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Antibody-conjugated ferromagnetic nanoparticles with lateral flow test strip assay for rapid detection of *Campylobacter jejuni* in poultry samples

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ABSTRACT

The aim of this study was to develop a nanoparticle-based cell capture system combined with a lateral flow test strip (LFT) assay for rapid detection of *Campylobacter jejuni* from poultry samples. The developed assay was bench-marked against the standard modified Charcoal Cefoperazone Deoxycholate Agar (mCCDA) method according to ISO16140:2003 procedures. The synthesized ferromagnetic nanoparticles (FMNs) were modified with glutaraldehyde, then functionalized with polyclonal antibodies for specific *C. jejuni* capture and concentration from poultry samples. After lysing captured cells, DNA from *C. jejuni* was amplified by PCR using the primers designed to target the *hipO* gene, and the PCR amplicons were detected with the lateral flow test strip assay. Following the ISO16140:2003 guidelines, the relative detection limit, and the inclusivity and exclusivity tests were determined. The results showed that the limit of detection (LOD) of the assay was 10^0 or 1 cfu/ml with *C. jejuni* in pure culture and 10^1–10^2 cfu/ml with target cells spiked in poultry sample. In addition, the inclusivity and exclusivity tests were found to be 100%. Using field chicken samples ($n = 60$), the assay showed relative accuracy, relative specificity, and relative sensitivity of 96.67%, 100% and 93.33%, respectively. The positive predictive values (PPV) and negative predictive values (NPV), and the kappa index of concordance (k) were calculated as 100% and 93.75%, and 0.93, respectively. The developed assay required approximately 3 h to complete and gave results comparable to those analyzed by the standard culture method, which required 5–7 days. The assay is rapid, easy-to-use, and has potential to be directly applied to *C. jejuni* detection in various categories of poultry samples.

1. Introduction

Campylobacter jejuni is considered the most common cause of human gastroenteritis in the world (Bolton, 2015; Centers for Disease Control and Prevention (CDC), 2017; Kaakoush et al., 2015). Every year, nearly one in ten people fall ill from Campylobacter or one of the other three key global causes of diarrhoeal disease (World Health Organization (WHO), 2018). The latest available data from 2016 reports 8547 cases of infection due to Campylobacter in the U.S. (Centers for Disease Control and Prevention (CDC), 2017), making it the most commonly reported foodborne illness. 80% of reported Campylobacter infections are caused by *C. jejuni*, the most important Campylobacter species with respect to both the number of infections and the impact on public health (Centers for Disease Control and Prevention (CDC), 2013; Kaakoush et al., 2015; Taylor et al., 2013). *C. jejuni* is gram negative, spiral shaped, grows optimally under microaerophilic conditions (Dasti et al., 2010), and readily colonizes in poultry, pigs, cattle, sheep, and goat hosts (De Boer et al., 2015). While most processors in the U.S. meet the current USDA performance requirements for Campylobacter (Sukted et al., 2017a, 2017b), poultry remains the most common source of *C. jejuni* infection.

The gold standard method of Campylobacter detection is ISO10272: 2006 (RSA, 2006), which takes 5–7 days for sampling, culture, enumeration, and biochemical confirmation of the presumptive bacterial colonies (Osiriphun et al., 2011; Sukted et al., 2017a). With this method, Campylobacter can be present in a viable and non-culturable

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state, which may cause false-negative determinations (Blackall, 2017). The conventional method is time consuming, requires various instruments and skilled labor. Recently, alternative methods have been developed for various applications, including sample testing in processing plants and hospitals. These are molecular techniques that offer rapid detection with improved sensitivity and specificity including PCR/real-time PCR (De Boer et al., 2015; Debretsion et al., 2007), immunoassays (ELISA) (Nauta et al., 2009; Reiter et al., 2005), and nanotechnology-based methods (Tansub et al., 2012). The rapid detection tests have been recently reported by Centers for Disease Control and Prevention (CDC) (2017), and the number of reported culture-independent diagnostic tests (CIDT) have been increasing because of the shorter time required to obtain results and the ease of obtaining them (Centers for Disease Control and Prevention (CDC), 2013). A primary goal of rapid Campylobacter detection is continuous monitoring to prevent shipping of contaminated products from slaughterhouses to local markets (Josefsen et al., 2015).

Nanotechnology has applications in many fields including water treatment, medicine, antimicrobial agents, and diagnostics (Chekli et al., 2013; Koedrith et al., 2014). Nanoparticles (NPs) have been used to recognize, capture, and enrich target bacteria from complex mixtures. For example, antibody-conjugated silica NPs (Tansub et al., 2012; Thepwiwatjit et al., 2014), and amino-functionalized ferromagnetic nanoparticles (FMNs) (Songvorawit et al., 2011) have been developed to increase cell concentration and improve the sensitivity of a detection assay. Recently, gold NPs (AuNPs) were developed as the biosensor for *C. jejuni* detection using surface plasmon resonance properties. NPs can enhance the sensitivity of rapid detection (Masdor et al., 2016, 2017). In addition, AuNPs can be used as signal on the lateral flow test strip (LFT). The ability of each NPs combination such as the FMNs was used for DNA extraction and DNA target can be detected with other NP including AuNP-LFT. The combination assay is rapid, easy to use for detection, low cost, and can be employed in many field situations (Nauta et al., 2009). Advantages of LFT are its versatility of detection formats, its simplicity, stability, less preparation with no power requirements, making it an ideal choice for point of care applications. It has high potential to commercialization (Sajid et al., 2015).

The objective of this work was to develop and validate a new test kit that combines antibody-conjugated FMNs (pAb-FMNs) with the LFT assay for rapid detection of *C. jejuni* in poultry products from the local market in Bangkok, Thailand. The FMN-LFT method was tested and validated with ISO16140:2003 to quantify the relative accuracy, relative specificity, relative sensitivity, ruggedness and consistency of the method. The successful validation of this rapid and simple *C. jejuni* detection method demonstrates its promising potential for use with various types of poultry samples and a variety of commercial applications.

2. Materials and methods

This section describes development of FMN-LFT starting from synthesis of amino functionalized FMN nanoparticles, surface modification, attachment of polyclonal antibodies, and test protocol for the target cells. DNA extraction of the captured cells, PCR amplification of *hipO* and DNA probe are described. Validation of the FMN-LFT method following ISO16140:2003 was also conducted.

2.1. Chemicals and reagents

Chemicals used for amino-FMNs synthesis were ethylene diamine (Sigma, U.S.A), ethylene glycol (Fisher Chemical, U.S.A), iron (III) chloride hexahydrate ($FeCl_3{\cdot}6H_2O$; Poch, Poland), sodium acetate (CH_3COONa; Merck, Germany), and sodium hydroxide (NaOH; Rankem, India). Polyclonal antibody for *Campylobacter jejuni* (PA1-7205) was purchased from Abcam (England). Phosphate buffer saline (PBS) and its solution containing 0.05% of Tween 20 (PBST) were prepared from 10× solution. Oligonucleotides and agarose were ordered from Bio Basic Inc. (Canada). 5× PCR master mix II from Molecules biology Tool (Taiwan) was used for PCR amplification. Bacterial media were obtained from Oxiod (England). Several chemicals and reagents used such as bovine serum albumin (BSA; Sigma, U.S.A), ethanol (95%), and others were of either molecular or analytical grades.

2.2. Microorganisms and growth media

C. jejuni strains ATCC 33291 and ATCC 33560 were obtained from the Faculty of Medical Technology, Mahidol University, Thailand. In addition, 50 different strains of *C. jejuni* were isolated from poultry samples obtained from local markets in Bangkok, Thailand and used as inclusive bacterial strains. These isolated *C. jejuni* strains were coded and confirmed by duplex PCR by our laboratory using the PCR primers targeting *16S rRNA* and *hipO* genes (Inglis and Kalischuk, 2003; Linton et al., 1996; Wang et al., 2002). All *Campylobacter* strains were grown on the modified cefperazone charcoal deoxycholate agar (mCCDA) plate at 42 °C for 48 h. The exclusive bacterial strains were obtained from the Faculty of Medical Technology, Mahidol University, Thailand and the Department of Medical Science, Ministry of Public Health, Thailand. These bacterial strains were grown in tryptic soy broth (TSB) media overnight at 37 °C. Please see the Supplementary Materials section for information of microorganism lists.

2.3. Method for FMN preparation

2.3.1. Preparation of amino functionalized FMNs

Amino-FMNs were prepared based on the polyol technique using ethylenediamine as an amino group donor according to previously described protocols (Rahman and Green, 2009; Songvorawit et al., 2011). Briefly, 2 g of $FeCl_3{\cdot}6H_2O$ were added to 40 ml of ethylene glycol and mixed thoroughly, giving the solution a yellow color. Then, 6 g of CH_3COONa, 1.6 g of NaOH and 20 ml of ethylenediamine were added and stirred for 30 min, followed by moist heat at 121 °C for 2 h/cycle in an autoclave, for 3 cycles. The $FeCl_3{\cdot}6H_2O$ and ethylene glycol were used as precursor and solvent, respectively.

After the reaction was completed, the amino-FMNs were isolated by a magnet, and washed with distilled water and ethanol (95%) several times to remove the solvents. The amino-FMNs were dried, ground with a mortar, and sonicated for at least 48 h prior to use. FMNs were examined for size and shape using TEM (Hitachi High tech 7700, Japan).

2.3.2. Surface modification and preparation of polyclonal antibody-functionalized FMNs

To produce the carboxyl group on the particle surface, the amino-FMNs (2 mg/ml) were treated with 5% glutaraldehyde at room temperature for 2 h with gentle stirring, washed with 1× PBS (pH 7.4), and then FMNs were determined by FT-IR. FMNs were conjugated with *C. jejuni*-specific polyclonal antibody (pAb) at 18 °C overnight with gentle shaking. The mass ratio was approximately 2 mg pAb/mg FMNs. To reduce non-specific binding, the pAb-conjugated FMNs were blocked with 1 ml of blocking solution (5% BSA in 1× PBS), and then stored at 4 °C until further use.

2.3.3. Determination of capture efficiency for pAb-conjugated FMNs

To determine the efficiency of pAb-conjugated FMNs towards *C. jejuni* capture, one ml of bacterial cell suspension was mixed with 20 µl of pAb-conjugated FMNs and incubated at 37 °C for 45 min. After incubation, the bacterial cells were separated from solution by a magnet, and washed once with PBST and twice with PBS, respectively. The captured cells were re-suspended in 100 µL PBS, spread on mCCDA plate, and incubated at 42 °C for 48 h. Typical colonies of *Campylobacter* were observed and enumerated on agar plate. The capture efficiency was calculated according to the formula: % Capture $= \frac{N\ capture}{N\ total} \times 100$,