1 **Highly specific and rapid immuno-fluorescent visualization and detection of *E.***
2 ***coli* O104: H4 with protein-A coated magnetic beads based LST-MUG assay –**
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Abstract

A method combining immunomagnetic separation and fluorescent sensing was developed to detect *Escherichia coli* (*E.coli*) O104:H4. The antibody specific to *E. coli* O104:H4 were immobilized on protein A-coated magnetic beads. This protein-A-anti *E. coli* O104:H4 complex was used to bind Fluorescein IsoThioCyanate (FITC) labeled *E. coli* O104:H4 antigen (whole cell) on it. The goal was to achieve a fluorescently detectable protein-A-anti *E. coli* O104:H4 - *E. coli* O104:H4 complex on the magnetic beads. Fluorescent microscopy was used to image the magnetic beads. The resulting fluorescence on the beads was due to the FITC labeled antigen binding on the protein-A-anti *E.coli* O104:H4 immobilized magnetic beads. This visually proves the antigen-antibody binding. The fluorescent imaging results were obtained in 2 hrs if the minimum available bacteria in the sample were at least 10^5 CFU/ml. If no fluorescence was observed on the magnetic beads during fluorescent imaging, it indicates the bacterial concentration in the sample to be too low for it to have bound to the magnetic beads and hence no detection was possible. To detect bacterial concentration less than 10^5 CFU/ml in the sample, an additional step was required for detection. The magnetic bead complex was added to the LST-MUG (Lauryl Sulfate Tryptose-4-methylumbelliferyl- β -D-glucuronide), a signalling reporter. The *E. coli* O104:H4 grows in LST-MUG and releases β -glucuronidase enzyme. This enzyme cleaves the MUG substrate that produces 4-methylumbelliferone, a highly fluorescent species. This fluorescence was detected using a spectrofluorometer. The emission peak in the fluorescent spectrum was found to be at 450nm. The lower and upper detection range for this LST-MUG assay was found to be 2.05×10^5 - 4.09×10^8 CFU/ml. The results for the LST-MUG assay for concentrations below 10^5 CFU/ml were ascertained in 8 hrs. The advantages of this technique include the specific detection of bacteria without an enrichment step and allowing the procedure to be completed in hrs rather than days.

Abbreviations: FITC, Fluorescein IsoThioCyanate, LST, Lauryl Sulfate Tryptose, MUG, 4-methylumbelliferyl- β -D-glucuronide

Keywords: *E. coli* O104:H4; MUG assay, optical sensing; magnetic beads; fluorescent microscopy

1. Introduction

Ingestion of Enterohaemorrhagic *Escherichia coli* strains (EHEC) from contaminated food causes Haemorrhagic Colitis (HC) and Haemolytic Uraemic Syndrome (HUS) in humans. EHEC O26, O103, O111, O118, O121, O145 and O157 strains are responsible for the majority of HC and HUS cases worldwide (Tzschoppe et al., 2012). More recently, the emerging aggregative EHEC O104:H4 strain caused a significant outbreak with high HUS incidence in Europe and the United States (Ahmed et al., 2012; Buchholz et al., 2011; Muniesa et al., 2012). According to the Food and Drug Administration, outbreaks of food-borne illnesses due to consumption of contaminated food have increased over the past decade. Foodborne pathogens cause millions of infections every year and are responsible for many fatalities worldwide. Along with the threat to human health and safety, there is also a major economic impact as a result of such outbreaks. It is noted that the cost related with foodborne illness in the US is estimated at \$77 billion annually (Scharff, 2012).

E.coli O104:H4 serotype outbreak which is relatively rare was a cause of serious concern in 16 countries recently, with more than 5000 people being affected and fatalities of upward of 50 (López-Campos et al., 2012). Pathogenic strains of *E.coli* are capable of causing a number of diseases. *E.coli* (EHEC) strains that produce Shiga toxins result in fever and bloody diarrhea (Rohde et al., 2015). The patients infected with *E.coli* O104:H4 could possibly develop hemolytic uremic syndrome (HUS) which was found to be more severe in nature in this serotype of *E.coli* than previously reported in patients with other Shiga-toxin producing *E.coli* strains. This strain of *E.coli* O104:H4 while expressing Shiga toxin typical of enterohemorrhagic (EH) shared features of enteroaggregative (EA) strains as well (López-Campos et al., 2012; Rohde et al., 2015).

The threat posed by foodborne pathogens can be monitored by a range of different detection methods. Some of which enable only the qualitative confirmation for the presence or absence of a pathogen, while others allow for the quantification of the bacterial load (Rohde et al., 2015). The latter feature might not be of significance for pathogens with a zero-tolerance-standard, but is especially vital for pathogens which are acceptable in food products if the concentration is below a certain limit (Pérez et al.,

2001). The standards for the detection of bacterial pathogens in food are still the culture and microbiological methods that follow standardized protocols developed years ago. Though accurate, these methods are time-consuming and do not provide results fast enough and thus are limited in their ability to protect consumers in time from potential microbial hazards.

Common methods for the detection of pathogenic *E. coli* include electrochemical-based detection (Peborde et al., 1989; Wang et al., 2008), enzyme-linked immunosorbent assay (ELISA) (Abdulmawjood et al., 2003) and perhaps most commonly, polymerase chain reaction (PCR) detection (Gilgen et al., 1998; Mukhopadhyay and Mukhopadhyay, 2007; Wang et al., 2014). These methods have their own advantages and limitations. Electrochemical sensors, for example, are highly adaptable for portability and field-deployment, but often suffer from poor sensitivity and can be quite expensive. ELISA techniques are often employed for laboratory-based detection of *E. coli*, but this method takes at least 48 hrs to obtain results. PCR-based methods are also relatively exhaustive and time consuming.

More advanced technologies like the Real-time PCR detection (Ivnitski et al., 1999), DNA extraction, PCR variants and amplicons analysis, Loop mediated isothermal amplification, Nucleic acid sequence based amplification, Biosensors, Electrochemical biosensors (Mortari and Lorenzelli, 2014), Flow cytometry (Shibajyoti Ghosh Dastider et al., 2013), Impedance microbiology (S. Ghosh Dastider et al., 2013; Dweik et al., 2012; Ghosh Dastider et al., 2012) and spectrofluorometry have been employed to detect bacteria (Peacock and Kudela, 2012). Fluorescence in situ hybridization (FISH) is a recent method that represents a rapid and highly specific technique for whole-cell detection (McNamara et al., 2014). However, lack of a protocol for quick detection could lead to significant infection and outbreaks that could rapidly take the form an epidemic, as was the case with the recent outbreak of EHEC O104:H4 infection (<http://www.cdc.gov/ecoli/2011/ecolio104/>, <http://www.euro.who.int/en/health-topics/emergencies/international-health-regulations/outbreaks-of-e.-coli-o104h4-infection>). The use of magnetic beads with PCR has also been studied earlier (Fu et al., 2005; Wang et al., 2012).

Most of the current methods for detecting trace amounts of bacteria need either amplification or enrichment of the target bacteria in the sample. Therefore, simple and sensitive methods, which do not need target amplification or enrichment, are currently being pursued. The fluorescence analysis we propose is a rapid, visually provable, technically simple and efficient method for directly isolating and detecting target bacteria from originals or pre-enriched samples.

In this study we propose a fluorescence measurement method for rapid detection of *E.coli* O104:H4. In this method, protein-A coated magnetic beads were used as a platform for immobilization of anti *E. coli* O104:H4 on it. This protein-A-anti *E. coli* O104:H4 complex was used to bind *E. coli* O104:H4 bacterial cell (antigen) on it. This technique ensures that the complex provides imagable results for the specific binding. The imaging process produced results if the concentration of the antigen was above 10^5 CFU/ml. If no discernable imaging results were obtained, it was concluded that the concentration of the antigen was below 10^5 CFU/ml. An extra step is required for detection of lower concentrations of the antigen. The magnetic beads complex was added to the LST-MUG broth, which utilizes 4-methylumbelliferyl- β -D-glucuronide (MUG) as a signaling reporter to indicate the presence of the pathogenic bacteria *E.coli* O104:H4. When the *E. coli* bacterium comes into contact with MUG, it cleaves the substrate via the enzymatic activity of β -glucuronidase. The resulting cleavage products include 4-methylumbelliferone, which is a highly fluorescent species. This fluorescence is detected using a spectrofluorometer. The advantages of this technique include, the specific (qualitative and quantitative) detection of bacteria, and versatility in terms of acting like a platform for detection of “any” bacteria should be especially useful. Using protein-A magnetic bead based purification and direct incubation in LST-MUG assay obviates the need for an enrichment step, allowing the procedure to be completed in hrs versus the tests like most-probable-number method and others that take 2-4 days (Moberg, 1985). While signaling process has been previously used for fluorogenic detection of *E. coli* (Cakir, 2001; Singh and Ng, 1986), it is also important to note that previous LST-MUG-based detection techniques have relied on surfactants that limit the growth of non-coliform organisms (Masuda-Nishimura et al., 2000). The technique proposed in this manuscript combines the unmatched selectivity of IgG antibodies with

the fluorescent reporter LST-MUG assay. There has been no study reported to best of our knowledge that shows either qualitative or quantitative response of *E.coli* O104:H4 using such a technique.

2. Experimental

2.1. Bacteria and inoculum preparation

The *E. coli* O104:H4 (ATCC BAA-2326) and *E. coli* O157:H7 (ATCC 700728) used in this study were obtained from American Type Culture Collection (ATCC, USA) For inoculum preparation, 18 hrs old culture at 37 °C in the Tryptic Soy Broth (TSB) (Fluka Analytical, USA) measuring 1 ml was centrifuged and the supernatant was pippered out. The resultant bacteria pellet was washed with Phosphate Buffer Saline (PBS). The washed pellet was re-dispersed in 1ml of LST-MUG (Fluka Analytical, USA) broth and used as inoculum throughout the experiment.

2.2. MUG Assay

2.2.1. Culturing bacteria in LST-MUG broth

From the prepared inoculum, (20 µl) was added to 20 ml of sterile LST-MUG broth and incubated at 37° C for 18 hrs (from prior experimental analysis it was determined that 18 hrs of incubation time was optimum for the experiment). The sterile media was incubated along with the culture to be used as a “control sample”. The prepared samples were subjected for fluorescent spectroscopy

2.2.2. Preparations for dilution assay

After incubation, many different dilutions of the culture were prepared using the sterile LST-MUG broth. For all dilutions the final volume to be tested was 2ml. Initially, the culture concentrations in the dilutions were kept between 500 µl and 50 µl, and for each subsequent dilution the culture volume was reduced by 50 µl. This dilution assay was used to find the lowest detection limit of the method.

2.2.3 Enumeration of the bacteria

Each dilution was subjected to Colony Forming Unit (CFU) analysis to determine the bacterial concentration. For serial dilutions, sterile deionized water was used and subsequent dilutions were plated on Tryptic Soy Agar (Fluka Analytical, USA). After inoculation, the plates were incubated at 37° C for 24 hrs. After incubation, the plates were observed for CFU's and the concentration determined.

2.2.4 Sample for detection time for *E.coli* O104:H4-LST-MUG assay

100 ml sterile LST-MUG broth was inoculated with overnight culture of *E. coli* O104:H4 (concentration with 1.96×10^5 cells / ml) and incubated at 37° C. The samples were analyzed at increasing intervals of 2 hrs of incubation until significant fluorescence was observed from the emission spectra. Bacterial concentration was determined by the plating method (CFU analysis) described previously.

2.3. Spectrofluorometer and data acquisition

The spectrofluorometry measurement was performed using Quanta Master-400 intensity based spectrofluorometer (Photon Technology International, NJ). The FlexGX software was used in conjunction with the Quanta Master-400 for acquiring the data. The emission scan was chosen and the instrument was set-up for an excitation wavelength of 366nm and emission range was selected between 400-500nm. The integration step size was chosen as 1nm for high resolution. The excitation and emission slit size on the spectrofluorometer were set at 6mm and 8mm and 8mm and 6mm respectively. For all performance metrics, data was analyzed using Origin software.

2.4. Protein-A magnetic bead based MUG assay

2.4.1. Labeling of bacteria with fluorescent dye

The Bacterial cells were labeled using Fluorescein IsoThioCyanate (FITC). 1 ml of 18 hr old culture (3.2×10^8 CFU/ml) was centrifuged and washed with PBS thrice and re-suspended in 1 ml of 2 mM FITC (Sigma, USA) in PBS. The contents were mixed by

208 pipetting briefly and then incubated for 30 min at 4 °C. The extra FITC was washed by
209 centrifugation using PBS. The FITC labeled pellet of the bacteria was re-suspended in 1
210 ml of PBS.

212 2.4.2. Immobilization of anti *E. coli* O104:H4 antibody on protein-A beads

213 20 µL of protein-A immobilized magnetic beads (LAB ON A BEAD AB, Sweden) were
214 taken into 2 ml microfuge tube to which 980 µL of PBS (15 mM Phosphate; 150 mM
215 NaCl; pH 7.4), containing 5 µg of anti *E. coli* O104:H4 Rabbit polyclonal antibody
216 (Abraxis, USA) was added and mixed well by pipetting. The contents were incubated
217 for 1 hr at room temperature. After incubation, the buffer was removed. The beads were
218 held in place by a magnet and washed (by pipetting) using PBS to remove any unbound
219 antibody.

220 2.4.3. Establishment of antigen-antibody complex

221 Antibody Immobilized protein-A magnetic beads were suspended in 950 µL of PBS and
222 the entire contents were transferred to a new microfuge tube. To this, 50 µL of
223 overnight cultured *E. coli* O104:H4 (3.2×10^8 CFU/ml) was added and mixed well by
224 pipetting. The resultant was incubated for 1 hr at room temperature. After incubation,
225 the buffer was removed and any unbound bacteria were washed out using PBS as
226 described previously. To confirm specificity of this test, anti - *E. coli* O157:H7 polyclonal
227 antibody (Meridian Life Science, USA) was immobilized on protein-A coated beads to
228 bind *E. coli* O104:H4. The same was repeated by interchanging the anti-*E.coli* and the
229 antigen.

230 2.4.4. Microscopic analysis

231 This FITC labeled antigen-antibody-protein-A bead complex was analyzed for
232 fluorescent microscopic analysis (Microscope - Leica DM IL LED; Camera - QIClick™
233 digital CCD; Fluorescence illumination system – PRIOR, Lumen 200) for possible green
234 fluorescence (FITC) on the bead. Captured images were processed using Q-Capture
235 Pro 7 software. The presence of fluorescence on the protein-A bead confirms the

antigen binding to the protein A-antibody **immobilized magnetic beads**. The imaging of the sample without antibody immobilized on protein will result in no fluorescence.

2.4.5. MUG assay for **immuno-complex**

The protein-A magnetic beads with antigen-antibody complex were transferred into the culture tube (5 ml). LST-MUG broth was added to the complex and the final volume of the medium was maintained at 2.5 ml and incubated at 37 °C for 8 hrs. After incubation, the culture was centrifuged to separate it from the supernatant. The supernatant was analyzed for possible fluorescent species of MUG using a **spectrofluorometer**. The instrument was set-up for an excitation wavelength of 366nm and the emission range was selected between 400-500nm. For comparison purposes, a separate set of experiments were performed simultaneously with exactly the same protocol as mentioned above, with the only exception being that the protein-A was not immobilized with the antigen specific antibody. LST-MUG cultures of *E. coli* O104:H4 incubated for 8 hrs were used as positive control and *E. coli* O157:H7 used as negative control. All the tests were performed in a sterile conditions and triplicates maintained.

2.5. Testing the proposed protocol with infected meat samples

The proposed technique was tested with food samples to prove its practical utility. Ground beef was purchased from local supermarke and 100 gms of the meat sample was sterilized by autoclaving (121 °C, 15 min at 15 psi). In one stomacher bag 25 gms of sterilized meat was taken and 10 ml of PBS was added to it. This sammple was inoculated with with 1 ml of the 18 hr old culture in *E.coli* O104:H4 and homogenized using a stomachar. This sample was incubated at 37 °C for 18 hrs. After incubation the sample was briefly homogenized again and 10 ml of solution was collected and centrifuged to get the bacteria "pellet". The pellet was then washed and suspended in 1 ml PBS. This prepared sample was subjected to the protein-A bead based MUG Assay as described previously. Controls were maintained by not using any antibody for all the tests. Pure culture of *E. coli* O104:H4 was used as a positive control and duplicates were maintained for all preparations. Similar procedure was adopted for a non-sterlized

267 meat sample concurrently. The intent of this test was to prove that, even if the non-
268 sterilized sample were contaminated with a coliform of *E.coli* other than *E.coli* 104:H4,
269 there would be no detection by the anti-*E.coli* O104:H4 ,

270 3. Results and discussion

271 3.1 Qualitative assay for detection of different *E.coli* serotypes with LST-MUG assay

272 The fluorescence inside the chamber was imaged before starting the scan for each
273 sample as shown in fig1. This fig indicates that even prior to the sample being scanned,
274 some fluorescence could be observed by the naked eye for the samples that have
275 positive reaction with the LST-MUG. The cleaving of the MUG substrate via the
276 enzymatic activity of β -glucuronidase was clearly visible with the resulting product, 4-
277 methylumbelliferone. It was noted that the control samples does not have the enzymatic
278 reaction with LST-MUG and does not indicate any fluorescence. The serotype *E.coli*
279 O104:H4 shows strong fluorescence.

280

Fig1. LST-MUG assay for different *E.coli* serotypes, tested after 18 hrs of incubation: a) tryptic soy broth control b) LST-MUG control c) *E.coli* O104:H4 LST-MUG assay.

3.2. Determination of *E. coli* O104:H4 concentration vs. comparative fluorescence

Various sets of dilutions of the *E.coli* O104:H4 were tested for determination of the lower and upper limit of the sensor. The dose response was found to be between 2.05×10^5 - 4.09×10^8 CFU/ml. The *E. coli* concentration vs. final fluorescence intensity was plotted to obtain the fluorogenic response correlation for the sensor as shown in fig 2. The standard error was found to be less than 2%, indicating very high repeatability for the technique.

Fig2. *E. coli* O104:H4 intensity for different concentrations vs. comparative fluorescence in (CFU/ml).

3.3 Effect of Incubation and refrigeration on *E.coli* O104:H4- LST-MUG assay

Imaging was performed on incubated and refrigerated samples of *E.coli* O104:H4-LST-MUG assay. The intent of this test was to compare, if or how much, the refrigeration effects the reaction of the bacterial serotypes to the enzymatic activity of β -glucuronidase as shown in fig 3.

Fig3. Effect of refrigeration on LST-MUG assay for *E. coli* O104:H4 tested after 18 hrs:
a1) incubated *E.coli* O104:H4 - LST-MUG assay, a2) refrigerated *E.coli* and O111:H8
LST-MUG assay.

3.4 Detection time for *E.coli* O104:H4-LST-MUG assay

The qualitative detection of *E.coli* O104:H4-LST-MUG can be achieved in just 8 hrs as shown in fig 4. Before the 8th hr there was no discernable intensity spectrum, which confirms completely with the “growth curve” of the bacteria. The viable reaction could only be possible in the presence of significant amount of bacteria and the enzymatic activity required for cleaving the “substrate”. As is the case with the growth curve, the exponential growth of bacteria can be attributed to times between 4-8 hrs (where the enzyme β -glucuronidase production starts), with the peak of the “knee” expected around 8 hrs. This duration presents itself as the cut-off point at which the spectrofluorometer begins to detect the fluorescence.

Fig4. Detection times for *E.coli* O104:H4- LST-MUG assay.

3.5. Protein-A magnetic bead based MUG assay

LST-MUG without immobilized antibody on Protein-A samples did not show any significant fluorescence, however, some level of fluorescence was observed as shown in fig 5. LST-MUG by its nature shows some fluorescence and that is considered as insignificant. LST-MUG culture incubated with only *E. coli* O104:H4 showed the highest intensity. This was a test for the positive control. The test sample (Protein-A-antibody-antigen complex) also indicated significant fluorescence peak. This fluorescence peak indicates the specific binding of *E. coli* O104:H4 to the anti *E. coli* O104:H4 which was immobilized on the Protein-A magnetic beads. Though, *E. coli* O157:H7 showed good growth in the LST-MUG broth, it did not show any fluorescence. Hence this is considered as negative control.

Fig5. Fluorescence scan results for protein-A magnetic bead based LST-MUG assay: a) LST-MUG, b) protein-A-*E.coli* O104:H4 (without antibody), c) protein-A-anti *E. coli* O157:H7 antibody-*E. coli* O104:H4, d) protein-A-anti *E. coli* O157:H7 antibody-*E. coli* O157:H7, e) protein-A-anti *E. coli* O104:H4 antibody- *E.coli* O157:H7, f) negative control (LST-MUG culture of *E. coli* O157:H7), g) protein-A-anti *E. coli* O104:H4 antibody- *E. coli* O104:H4 and h) positive control (LST-MUG culture of *E. coli* O104:H4).

3.6. Fluorescent microscopy

Images of fluorescent microscopy as shown in fig 6 indicate fluorescence (green hue due to labeling with FITC) on the beads when the specific antigen was bound to the protein A-antibody complex, whereas, the samples processed without antibody did not show any fluorescence on the bead, which means that the *E. coli* O104:H4 did not bind to the protein-A magnetic beads when the specific antibody was not immobilized on the bead. This method can be used as a rapid qualitative analysis technique for any bacteria with its specific antibody. This method also proves the binding of *E. coli* O104:H4 with its specific antibody on the magnetic beads.

Fig6. Microscopic images of immune-complex formation: a) light microscopic image of protein-A magnetic bead-anti *E. coli* O104:H4 antibody-*E.coli* O104:H4 complex b) fluorescent microscopic image of protein-A magnetic bead- anti *E. coli* O104:H4 antibody-*E.coli* O104:H4 complex c) light microscopic image of the test without adding anti *E. coli* O104:H4 antibody d) fluorescent microscopic image of the test without adding anti *E. coli* O104:H4 antibody.

3.7. Testing the proposed protocol with infected food samples

Significant fluorescence from MUG assay of infected meat samples proves that the method was able to capture the target bacteria from the infected food sample. The purified bacterium was able to grow in LST-MUG broth and produced fluorescent species 4-methylumbelliferone on cleaving the MUG substrate. The reason for incubating the infected meat sample was to mimic the natural contamination of food, where the bacterium stays alive in the meat sample. If the bacteria die during incubation, there would not have been any significant fluorescence in the MUG assay. This technique worked equally well for the meat sample and well as the pure bacterial culture.

Fig7. Fluorescent values of MUG Assay with infected food sample.

Conclusion

In this study we have presented a very reliable technique for the specific detection of *E.coli* O104:H4. The same technique could be used for detection of any serotype or species of bacteria with its antibody. The qualitative determination of the bacteria could be obtained by imaging the fluorescently labeled bacteria in 2 hrs, though it should be stated here that the minimum concentration of bacteria available in the test sample should at least be 10^5 CFU/ml. Detection using MUG assay spectrofluorometry, as an additional step with fluorescent imaging will result in positive identification of bacteria in 8 hrs for even the smallest concentrations using this technique. This method should be extremely useful for specific detection of any kind of bacteria in very short amounts of time.

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490

Figures

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7