of target antigens, e.g. HIV and hepatitis in blood, bacterial toxins (e.g. *Staphylococcus enterotoxins*), bacteria (e.g. *Salmonella*, *listeria*) in multiple environments.

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