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Article

Surface Plasmon Resonance Immunosensor for the Detection of *Campylobacter jejuni*

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Abstract: Campylobacteriosis is an internationally important foodborne disease caused by *Campylobacter jejuni*. The bacterium is prevalent in chicken meat and it is estimated that as much as 90% of chicken meat on the market may be contaminated with the bacterium. The current gold standard for the detection of *C. jejuni* is the culturing method, which takes at least 48 h to confirm the presence of the bacterium. Hence, the aim of this work was to investigate the development of a Surface Plasmon Resonance (SPR) sensor platform for *C. jejuni* detection. Bacterial strains were cultivated in-house and used in the development of the sensor. SPR sensor chips were first functionalized with polyclonal antibodies raised against *C. jejuni* using covalent attachment. The gold chips were then applied for the direct detection of *C. jejuni.* The assay conditions were then optimized and the sensor used for *C. jejuni* detection, achieving a detection limit of 8×10^6 CFU·mL^{-1}. The sensitivity of the assay was further enhanced to 4×10^4 CFU·mL^{-1} through the deployment of a sandwich assay format using the same polyclonal antibody. The LOD obtained in the sandwich assay was higher than that achieved using commercial enzyme-linked immunosorbent assay (ELISA) (10^6–10^7 CFU·mL^{-1}). This indicate that the SPR-based sandwich sensor method has an excellent potential to replace ELISA tests for *C. jejuni* detection. Specificity studies performed with Gram-positive and Gram-negative bacteria, demonstrated the high specific of the sensor for *C. jejuni*.

Keywords: *Campylobacter jejuni*; campylobacteriosis; Surface Plasmon Resonance (SPR); immunosensor; sandwich assay

1. Introduction

Campylobacter spp. are a major cause of gastroenteritis in humans and have led to economic losses by decreasing the productivity and requiring additional medical costs in developed and developing countries. Foodborne illness causes significant economic losses globally. The largest outbreak of *Campylobacter* was in 1978 in Bennington (VT, USA) where approximately 3000 people were infected through contaminated water [1]. In the UK, there were 72,571 confirmed cases in 2012 but the available statistics suggest more cases than these. Underreporting is estimated to occur in these instances since the disease do not require urgent hospitalization upon infection [2]. Poultry-based food forms the largest pool of *C. jejuni* [3]. The bacterium itself is a largely zoonotic and does not usually reproduce in foods [4]. The host, in which it colonizes, ranges from wild birds to domestic animals [5,6]. In most cases, the bacterium multiplies in chicken and the animal become a reservoir of the pathogen [7]. *Campylobacter* especially *C. jejuni* colonizes well in chicken since the microaerophilic conditions of chicken's intestine and its body temperature (41–42 °C) are the optimum conditions for

the growth of this bacterium [8]. Intestinal colonization is the main source of contamination of the final product and is usually rampant in many poultry processing plants [5]. In the past three decades, *Campylobacter* spp. are the focus of numerous research groups in the world. Routine food monitoring, screening, and identification of the bacteria require reliable and rapid diagnostic methods, and this has been recognized as major objective in order to reduce the impact of these pathogenic bacteria on the human and animal's health [9].

The gold standard for the detection of *C. jejuni* in food products is the culturing method, which takes more than 48 h to get results. Food products such as poultry are usually consumed a few days after the production, and research on the potentially rapid detection methods using new technologies such as quartz crystal microbalance (QCM), polymerase chain reaction (PCR), and surface plasmon resonance (SPR) is intensely being conducted. Among these techniques, biosensors (SPR and QCM) have been considered as fast, easily applicable and sensitive methods for the detection of *C. jejuni* [10–12]. Of all of the biosensor methods developed for pathogen detection, optical-based methods including SPR have become the most dominant method over the years followed by the electrochemical and acoustic-based methods. SPR has been investigated for the detection of *C. jejuni* using antibody-based bioreceptors. Although both monoclonal and polyclonal antibodies have been used, the polyclonal antibodies have been reported to be the dominant form of antibody utilized in several works [9,10,12]. Advantages of SPR over the other methods include shorter detection time, reliable and reproducible results. However, sensitivity is an issue due to the limited evanescent wave penetration depth in SPR, particularly in the case of studying a large size pathogen as *C. jejuni* [12].

The SPR biosensor is a well-established unique sensing platform widely employed for a large number of analytes' detection including bacteria. In SPR measurement of an analyte, the ligand or bioreceptor is initially immobilized on the sensor surface, and then the analyte passes through a microfluidic channel over the ligand. The direct interaction between the analyte and ligand gives rise to a measurable signal, which is detected and quantified. Since SPR is very sensitive to the refractive index of the solution, it is important to obtain a stable baseline prior to the interaction between ligand and analyte [13]. In conventional SPR, the interaction between the analyte and the sensor surface results in a rapid adsorption leading to an initial increase in the SPR angle (association phase) followed by a saturation of the surface (dissociation phase) observed as the emergence of a plateau in the sensorgram. The final stage involved the replacement (regeneration phase) of the analyte or biomolecule with buffer to remove loosely bound materials and regenerate the surface [12]. To date, the detection of *C. jejuni* via SPR only utilize a direct assay format, and other formats such as sandwich assay and sandwich assay with nanomaterial amplification have not been attempted, although these formats have been successful in improving bacterial detection in the other immunosensor works such as electrochemical [14] and QCM [10]. In this study, several potentially rapid assay formats were investigated, and the improvements for the detection of *C. jejuni* were explored which include direct, sandwich and nanomaterial-modified sandwich assays.

2. Materials and Methods

2.1. Materials

Rabbit polyclonal antibody against *C. jejuni* was obtained from the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. Mouse IgG was from a commercial source (Abcam Ltd., Cambridgeshire, UK). N-hydroxysuccinimide (NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) were sourced from Thermo Scientific, Paisley, UK. 11-mercaptoundecanoic acid (11-MUDA), 40 nm gold colloidal (AuNPs), sodium acetate, ethanolamine hydrochloride, PBS (phosphate buffered saline tablet: 0.0027 M potassium chloride, 10 mM phosphate buffer and 0.137 M sodium chloride, pH 7.4), sulfuric acid (H_2SO_4), hydrogen peroxide (H_2O_2), Tween-20, bovine serum albumin (BSA), and ethanol were sourced from Sigma-Aldrich, Dorset, UK.

2.2. Instrumentation

A fully automated SPR-4 biosensor (Sierra Sensors GmbH, Hamburg, Germany) with its amine coated gold sensor chips was employed in this study. The instrument can carry out four separate assays simultaneously as it is equipped with four sensing spots. SPR-4 data were analyzed with the R2 software from Sierra Sensors (Hamburg, Germany) and Microsoft Excel.

2.3. Bacterial Strains and Preparation of C. jejuni Cells

MacConkey sorbitol agar, Xylose lysine deoxycholate agar (XLD) and Oxford medium were used for the enumeration of *E. coli* O157:H7, *S.* Typhimurium and *L. monocytogenes*, respectively, and were purchased from Acumedia Manufacturers Inc. (Baltimore, MD, USA). *Campylobacter jejuni* subsp. *jejuni* ATCC® 33291 was purchased from Oxoid Ltd, Basingstoke, UK. Other bacteria such as *Listeria monocytogenes*, *Salmonella* Typhimurium and *E. coli* O157:H7 were used to verify the selectivity of the developed *C. jejuni* sensor, and were sourced from the culture collection of MARDI. The growth, maintenance and preparation of heat-killed *C. jejuni* for biosensing works are carried out as before [10].

2.4. Sensor Chip Preparation and SAM Formation

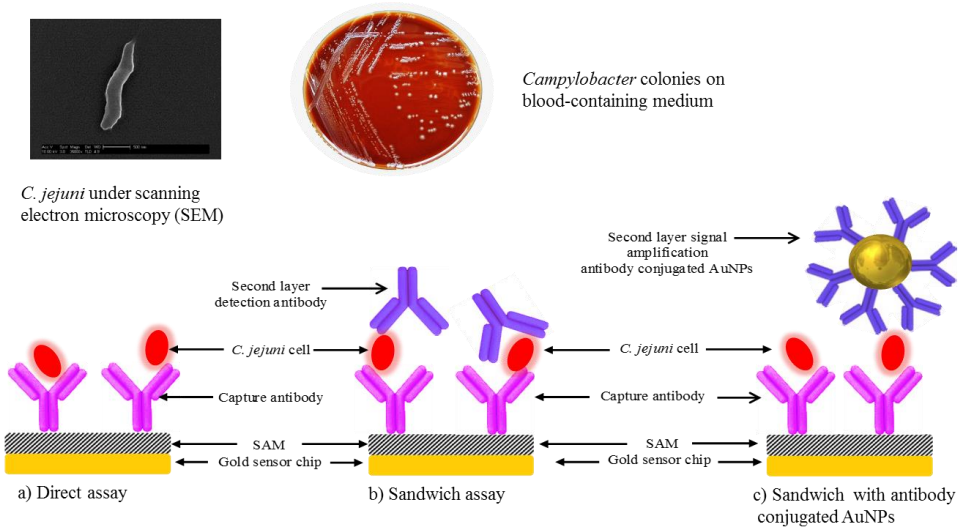
The gold surface of SPR chip needs to be cleaned prior to SAM formation. The surface was cleaned using a piranha solution (H_2SO_4:H_2O_2/3:1) [15]. Briefly, the sensor chip was initially washed with deionized water. This was followed by a second wash with ethanol. Finally, the chip was dried under a stream of nitrogen gas. A glass pipette was utilized to deposit the piranha solution over the entire sensor surface and the solution was left on the chip for 20 min. The sensor chip was then washed thrice with deionized water. Finally, the chip was rinsed with absolute ethanol. This was followed by an immersion of the sensor chip in 20 mM solution of thiol (11-MUDA) dissolved in absolute ethanol. The chip was left at ambient temperature overnight to ensure a complete formation of the carboxy-terminated thiol layer [15]. Before use, the sensor chip was rinsed with ethanol followed by deionized water, and then finally dried using nitrogen gas. MUDA coated chip can be used immediately, or stored at 4 °C in a container sealed with parafilm until further use.

2.5. Surface Activation and Antibody Immobilization

The experiment was started by docking an SPR sensor chip onto the SPR-4 instrument. A filtered and degassed PBS was utilized to prime the instrument at a constant flow rate of 25 $\mu L \cdot min^{-1}$. PBS buffer was also utilized as the running buffer throughout the study. Priming was carried out until a stable baseline was achieved. A 1:1 mixture of 100 mM NHS and 400 mM EDC, prepared fresh in deionized water, was injected (3 min at a flow rate of 25 $\mu L \cdot min^{-1}$) across the sensor surfaces to activate the surface. The mixture converts the carboxylic terminal groups of self-assembled monolayer (SAM) into the active ester of NHS [16]. Immediately, the different concentrations of rabbit polyclonal antibody against *C. jejuni* (50, 70, 100 and 150 $\mu g \cdot mL^{-1}$) were injected (3 min at a flow rate of 25 $\mu L \cdot min^{-1}$) on the parallel spots to determine the optimum concentration of the surface antibody. The antibody was prepared in sodium acetate buffer (100 mM, pH 5.0). Once the optimal conditions of the capture antibody for *C. jejuni* assay was determined, a 70 $\mu g \cdot mL^{-1}$ of the control (mouse IgG) and capture antibodies were injected (3 min at a flow rate of 25 $\mu L \cdot min^{-1}$) onto separate sensor spots. Blocking of non-specific binding on both sensor spots was achieved by an injection of 50 $\mu g \cdot mL^{-1}$ of BSA in PBS (3 min at a flow rate of 25 $\mu L \cdot min^{-1}$). Finally, unreacted NHS esters were capped with 1 M ethanolamine, pH 8.5 (a 3 min injection at a flow rate of 25 $\mu L \cdot min^{-1}$) [17]. A 1×10^7 $CFU \cdot mL^{-1}$ of *C. jejuni* cells were then injected over the sensor surface for 3 min (75 μL) to measure the optimal interaction resulting in the highest sensor response.

2.6. Optimization of C. jejuni Detection

This study reports on three different assays formats: direct, sandwich and sandwich assay with antibody-functionalized gold nanoparticles (AuNPs) (see Scheme 1). In the direct detection assay, *C. jejuni* cells at various concentrations from 1×10^4 to 1×10^9 Colony Forming Unit (CFU) mL^{-1} were injected over sensor surfaces functionalized with mouse IgG on the control channel and *C. jejuni* capture polyclonal antibody on the active channel. After *C. jejuni* binding, a regeneration solution consisting of 100 mM HCl (1 min, 25 µL) was injected over the surface to regenerate and reuse the surface. The regeneration solution was also injected before a sandwich assay was performed. The mild regeneration conditions coupled with a short contact time regenerated the surface, and at the same time allowing for the preservation of the biological activity of the ligand.



Scheme 1. *C. jejuni* detection using three different assays formats: (**a**) direct; (**b**) sandwich and (**c**) sandwich assay with antibody-functionalized gold nanoparticles (AuNPs).

In the sandwich detection assay, injections of *C. jejuni* cells to the sensor surface via the *C. jejuni* capture polyclonal antibody (active channel) and over mouse IgG functionalized sensor surfaces (control channel) were carried out as before. The calibration curve was prepared by injecting various concentrations of *C. jejuni* cell suspensions (1×10^4 to 1×10^9 CFU·mL^{-1}) for 3 min to allow binding interactions (75 µL). At each concentration of *C. jejuni* tested, an injection of 75 µL of polyclonal antibody (30 µg·mL^{-1}) for 3 min was carried out as the detector antibody to allow binding.

2.7. Modification of AuNPs with Anti-C. jejuni Detection Antibody

Gold nanoparticles (AuNPs) is often used in a further amplification of signal obtained from a sandwich assay. Therefore, AuNPs were also investigated in this study. These nanomaterials are available as a colloidal gold solution and can be used directly. The method described previously [10] was utilized to prepare the antibody-colloidal gold conjugate.

2.8. Antibody-Modified AuNPs Sandwich Detection Assay for C. jejuni

The antibody-conjugated AuNPs stock in PBS was first diluted 20 times and this dilution was utilized throughout the study. *C. jejuni* cells were first captured by the immobilized polyclonal antibody (active channel). The sandwich detection assay started with a 75 µL injection of antibody-conjugated AuNPs to allow binding. After the binding response was obtained the surface, which was immobilized with polyclonal antibody, was regenerated with a solution of 100 mM HCl (1 min, 25 µL). Different concentrations of *C. jejuni* cell suspensions (1×10^4 to 1×10^9 CFU·mL^{-1}) were injected for 3 min

(75 μL) each to construct the bacterial calibration curve, and the regeneration solution was injected after each binding interaction occurred.

2.9. Specificity Studies

The developed immunosensor assay (sandwich assay) was tested for its specificity against *C. jejuni*. For this, binding response of non-specific bacteria, a Gram-positive bacterium (*Listeria monocytogenes*) and two Gram-negative bacteria (*Escherichia coli* O157:H7 and *Salmonella* Typhimurium), were investigated. The negative control of this experiment was PBS. A 75 μL of cell suspension of each different bacterium (1×10^9 CFU·mL^{-1}) was injected on the sensor surface which was immobilized with *C.jejuni* specific surface antibody. After a binding response was obtained, the antibody immobilized surface was regenerated using HCI (100 mM) injection for 1 min.

2.10. Limit of Detection and Statistical Analysis

The limit of detection (LOD) and statistical analysis was determined using GraphPad Prism version 5.0. A Student's *t*-test was utilized for comparison of means between two groups while a one-way analysis of variance with Tukey's post hoc analysis was utilized for comparison between more than two groups. A p value of <0.05 was deemed statistically significant. *C. jejuni* standard curves were fitted to a four-parameter logistic equation [18] as follow:

$$y = \frac{a - d}{1 + \left(\frac{x}{c}\right)^b} + d$$

where y is the response signal obtained (RU), a and d the maximum and minimum signal response (RU) of calibration curve respectively, c is the concentration of bacterial cells (log CFU·mL^{-1}) that produces a 50% signal response (EC_{50}) value, x is the bacterial cell concentration (log CFU·mL^{-1}), and b is the slope-like parameter (Hill coefficient). The limit of detection (LOD) was calculated as the mean value of absorbance at a blank concentration of bacteria at three standard deviations (SD). LOD and regression analysis were calculated using the four-parameter logistics model available from PRISM non-linear regression analysis software from www.graphpad.com.

3. Results and Discussion

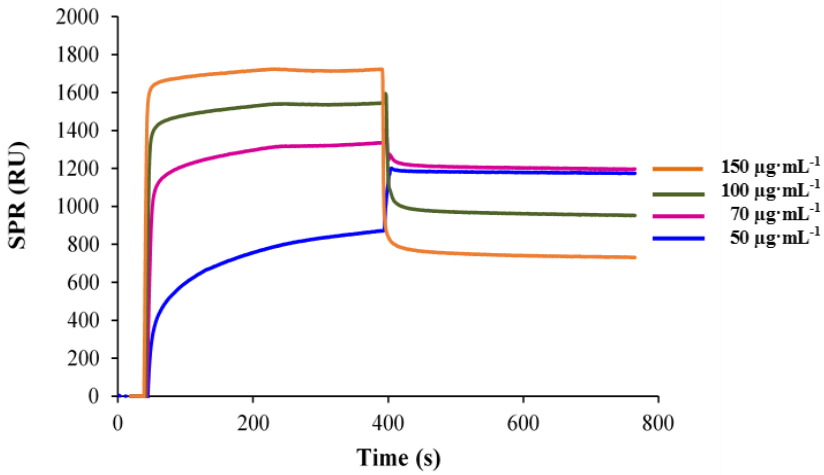
The advantages of surface plasmon resonance (SPR) spectroscopy are numerous including rapid, label-free, sensitive detection as well as providing real-time data. In medical diagnosis, the demand on SPR biosensors is growing since these systems allow researchers to obtain vital parameters such as specificity, binding kinetics, affinity and analyte levels. In SPR, the analyte binding to the ligand immobilized surface leads to changes in the refractive index of the dielectric; therefore, this analog signal is turned into a readable digital signal. Surface plasmon excitation-based detection of analyte consists of three methods including an optical fibre-based architecture, waveguide-based approaches, and prism-based configuration. In optical-fibre or waveguide based methods, an excitation of surface plasmons by a broadband light source takes place. The transmitted light is analyzed for a dip in the spectrum that represents resonance change.

The prism-based configuration is the subject of the current study. In this configuration, the resonance change due to the analyte-ligand interaction is monitored by amassing the light reflected as a function of the incident wavelength at a fixed angle or as a function of the incidence angle at a fixed wavelength.

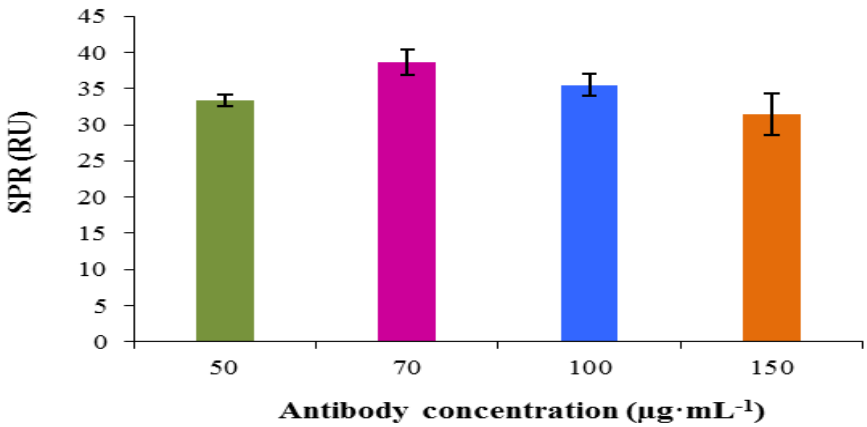
3.1. Immobilization of the Antibody on SAM

Antibody immobilization on the sensor surface must be optimized as it has a pivotal role in the development of an efficient immunoassay method [17]. Therefore, different concentrations of the capture antibody (50, 70, 100 and 150 μg·mL^{-1}) were investigated on the sensor surface using

a standard concentration of bacterial cells (1×10^7 CFU·mL^{-1}). The changes in response unit by increasing concentrations of polyclonal antibody immobilized on the SPR sensor chip are shown in Figure 1a, while their corresponding binding responses for a fixed concentration of *C. jejuni* at 1×10^7 CFU·mL^{-1} are shown in Figure 1b. The most optimal polyclonal antibody concentration was 70 μg·mL^{-1}, which gave the highest signal response for both antibody immobilization (1196 RU) and the bacterial capture (38.66 RU). It was discovered that higher concentrations of the polyclonal antibody did not lead to further increase in signal. In addition, the analyte binding and immobilization level were decreased, possibly due to steric hindrance. Furthermore, high concentrations of the antibody could also promote nonspecific adsorption and increase the cost of the test [19]. Hence, the polyclonal antibody concentration of 70 μg·mL^{-1} was chosen for further sensor studies.



(**a**)



(**b**)

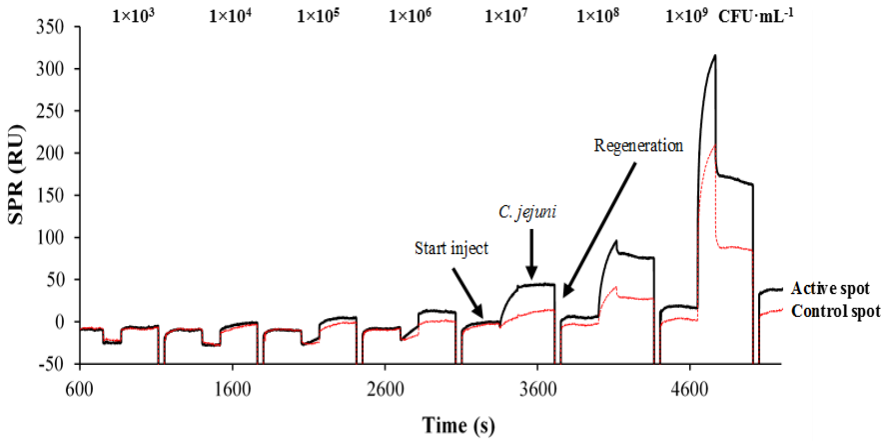
Figure 1. Concentration dependent sensorgram of the capture antibody immobilization. Mercaptoundecanoic acid (MUDA)-coated surface of gold sensor chip was activated with 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide-N-hydroxysuccinimide (EDC-NHS) prior to the antibody immobilization. Four different concentrations of the capture antibody (50, 70, 100 and 150 μg·mL^{-1}) were investigated (**a**). The binding responses obtained for 1×10^7 CFU·mL^{-1} of *C. jejuni* based on the varying concentration of the capture antibody immobilized on the sensor surface. Bars represent mean ± standard deviation of triplicates (**b**).

3.2. Optimization of *C. jejuni* Binding Assay

Three different assay formats for *C. jejuni* detection were investigated and utilized in this study: direct assay (1), sandwich assay (2) and gold-nanoparticles (AuNPs) based signal amplification assay using conjugated detector antibodies (3).

3.2.1. Direct Assay

In this assay, the polyclonal antibody against *C. jejuni* was first immobilized on the surface as the capturing antibody. Various concentrations of *C. jejuni* cells were then injected onto the ligand and the binding responses were recorded as response unit (RU). The real-time sensorgram showed a gradual increase in the response with the raising concentration of *C. jejuni* in the calibration range from 1×10^4 to 1×10^9 CFU·mL^{-1} (Figure 2a). There was no increase in binding response observed at *C. jejuni* concentrations from 1×10^4 to 1×10^5 CFU·mL^{-1}; however, there was a slight increase in the binding response observed (9.9 RU) at *C. jejuni* concentration of 1×10^6 CFU·mL^{-1}. The increase in response unit plotted against *C. jejuni* concentrations showed a partial sigmoidal profile (Figure 2b).



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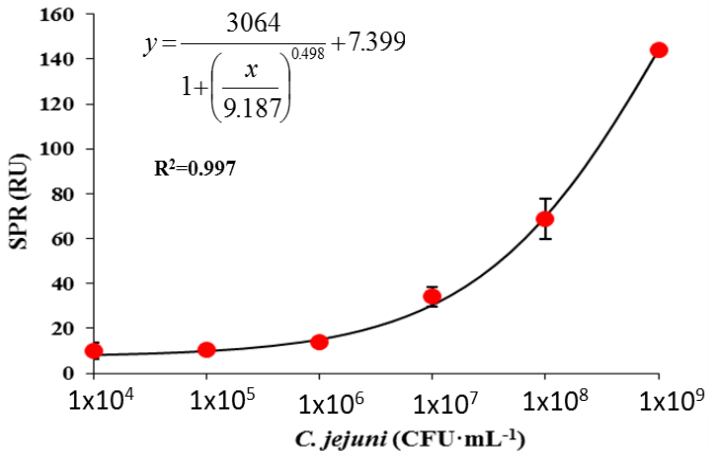


(**b**)

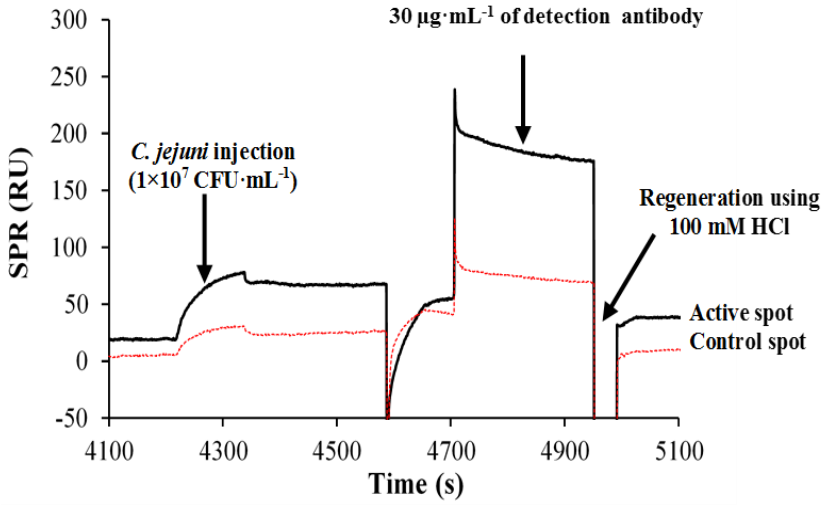
Figure 2. The SPR sensorgram showing the binding response of *C. jejuni* in a direct assay format ranging from 1×10^3 to 1×10^9 CFU·mL^{-1} prepared in PBS buffer injected (3 min) onto 70 μg·mL^{-1} of capture antibody (**a**), and the corresponding standard curve for the direct assay (**b**). The surface was regenerated by 100 mM HCl after every injection of *C. jejuni*. Bars represent mean ± standard deviation of triplicates.

The highest response obtained was at 1×10^9 CFU·mL^{-1} with a binding response of 144.34 RU. The calculated LOD value was 8×10^6 CFU·mL^{-1} (with PBS injection as a control). The value of the LOD was poor compared to the LOD value obtained in a previous work for *C. jejuni* detection on a QCM instrument with a LOD value of 1×10^5 CFU·mL^{-1} using a direct format [10]. Generally, as far as pathogen detection is concerned, QCM instrument is more sensitive than SPR, due to the latter limited evanescent wave penetration depth. LOD values of between 10^3 and 10^7 CFU·mL^{-1} are reported for SPR-based assays of pathogens, while LOD values of between 10^1 to 10^3 CFU·mL^{-1} are reported for QCM- or piezoelectric-based assays, all in a direct format. For an instance, Son et al. [20] reported a LOD value of 10^5 CFU mL^{-1} for detecting *S. enteritidis* in chicken samples, while Meeusen et al. [21] reported a LOD value of 10^7 CFU mL^{-1} for both *E. coli* O157:H7 and *S.* Typhimurium [21]. A QCM-based direct detection method for *Escherichia coli* O157:H7 reported a LOD value of 10 cells [22], in a piezoelectric-excited millimeter-size cantilever (PEMC) while Salam et al. [23], reported a direct detection of *S.* Typhimurium with a LOD value of 20 cells in a Sierra sensor QCM. Another QCM-based direct detection assay for *S.* Typhimurium reported a LOD value of 10^2 [24].

3.2.2. Sandwich Assay

Several researchers have reported an improvement of LOD [25] or an improvement of the detection range in a sandwich format compared to a direct format [26]. Hence, the detection sensitivity of the assay using a sandwich approach was investigated. The results in Figure 3a show the real–time binding sensorgram of the sandwich assay for 1×10^7 CFU·mL^{-1} on the capture antibody (70 μg·mL^{-1}), and control mouse IgG (70 μg·mL^{-1}) immobilized surfaces, followed by the injection of optimized detection antibody (30 μg·mL^{-1}) for 3 min. The regeneration of the sensor surface was also successful (100 mM HCl, 1 min injection) judging by the binding response that returned to the baseline after regeneration. The standard calibration curve for *C. jejuni* in a sandwich format over the entire calibration range from 1×10^4 to 1×10^9 CFU·mL^{-1} is shown in Figure 3b. There was an increase in binding response observed at *C. jejuni* concentration as low as 4×10^4 CFU·mL^{-1}. The increase in response unitplotted against *C. jejuni* concentrations showed a sigmoidal profile. The highest response obtained was at 1×10^9 CFU·mL^{-1} with a binding response of 131.5 RU, with a calculated LOD value of 4×10^4 CFU·mL^{-1} and a good correlation coefficient value of 0.997. This is a significant improvement over the direct format observed previously with a LOD of 8×10^6 CFU·mL^{-1}. The LOD achieved in the SPR assay was similar to the LOD value obtained in the QCM instrument with a LOD value of 2×10^4 CFU·mL^{-1} using a sandwich format [10]. This assay was two to three orders of magnitude sensitive compared to the available commercial ELISA kits that range from 10^6 to 10^7 CFU·mL^{-1} [27].

In this work, the LOD value for the detection of *C. jejuni* in a direct format with a polyclonal as the capturing antibody was 8×10^6 CFU·mL^{-1} while a sandwich format using a polyclonal as the capturing and secondary antibodies resulted in an improvement of LOD to 4×10^4 CFU·mL^{-1}. On the other hand, the development of a sandwich assay format for SPR demonstrates mixed results with several researchers reporting no increase in LOD using a direct or sandwich format [28]. While several researchers reported either an improvement of LOD [25], as similarly reported in this work, or improvement of the detection range with the transition from a direct into a sandwich format [26]. Other researchers, who used SPR for assaying bacterial pathogens, only report a detection in a direct format without reporting on a sandwich format, and all mentioned on the limited penetration depth of the evanescent wave as a problem for sensitive detection of bacterial pathogen in SPR-based assay methods [12,20]. Interestingly, in one report where the sandwich format gave a more sensitive LOD than a direct assay, the authors suggested that portion of the secondary antibody used can bind to the bacterial cells in areas that are within the penetration depth of the evanescent wavelengths especially near the area of the capture antibody. The authors gave this as the reason for the observed improvement of LOD in the sandwich assay seen in a particular study [25].



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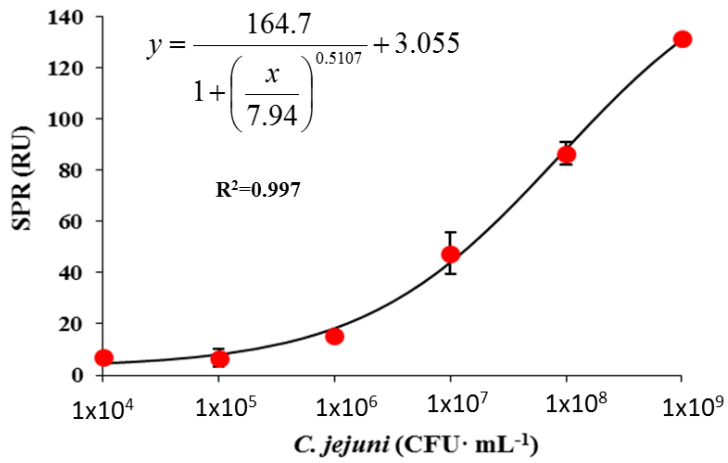


(**b**)

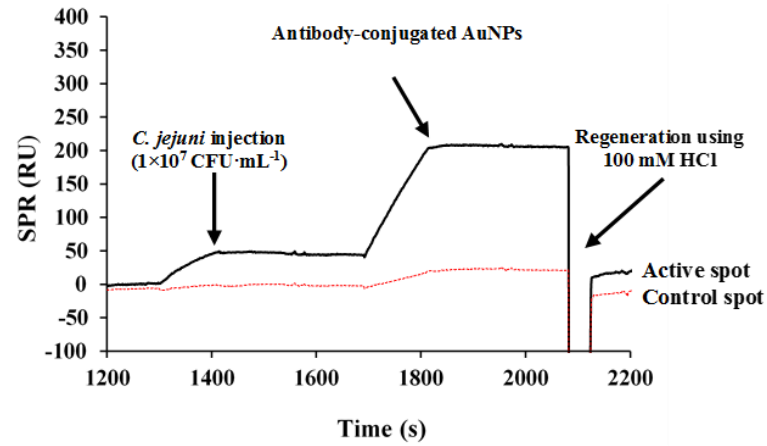
Figure 3. The surface plasmon resonance (SPR) sensorgram showing the binding response of 1×10^7 CFU mL^{-1} *C. jejuni* on the target (black line) and control surfaces (red line) followed by an injection of the detector antibody in a sandwich assay format (**a**), and the corresponding *C. jejuni* standard curve ranging from 1×10^4 to 1×10^9 CFU·mL^{-1}. Bars represent mean ± standard deviation of triplicates (**b**). (The same concentration (70 µg·mL^{-1}) of the surface antibody was used for the immobilization of target (*C. jejuni* specific antibody) and control (mouse IgG antibody) antibodies).

Direct detection format shows a higher LOD value compared to a sandwich or nanoparticles enhanced formats. For example, in the detection of *S.* Typhimurium in chicken meat sample, a 10^5 CFU·mL^{-1} LOD value was obtained with a direct format while the use of anti-*Salmonella*-magnetic beads as a marker for signal amplification resulted in an improvement of the LOD to 10^2 CFU·mL^{-1} [24]. In another example, the detection of *L. monocytogenes* via a piezoelectric cantilever shows a LOD of 10^3 CFU·mL^{-1} in a direct format whilst a sandwich format improves the LOD to 10^2 CFU·mL^{-1} [29].

The LOD obtained using this format was comparatively less sensitive than a direct format reported by Wei et al. [12], at 10^3 CFU·mL^{-1}. However, the SPR-based direct format method reported by them showed very high cross-reactivity (about 70%) to *S.* Typhimurium when challenged with the bacterium at 10^6 CFU·mL^{-1}. Despite this general observation, the fact that a sandwich assay can improve the detection of bacteria on an SPR suggests that this format should be included in any SPR-based methods for the detection of bacterial pathogens.

3.2.3. Sandwich Assay with Signal Amplification Using AuNPs

A further possible signal enhancement method for the *C. jejuni* detection on the SPR-4 sensor device was employed through the application of gold nanoparticles (AuNPs) conjugated secondary antibody. The antibody-conjugated AuNPs were injected over the captured bacteria increase the refractive index, thus the binding response. A 20 times dilution of the antibody-conjugated AuNPs stock in PBS was utilized throughout the entire *C. jejuni* binding assay. The time of the assay for each *C. jejuni* concentration test was about 9 min. Figure 4a shows the resulting response obtained from a 3 min injection of 1×10^7 CFU·mL^{-1} *C. jejuni* cells followed by the injection of 20 times dilution of the antibody-AuNPs conjugate. Moreover, the regeneration of the sensor surface could be successfully achieved which allowed reusing the same surface for subsequent analysis.



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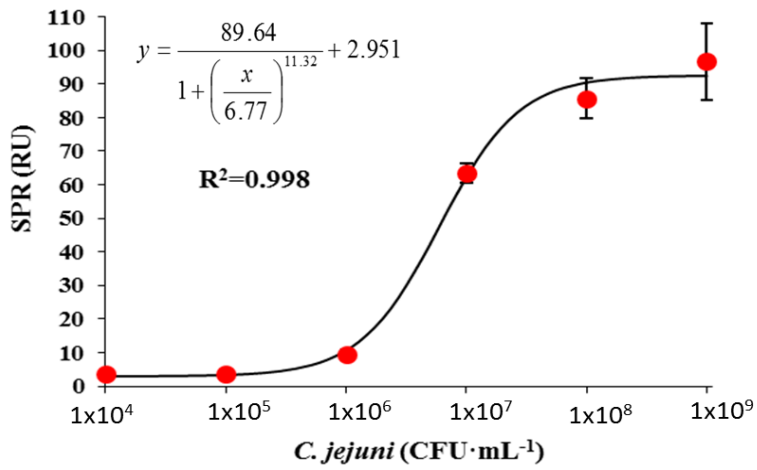


(**b**)

Figure 4. The SPR sensorgram showing the binding response of 1×10^7 CFU·mL^{-1} *C. jejuni* on the target (*C. jejuni* antibody) and control (mouse IgG antibody) antibody immobilized surfaces, followed by an injection of AuNP conjugated detector antibody for signal amplification assay using nanomaterials (**a**). The corresponding *C. jejuni* standard curve ranging from 1×10^4 to 1×10^9 CFU·mL^{-1}. Bars represent mean ± standard deviation of triplicates (**b**).

The standard calibration curve for *C. jejuni* in a sandwich format is shown in Figure 4b. The increase in response unit plotted against *C. jejuni* concentrations showed a sigmoidal profile. There was no increase in binding responses observed at *C. jejuni* concentrations from 1×10^4 to 1×10^5 CFU·mL^{-1}.

A slight increase in binding response was only observed at 1×10^6 CFU·mL^{-1} (9.33 RU). The highest response obtained was at 1×10^9 CFU·mL^{-1} with a binding response of 96.6 RU, with a calculated LOD value of 8×10^5 CFU·mL^{-1} and an acceptable correlation coefficient value of 0.998.

The sandwich immunoassay with signal amplification is more sensitive than the direct format observed previously. However, the LOD value is one order of magnitude less sensitive than a sandwich format without signal amplification AuNPs (Table 1). The value of the LOD was about three orders of magnitude less sensitive compared to the LOD value obtained previously in the QCM instrument for *C. jejuni* detection with a LOD value of 150 CFU·mL^{-1} using a sandwich format with AuNPs amplification [10]. The results indicate that the use of signal amplification using antibody-conjugated AuNPs did not improve the sensitivity of the sandwich format. The sensor surface could be regenerated for more than 10 times with about only 5.0% decrease in the surface activity from the first to the last binding cycle.

Table 1. Limit of detection (LOD) values for various assay formats.

Assay	LOD (CFU·mL^{-1})
Direct	8×10^6
Sandwich assay	4×10^4
Sandwich assay AuNPs signal amplification	8×10^5

Comparison with other bacterial pathogen detection on an SPR platform (Table 2) shows that the method is comparable in sensitivity to other bacterial pathogen but was less sensitive than Singh et al. [30] who reported a LOD value of 1×10^2 CFU mL^{-1} for *C. jejuni*. However, Singh et al. [30] uses a *C. jejuni* bacteriophage for the detection of the bacterium in a direct format and to date commercialization using this type of bioreceptor is not widely available. In addition, there is no cross-reactivity work in their studies.

Table 2. Comparison of LOD values on SPR for various bacterial pathogen.

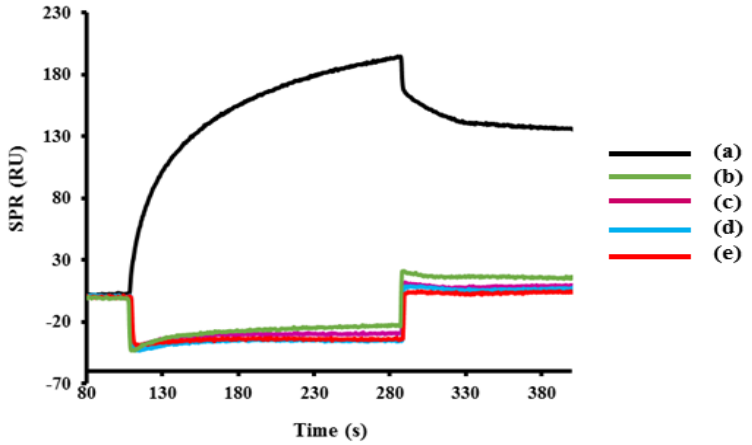
Instrument	Pathogen	Limits of Detection (CFU·mL^{-1})	Format of Detection Assay	Reference
BIAcore (USA)	*B. cereus*	10^5–10^8	Direct	[31]
BIAcore (USA)	*B. cereus*	10^2	Subtractive	[31]
BIAcore (USA)	*Salmonella*	8×10^7	Direct	[32]
BIAcore (USA)	*B. atrophaeus*	1×10^5	Sandwich	[33]
GWC technologies (USA)	*C. jejuni*	1×10^2	Direct	[30]
BIAcore (USA)	*E. coli* O157:H7	3×10^4	Subtractive	[34]
BIAcore (USA)	*E. coli* O157:H7	3×10^5	Direct	[34]
BIAcore (USA)	*Porphyromonas gingivalis*	7.8×10^6	Subtractive	[35]
BIAcore (USA)	*B. anthracis*	1×10^4	Subtractive	[36]
Spreeta (USA)	*C. jejuni*	1×10^3	Direct	[12]
Reichert SR7000(USA)	*E. coli* O157:H7	1×10^3	Sandwich	[37]
Spreeta (USA)	*E. coli* O157:H7	8.7×10^6	Direct	[21]

The use of nanomaterials in signal enhancement for the detection of an analyte in SPR has been intensely studied. Most commonly applied nanomaterials include latex nanoparticles [38], metallic nanoparticles e.g., gold and silver nanoparticles [39,40], magnetic nanoparticles [41,42], carbon-based nanostructures e.g., graphene [40,43] and liposome nanoparticles [41,44]. However, the use of the above nanoparticles in the enhancement of SPR-based assays of biological materials are predominantly for protein and small molecules. Only a few number of reports are available on the use of nanoparticles for the enhancement of SPR-based detection of bacteria. SPR-based detection of bacteria is limited by the short penetration depth of the evanescent wave. In addition, the similar refractive index of most bacteria to the running buffer in SPR presents a problem for sensitive detection [25]. However,

the reason the sandwich assay alone without signal amplification is more sensitive than the sandwich assay with signal amplification in this work could be due to stearic hindrance, which may cause lower signal.

3.3. Cross-Reactivity Studies Against Other Bacteria

Cross-reactivity is an important aspect of the developed immunosensor in detecting targeted bacterial pathogen in the presence of other pathogens. Thus, the specificity of the sandwich immunosensor for the detection of *C. jejuni* was investigated using three different foodborne pathogens including *E. coli*, *L. monocytogenes*, and *S.* Typhimurium. The lowest binding was exhibited by *Escherichia coli* with 5.3% of cross-reactivity, whilst the highest was *S.* Typhimurium with 10.4% cross-reactivity (Figure 5). The specificity analysis demonstrated that the developed method is suitable for routine monitoring of *C. jejuni* in samples containing other bacterial pathogens.



Bacteria Tested (1×10^9 CFU·mL^{-1})	SPR (RU)	Percentage (%)
(a) *Campylobacter jejuni*	138.8 ± 0.14	100
(b) *Salmonella* Typhimurium	14.4 ± 1.42	10.4
(c) *Listeria monocytogenes*	9.1 ± 1.30	6.6
(d) *Escherichia coli*	7.4 ± 0.20	5.3
(e) Control	0.8 ± 1.60	0.5

Figure 5. The SPR sensorgram of cross-reactivity analysis showing the binding response of *C. jejuni* compared with other foodborne bacteria (*E. coli*, *L. monocytogenes*, *S.* Typhimurium) and also negative control (PBS) using the sandwich assay format. The concentration of all bacteria was fixed at 1×10^9 CFU·mL^{-1} and the percentage of the cross-reactivity is shown below. The surface was immobilized with *C. jejuni* specific antibody.

The preparation and batch of antibody used dictates the results of cross-reactivity tests [45]. The detection of *C. jejuni* in other methods developed for the detection of pathogen in various methods such as ELISA, electrochemical and QCM shows varying cross-reactivity results. For example, in an indirect ELISA assay for *C. jejuni* by Hochel et al. [46] a polyclonal antibody preparation using heat-killed antigen from *C. jejuni* O:23 shows little cross-reaction (<10%) to other bacterial genus including *E. coli*, *S.* Typhimurium, *S. enteritidis*, *Yersinia pestis*, *Enterococcus faecalis*, *Bacillus subtilis* and *Bacillus cereus*. A SPR-based method for the detection of this bacterium showed very high cross-reactivity to *S.* Typhimurium (about 70%) in the concentration of 10^6 CFU·mL^{-1} [12]. Therefore, our work has shown superiority over all other existing methods for *C. jejuni* detection in terms of high specificity.

4. Conclusions

In this study, three different immunoassays (direct, sandwich and sandwich with AuNPs amplification) were developed for the detection of *C. jejuni* on an SPR device. The best assay was the sandwich, and the poorest was the direct assay. The detection limit obtained for the detection of *C. jejuni* cells using the sandwich assay was 4×10^4 CFU·mL^{-1}. In addition, the comprehensive cross-reactivity investigations against the other foodborne pathogens demonstrated a very little non-specific binding and the developed assay is the most specific among the existing methods. The improvement of sensitivity over the direct method displayed the feasibility of the sandwich assay to be employed as a rapid method for the detection of *C. jejuni*.

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Author Contributions: Noor Azlina Masdor performed all the experimental work and wrote the paper draft; Zeynep Altintas assisted in the laboratory work supervision; Ibtisam E. Tothill1 supervised and directed the research and finalised the paper.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

11-MUDA	11-mercaptoundecanoic acid
CFU	Colony forming unit
EDC	1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide
ELISA	Enzyme-linked immunosorbent assay
AuNPs	Gold colloidal
IgG	Immunoglobulin G
LOD	Limit of detection
NHS	*N*-hydroxysuccinimide
PBS	Phosphate buffered saline
QCM	Quartz crystal microbalance
RU	Response unit
SAM	Self-assembled monolayer
SD	Standard deviations
SPR	Surface plasmon resonance

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