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Rapid and Simultaneous Detection of *Salmonella* and *Campylobacter* in Poultry Samples Using Quantum Dots Based Fluorescent Immunoassay Coupled with Magnetic Immunoseparation

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Abstract: *Salmonella* Typhimurium and *Campylobacter jejuni* are the most important bacterial pathogens associated with food borne diseases caused by consuming undercooked poultry or handling raw poultry and poultry products. Because of their low infectious dose of pathogens, a rapid, sensitive, simultaneous detection method is urgently needed. The objective of our research was to develop a sensitive biosensing method for rapid and simultaneous detection of *S.* Typhimurium and *C. jejuni* in chicken and ground turkey meat using magnetic nanobeads (MNBs) to capture and separate the target bacteria and quantum dots (QDs) to label the captured bacteria. In this research, both streptavidin conjugated QDs 530 and QDs 620 were coated with the specific biotin conjugated anti-*S.* Typhimurium and anti-*C. jejuni* antibodies, respectively. The MNBs were separately coated with the specific biotin conjugated anti-*S.* Typhimurium and anti-*C. jejuni* antibodies. The inoculated poultry samples were mixed with conjugated MNBs to capture the two target bacteria. After magnetic immunoseparation, the MNB-cell complexes were mixed with the conjugated QDs 530 and QDs 620 to form the MNB-cell-QD complexes. Unattached conjugated QDs were removed using magnetic separation. Finally, the fluorescence intensities of the MNB-*S.* Typhimurium-QD and MNB-*C. jejuni*-QD complexes were measured and correlated to the cell number of two target pathogens. The results showed that *S.* Typhimurium and *C. jejuni* in pure culture, chicken carcass and ground turkey wash solutions could be simultaneously separated and detected using the developed immunoassay. The fluorescence intensities at 530 and 620 nm wavelengths increased linearly with the increasing cell numbers of *S.* Typhimurium and *C. jejuni*, respectively. The assay detection limit was 30-50 cfu/ml and the assay time was less than 2 h.

Key words: Fluorescent immunoassay, *Salmonella* typhimurium, *campylobacter jejuni*, simultaneous detection, magnetic nanobeads, quantum dots

INTRODUCTION

Salmonella Typhimurium and *Campylobacter jejuni* are two of the most important bacterial pathogens associated with food borne diseases reported in the U.S. and worldwide. While numerous potential vehicles of transmission exist, poultry and poultry products have been identified as the most important food vehicles for these two bacterial species (Pires *et al.*, 2010). *Campylobacter jejuni* is estimated to cause 2.4 million cases of food borne illness in the U.S. each year (CDC, 2010). Many of the previous cases have been linked to eating undercooked poultry products or handling raw poultry and poultry products (Murphy *et al.*, 2006; Melero *et al.*, 2011). Studies have found *Campylobacter* contamination of up to 88% of chicken carcasses (Clark, 2013; FDA, 2013). *Salmonella* Typhimurium is the second most common human intestinal infection and number one leading causes of hospitalization in the U.S. (Scallan *et al.*, 2011). Foods that are most likely to be contaminated with *Salmonella* include raw or undercooked eggs, raw milk, contaminated water and raw or undercooked meats (Jones *et al.*, 2012; Clark, 2013). For the past decade, total food borne illness cases caused by *E. coli* O157:H7, *Listeria monocytogenes* and *C. jejuni* have dropped by 23%. However, the *Salmonella* infections have increased by 10% (CDC, 2012).

Conventional culture methods to identify *S.* Typhimurium and *C. jejuni* are extremely time-consuming, typically requiring at least 20-24 h for *Salmonella* and 40-48 h for *Campylobacter* and complicated multi-steps to confirm the analysis. Standard culture methods cannot detect viable-but-nonculturable (VBNC) pathogenic bacteria effectively, including *Salmonella* and *Campylobacter*. In VBNC state of bacteria, cells lose culturability but remain viable and potential pathogenic under favorable conditions (Oliver, 2005; Oliver *et al.*, 2005; Lin *et al.*, 2008; Melero *et al.*, 2011; Fakruddin *et al.*, 2013).

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Currently well studied rapid methods based on immunochemistry and molecular biology, such as enzyme linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) methods including real-time PCR and multiplex PCR, may provide an alternative to culture methods for the detection of *S.* Typhimurium and *C. jejuni*. However, ELISA and PCR assays need 10 to 24 h or 4 to 6 h, respectively, with detection limits varying from 10^3 to 10^6 cfu/ml (LaGier *et al.*, 2004; Debretsion *et al.*, 2007; Leblanc-Maridor *et al.*, 2011) or need 48 h pre-enrichment (Bonjoch *et al.*, 2010; Mayr *et al.*, 2010; Melero *et al.*, 2011; Galikowaka *et al.*, 2011). Immunoassays due to their better specificity and simplicity, represent the mainstream of the biosensors reported for detection of food borne pathogens.

Magnetic nanobeads (MNBs) are well-established core-shell nanoparticles with controlled size. The magnetic core consists of Fe_3O_4 and has an external magnetic force in an external magnetic field. The dimensions of MNBs are usually smaller than or comparable in size to most biological entities including cells (10-100 µm), bacteria (1-10 µm), or viruses (20-450 nm). When MNBs are coated with biological molecules, they can fully interact with or bind to target biological entities to isolate, separate, purify and concentrate the targets from complex matrices (Pankhurst *et al.*, 2003; Colombo *et al.*, 2012; Wierucka and Biziuk, 2014). Reports have shown the use of MNBs conjugated with antibodies (Ab), peptides, aptamers, antibiotics, probes and proteins for bacteria separation and detection (Varshney and Li, 2007; Goransson *et al.*, 2010; Chen and Zhang, 2012; Wu *et al.*, 2014; Yoo *et al.*, 2014).

Following the advances in nanotechnology, quantum dots (QDs) as highly luminescent semiconductor nanocrystals have been reported in biological applications (Jin *et al.*, 2011; Barroso, 2011; Rosenthal, *et al.*, 2011; Baba and Nishida, 2012; Kirsch *et al.*, 2013; Chinnathambi *et al.*, 2014). QDs are composed of CdSe (CDs, CdTe, InP, InAs, or PbSe)/ZnS core/shell, nanometer-sized (2-10 nm) nanoparticles (Jamieson *et al.*, 2007; Samir *et al.*, 2012). The inorganic fluorophores QDs have advantages over traditional fluorescent organic dyes and green fluorescent proteins due to their broad absorption spectra, narrow emission spectra, superior photostability and a long fluorescent lifetime. The water soluble and biocompatible QDs are flexible for biological detection through variation of surface coating or bioconjugation. Desirable biological molecules can be coupled to QDs and retain their biological activities, which leads to the development of molecular conjugates of QDs that are biocompatible and suitable for applications in biosensing. Researchers have investigated the applications of QDs for detection of pathogenic bacteria (Wang *et al.*, 2007, 2011a, 2014; Hu *et al.*, 2014) and viruses (Zeng *et al.*, 2009; Tian *et al.*, 2012). Because QDs of different sizes can emit at different wavelengths upon excitation with the same light, they have been exploited to simultaneously excite multiple colored QDs using a single wavelength. QDs-based assays have been investigated specifically for simultaneous detection of multiple pathogenic bacteria (Xue *et al.*, 2009; Zhao *et al.*, 2009; Wang *et al.*, 2011b). The objective of this research was to develop a sensitive biosensing method for rapid and simultaneous detection of *S.* Typhimurium and *C. jejuni* in chicken and ground turkey wash solutions using MNBs to capture and separate the target bacteria and fluorescent QDs to label the captured bacteria.

MATERIALS AND METHODS

Bacterial cultures and surface plating methods: Stock cultures of *S.* Typhimurium (ATCC 14028) purchased from American Type Culture Collection (Manassas, VA) were grown at 37°C for 18-20 h in brain heart infusion (BHI) broth (Remel, Lenexa, KS). Serial 10-fold dilutions in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) and the cultures were for further use. The viable cell numbers of *S.* Typhimurium were determined by surface plating on XLT_4 agars at 37°C for 20-24 h (HiMedia, Mumbai, India).

Stock cultures of *C. jejuni* (ATCC 29428) purchased from American Type Culture Collection (Manassas, VA) were grown at 42°C for 20-24 h in *Campylobacter* enrichment broth (Neogen, Lansing, MI) under low oxygen (5%) condition. Serial 10-fold dilutions were made in PBS and the cultures were ready for further used. The viable cell numbers of *C. jejuni* were determined by surface plating on *Campylobacter* enrichment selective agar (Neogen, Lansing, MI) and incubating at 42°C for 40-48 h in an anaerobic container with a BD GasPak™ EZ gas generating systems (Becton, Dickson and Company, Sparks, ML).

Preparation of immuno-MNBs and immuno-Qds: Biotinylated rabbit anti-*S.* Typhimuriun and biotinylated rabbit anti-*C. jejuni* antibodies (4-5 mg/ml) were obtained from Thermo Scientific (Rockford, IL) and diluted 1:10 with 0.01 M PBS for further use. Streptavidin captivate ferrofluid conjugated MNBs (150 nm diameter) purchased from R and D Systems (Minneapolis, MN) were coated with the anti-*S.* Typhimurium antibody or anti-*C. jejuni* antibody separately. To avoid using extra reagents, the ratio of the streptavidin conjugated MNBs and biotinylated antibodies were determined according their binding sets. At this step, each of 20 µl of MNBs was mixed with 20 µl biotin-conjugated rabbit anti-*S.* Typhimurium antibody or anti-*C. jejuni* antibody (0.5 mg/ml). The MNBs-antibody conjugates were separated from the solution by placing the test tubes on a magnetic separator and then washing them to remove un-conjugated antibodies. Streptavidin conjugated QDs (1 µM) with emission wavelengths of 530 and 620 nm

purchased from Ocean Nanotech (San Diego, CA) were coated with the biotin labeled anti-*S*. Typhimurium and anti-*C. jejuni* antibodies, respectively, by biotin-streptavidin binding. The ratio of the streptavidin conjugated QDs and biotinylated antibodies were tested to minimize the materials used. The 20 µl of QDs 530 or 1:3 diluted QDs 620 were mixed with 20 µl of biotin-conjugated rabbit anti-*S*. Typhimurium antibody or anti-*C. jejuni* antibody (0.5 mg/ml). Both MNBs-antibodies and Qds-antibodies conjugates were shaken on a RKVSD 10101 mixer (ART, Laurel, MD) at a speed of 10 rpm at room temperature for 30 min.

Inoculation of chicken carcass wash and ground turkey wash solutions: Post-chilled chicken carcasses obtained from a poultry processing plant were individually washed with 100 ml of 0.1% buffered peptone water (BPW) by hand shaking for 1 min and the wash solutions were collected. Each of 25 g samples of ground turkey (purchased from a local grocery store) was mixed with 225 ml of 0.1% PBW and stomacher washed for 1 min. The wash solutions were collected. All chicken carcass wash and ground turkey wash solutions were divided into two portions. One portion was inoculated with various dilutions of *S*. Typhimuriun and *C. jejuni* broth cultures to obtain final inoculation concentrations of 10^0 to 10^3 cfu/0.1 ml in the wash solution. The second portion was not inoculated with the bacterial culture and served as negative controls. The final inoculation concentrations and controls were confirmed with the tests using bacterial plating methods.

Assay procedure: The assay procedure is outlined in Fig. 1. First, each of samples inoculated with *S*. Typhimurium and *C. jejuni* at different concentrations of 10^0-10^3 cfu/0.1 ml in tubes was mixed with 40 µl of the antibody coated MNBs (MNB-Ab) for 30 min to form the MNB-Ab-cell complexes. These complexes were separated from the sample solution by putting the tube on a magnetic separator for 2 min and then removing the solution. After washing twice with PBS, the captured MNBs-Ab-cells complexes were mixed with 40 µl of anti-*S*. Typhimurium antibody-coated QDs 530 and the anti-*C. jejuni* antibody coated QDs 620 (QDs-Abs) for 30 min. Then magnetic separation was done for removing excess QDs solution and the samples were ready for detection of target bacteria. The fluorescence measurement was performed using a laptop-controlled portable system which included a USB2000 miniature fiber-optic spectrometer with a LS-450 LED light source module and a R400-7 UV-vis optical probe (Ocean Optics, Dunedin, FL) and a probe/cuvette holder housed in a dark box. When they were excited at a single 395 nm wavelength, QDs 530 and QDs 620 in the MNB-Ab-Cell-Ab-QD complexes gave two peak values of fluorescent intensity at 530 nm and 620 nm wavelengths which was correlated with the cell number of two target bacteria. All tests were duplicated.

Scanning electron microscopy (SEM) observation: The mixture of *S*. Typhimuriun and *C. jejuni* cultures before and after they bound with MNB-Ab and QD-Ab conjugates were treated as follows for SEM observation.

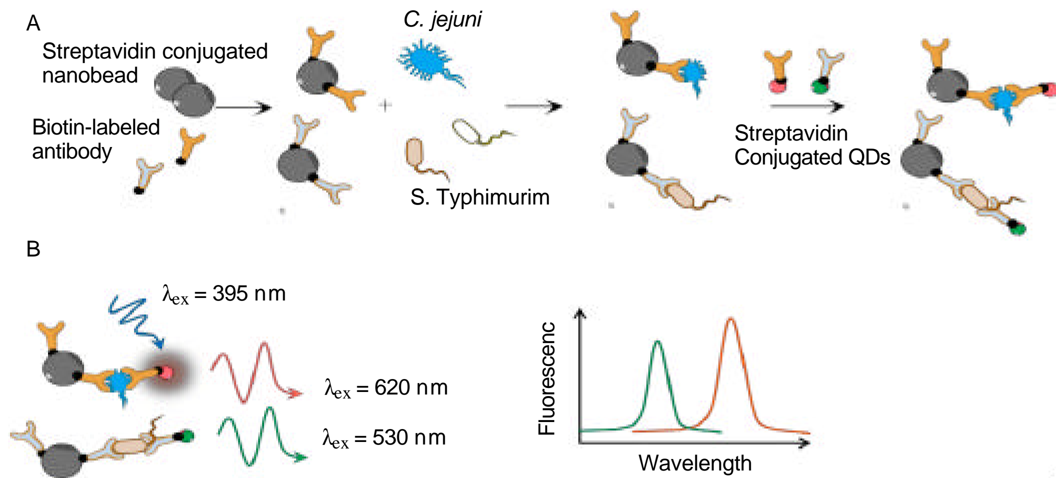

Fig. 1: Procedure of the immunoassay for simultaneous detection of *Salmonella* and *Campylobacter*: (A) Schematic diagram of the detection procedure. Biotinylated rabbit anti-*S*. Typhimurium and anti-*C. jejuni* antibodies coated streptavidin captivate MNBs were used for capturing and separating *S*. Typhimurium and *C. jejuni* cells from the samples. Biotinylated rabbit anti-*S*. Typhimurium and anti-*C. jejuni* antibodies coated streptavidin conjugated QDs 530 and QDs 620 were used as fluorescence labels. (B) *S*. Typhimurium and *C. jejuni* were detected through the measurement of fluorescence intensity at 530 and 620 nm wavelengths produced by bound QDs

Samples were fixed by immersing in Karnovsky's fixative for 2 h in a weak vacuum and then post-fixed by soaking in 1% osmium tetroxide for 2 h. The cells were dehydrated in a graded ethanol series of 30, 50, 70, 80, 95 and 100%. After gold-coating, the samples were observed by SEM (FEI, Nova NanoSEM, Hillsboro, OR).

Statistical analysis: The results of experiments on the simultaneous detection of *S*. Typhimurium and *C. jejuni* at different concentrations in pure culture and poultry samples were compared individually to those of uninoculated negative controls. The means and standard deviations of measured florescent intensity in each test were calculated and statistically analyzed by paired two samples for means of t-Test. In comparison, the means were considered to significantly different when $p<0.01$. Linear regression with the coefficient of determination R^2 between fluorescent intensity and bacterial cell concentrations was calculated using Microsoft Excel (Microsoft Redmond Campus, Redmond, WA).

RESULTS AND DISCUSSION

Figure 2a shows the representative fluorescent spectra obtained from uninoculated negative control and the samples containing a mixed target bacteria, *S*. Typhimurium and *C. jejuni*, at different concentrations in pure culture. Compared with background fluorescent signal (or negative control), the fluorescence for the lowest concentration (3-5 cells/0.1 ml) of *S*. Typhimurium and *C. jejuni* cells increased by 650 counts at 530 nm and 900 counts at 620 nm, respectively. The two peaks at 530 and 620 nm wavelengths represent the fluorescence signals produced by Qds 530 and QDs 620 in the MNB-Ab-cell-Ab-QD complexes and specifically recognize *S*. Typhimurium and *C. jejuni*, respectively. The means of peak values are significantly different between the samples for *S*. Typhimurium and *C. jejuni* cells and their negative controls. The results confirm that these QDs-antibodies complexes used in this study can specifically bind to their targets in the mixed bacterial samples and simultaneously produce different fluorescence signals. The results of the experiments on *S*. Typhimurium and *C. jejuni* in ground turkey wash solution are shown in Fig. 2b. The group of fluorescent spectra represents the fluorescent intensities from uninoculated negative control and samples containing different concentrations of two target bacteria. The spectra peaks of the lowest concentrations (3-5 cells/0.1 ml) of both *S*. Typhimurium and *C. jejuni* cells were 300 counts above the background (or negative control) at both 530 and 620 nm wavelengths. Fig. 2c shows the representative fluorescent spectra obtained from negative control and the samples containing mixed *S*. Typhimurium and *C. jejuni* at different concentrations in chicken carcasses wash solution. The difference in fluorescent intensities between the lowest concentrations of *S*. Typhimurium and *C. jejuni* cells (3-5 cells/0.1 ml) and the background is over 250 counts at 530 nm and 300 counts at 620 nm, respectively.

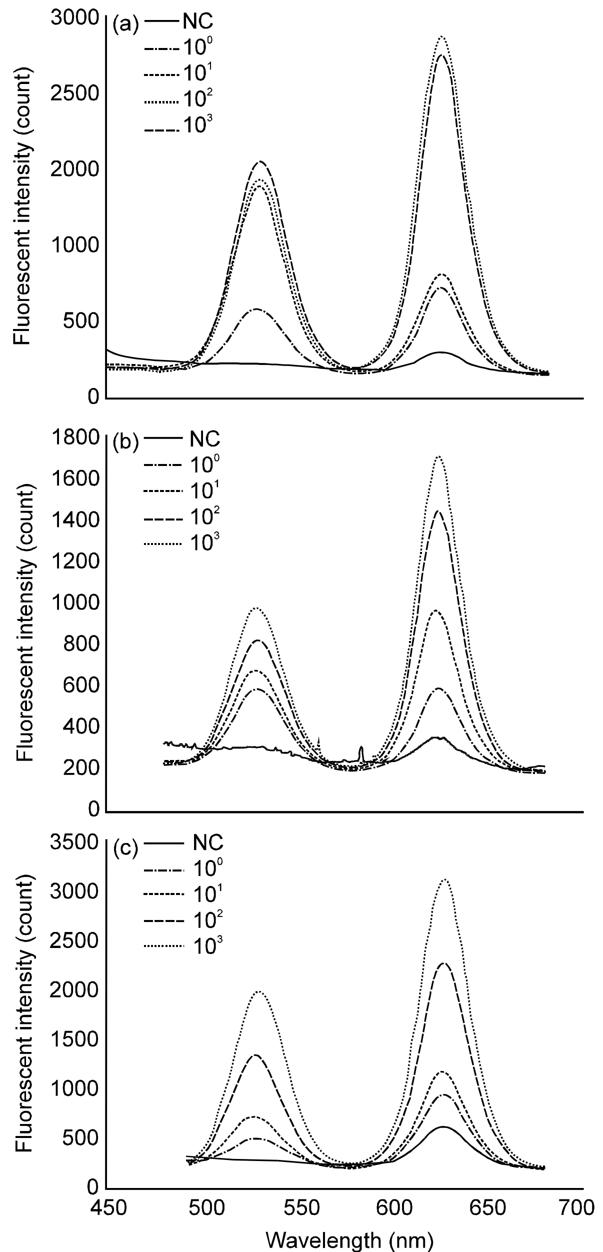

Fig. 2: Representative fluorescence spectra obtained from uninoculated negative control and the samples containing *S*. Typhimurium and *C. jejuni* at concentrations of 3-5 x 10^0, 10^1, 10^2 and 10^3 cfu/0.1 ml in (a) pure culture, (b) in ground turkey wash solutions and (c) chicken carcass wash solutions

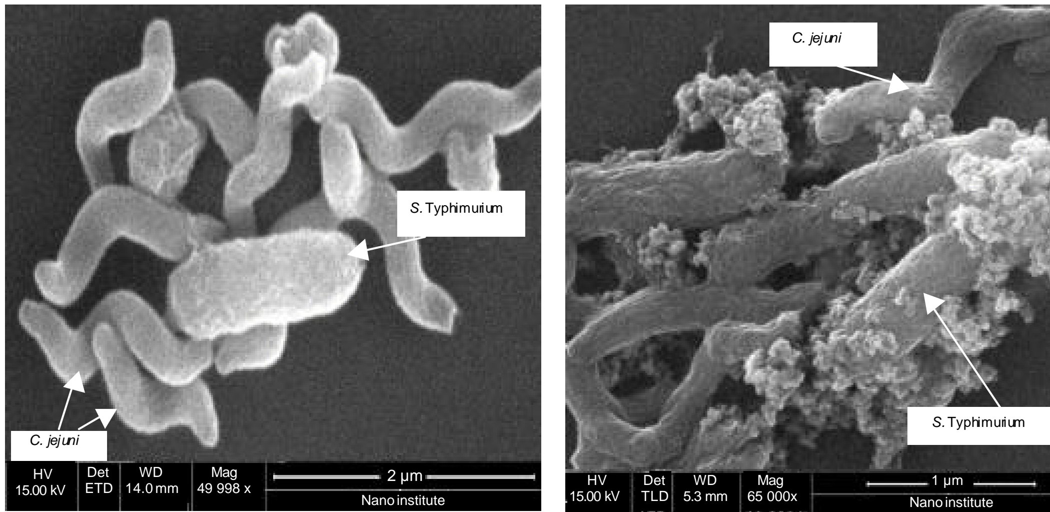

Fig. 3: SEM images of (a) Mixture of *S.* Typhimurium and *C. jejuni* cells and (b) Mixture of the MNBs and QDs attached *S.* Typhimurium and *C. jejuni* cells

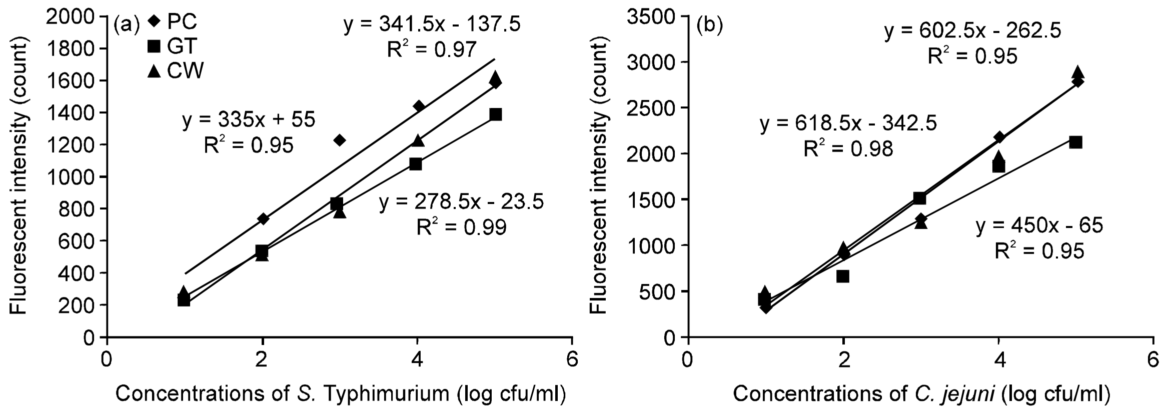

Fig. 4: Linear relationships between fluorescent intensity and the logarithmic value of cell concentrations for (a) *S.* Typhimurium and (b) *C. jejuni* in pure culture (PC), ground turkey wash (GT) and chicken carcasses wash (CW)

The morphologies of the mixture of *S.* Typhimurium and *C. jejuni* before and after binding with the MNB-Ab and QD-Ab conjugates are shown in Fig. 3a,b, respectively. It was observed that MNB-Ab conjugates tend to aggregate together to form large clusters during magnetic separation (Fig. 3b). Figure 4a,b show the means of fluorescence intensities measured at different concentrations of *S.* Typhimurium and *C. jejuni* in pure cultures and different poultry samples. Linear relationships of the changes in fluorescence intensity versus the logarithmic value of target bacterium concentrations of *S.* Typhimurium in different samples are shown in Fig. 4a. The correlation coefficient (R^2) of 0.95, 0.99 and 0.97 vs. *Salmonella* at concentrations ranging from 0 to 10^3 cells/0.1 ml in pure culture, ground turkey and chicken wash solution samples are statistically calculated. The correlation coefficient (R^2) of 0.98, 0.95 and 0.95 represent the linear relationships of the change in fluorescent intensity vs. *C. jejuni* in above samples, respectively, shown in Fig. 4b.

The use of MNBs for bacterial separation and concentration in combination with QDs as fluorescent labels provides a method that gives the quantitative and simultaneous detection of *S.* Typhimurium and *C. jejuni*. MNBs and QDs Conjugated with the antibodies that are specific to target bacteria resulted in a detection method that is both sensitive and specific for *S.* Typhimurium and *C. jejuni* in pure culture, chicken wash and ground turkey wash solutions at a concentration as low as 3-5 cells/0.1 ml (30-50 cfu/ml) samples. The difference in fluorescent intensities for the lowest detection concentrations, 3-5 cells/0.1 ml of *Salmonella* cells,

above their background fluorescent signal at 530 nm wavelength are 250-650 counts and *Campylobacter* at 620 nm wavelength are from 300 to 900 counts. With the increasing of inoculated concentrations of both *S.* Typhimurium and *C. jejuni* in the mixed samples from 3-5 to 10^3 cfu/0.1 ml, the fluorescent intensity at 530 nm increases from 700 to 2100 counts, from 600 to 1000 counts and from 500 to 2000 counts in pure culture, ground turkey wash and chicken wash, respectively; While the fluorescent intensity at 620 nm also increases from 900 to 2700 counts, from 600 to 1700 counts and from 900 to 3000 counts in pure culture, ground turkey wash, chicken wash, respectively. Both fluorescent intensities produced by *S.* Typhimurium bound QD 530 and *C. jejuni* bound QD 620 increase by 3 times. The total detection time from sampling through a result report was within 2 h.

Immunoassays using QDs as a fluorescent label in combination with MNBs to capture bacteria from food matrices have been investigated for detection of *Listeria monocytogenes* in different meats and vegetable samples and detection of *C. jejuni* in poultry samples with a detection limit of 20-30 cfu/ml (Wang *et al.*, 2011a, 2014). Hu *et al.* (2014) used the same assay to detect *Staphylococcus aureus* with a detection limit at 2.4 x 10^3 cfu/ml in pure culture and 10^5 cfu/ml in lamb wash. For simultaneous detection method, researchers used QDs with microscopic beads for detection of *E. coli* O157:H7 and *Salmonella* Typhimurium and the detection limits were 10^3-10^4 cfu/ml in culture (Yang and Li, 2006). Zhao *et al.* (2009) reported that using QDs and MNBs for simultaneous detection *Shigella flexneri*, *E. coli* O157:H7 and *S.* Typhimurium from apple juice with detection limit at 10^4 cfu/ml. Recently, our research reported simultaneous detection of *S.* Typhimurium, *E. coli* O157:H7 and *L. monocytogenes* in meat and fresh vegetable samples with detection limit 20-50 cfu/ml (Wang *et al.*, 2011b). The QDs and MNBs based immunoassay described in this study is the first one for simultaneous detection of *S.* Typhimurium and *C. jejuni* in poultry samples and the detection limits and detection time for both target bacteria are comparable to those in previous reports.

Conclusions: Our research demonstrated a rapid, immunoassay method to simultaneously detect *S.* Typhimurium and *C. jejuni* in poultry products using the MNBs to capture, separate and concentrate the target bacteria and the QDs to label the captured bacteria. We found this method could simultaneous detect both bacterial pathogens in pure culture, ground turkey and chicken wash solutions at concentrations as low as 30-50 cells/ml within 2 h. This study could provide the poultry industry a more effective rapid method for simultaneous detection of two major food borne pathogens in poultry products to ensure food safety.

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