The development of indirect and sandwich ELISA-based detection methods for the detection of *Campylobacter jejuni* using monoclonal and polyclonal antibody preparations

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ABSTRACT

Aims: The current gold standard method for the detection of *Campylobacter jejuni* is the culturing method followed up by immuno-based detection method, of which, the ELISA is the most often used. Many commercial detection methods based on ELISA use monoclonal antibody preparations although polyclonal antibody can be more sensitive and cheaper to produce. In this study, a comparison of indirect and sandwich ELISA-based detection methods for the detection of *C. jejuni* using a commercial monoclonal and polyclonal antibody preparations was explored.

Methodology and results: An indirect and sandwich ELISA-based methods for the detection of *C. jejuni* was carried out using the same concentration of antibody (5 µg/mL) and the same concentration of the bacterium at 1×10^9 CFU/mL. At the pre-screening for optimum concentration of antibody to be used for both assay formats, the commercial monoclonal preparation gave a poor absorbance value of about 0.112 compared to 1.582 for the polyclonal antibody preparation. Hence, the use of the monoclonal antibody was not pursued further. Using the polyclonal antibody, the calculated Limits of Detection (LOD) value obtained for the indirect and sandwich ELISA methods were at 1.6×10^4 CFU/mL and at 1.29×10^4 CFU/mL, respectively, which are more sensitive than commercially used methods. The results of the specificity test obtained from the developed polyclonal antibody were then tested against other common food borne bacterial pathogens such as *Salmonella* Typhimurium, *Listeria monocytogenes* and *Escherichia coli* tested using the sandwich ELISA format indicated that the responses by other bacterial genus were relatively low with the translated cross-reactivity percentages of 1.78, 2.36, and 6.87%, respectively.

Conclusion, significance and impact of study: The results indicated that the developed system using a polyclonal antibody preparation can be more sensitive than monoclonal preparation. In addition, it is also specific towards *Campylobacter* while the monoclonal antibody preparation fares poorly.

Keywords: *Campylobacter jejuni*, campylobacteriosis, ELISA, polyclonal antibody, monoclonal antibody

INTRODUCTION

Campylobacteriosis is a food-borne illness caused by bacteria of the genus *Campylobacter* (Vencia *et al.*, 2014). The genus *Campylobacter* comprises of 18 species and 6 subspecies with *Campylobacter jejuni* being the most frequently reported species contaminating poultry, and the main cause of food-borne illnesses in humans. Approximately 214,268 cases of food-borne illness and 100,000 deaths due to *Campylobacter* spp. infection in the EU countries has been reported. Although epidemiological data from Malaysia is still incomplete, the available data indicate that *Campylobacter* infection and occurrence is on the rise (Nor Faiza *et al.*, 2013; Khalid *et al.*, 2015; Premarathne *et al.*, 2017). Guillain-Barré syndrome (GBS) is a neurological disease caused by post-infection of *C. jejuni* that leads to death in a small

number of cases (1 in 1,000 infections) (Grozdanova *et al.*, 2010). Patients afflicted by GBS are likely to require mechanical support for normal breathing, often afflicted by extensive axonal injuries, and suffer from irreversible nerve damage (Allos, 2001).

There are numerous quantification methods available for the detection of *Campylobacter* in food products such as culturing, microscopy, enumeration and biochemical testing, which include new emerging methods such as polymerase chain reaction (PCR), immunoassays and deoxyribonucleic acid (DNA) probes (Saiyudthong *et al.*, 2015). Of all these methods, immuno-based methods are increasingly being used by developed countries such as the EU and the US in detecting *Campylobacter* (Ali *et al.*, 2016). It is estimated that about 25 and 50% of screening methods for *Campylobacter* spp. are being used in the EU and US, respectively, as compared to relatively low

usage in Asian countries (Weschler, 2013). Immuno-based methods have been successfully employed for the detection of bacterial cells, spores, toxins and viruses. After the pre-enrichment step, immuno-based methods are faster, cheaper and required lesser operator skill than nucleic-acid based methods (Cho *et al.*, 2014). Thus, immuno-based diagnostic methods in conjunction with culturing method for the detection of *C. jejuni* will continue to be the gold standard method for years to come (Oyarzabal and Battie, 2012; Morsy *et al.*, 2017).

ELISA is the most used immuno-based method for detection of food-borne pathogen detection. Four such enzyme immunoassays (EIAs) techniques that are commercially available include the ProSpecT *Campylobacter* EIA from Remel, Lenexa, Kansas (US), Premier CAMPY EIA and the ImmunoCard STAT! CAMPY test from Meridian Bioscience, Cincinnati, Ohio (US) and the Campylobacter test kit from Diagnostic Automation, Inc. (California, USA). All four EIAs had a very satisfactory overall performance with the percentage of sensitivity greater than 98% (Granato *et al.*, 2010). However, the biggest issue regarding the use of these commercial kits is the insensitive limit of detection which ranges from 10^6 - 10^7 CFU/mL (Ramírez *et al.*, 2009). As the dose for *Campylobacter* spp. infection is from 400 to 500 number of cells depending on the strain, the lowering of the detection limit is being intensively pursued (Velusamy *et al.*, 2010a). Many of the commercial detection kits employ both monoclonal and polyclonal preparation and for *Campylobacter* spp., especially *C. jejuni*, it has been reported that many monoclonal preparations are less sensitive compared to polyclonal preparations in detecting this bacterium (Oyarzabal and Battie, 2012; Masdor *et al.*, 2016). Very few studies have compared the sensitivity of polyclonal and monoclonal preparation for the detection of this bacterium in ELISA-based formats. Since many commercial detection methods based on ELISA use monoclonal antibody preparations, a comparison of indirect and sandwich ELISA-based detection methods for the detection of *C. jejuni* using a commercial monoclonal and polyclonal antibody preparations was explored.

MATERIALS AND METHODS

Purified rabbit polyclonal antibody raised against *C. jejuni* was obtained as before (Masdor *et al.*, 2016) from the Malaysian Agricultural Research and Development Institute (MARDI), Malaysia. Culti-loops™ of *Campylobacter jejuni* subsp. *jejuni* (ATCC® 33291™), Bolton broth, Bolton broth selective supplement, laked horse blood, Oxoid anaerobar and CampyGen™ were purchased from Fischer Scientific (Loughborough, UK). The mouse monoclonal antibody against *C. jejuni* (7721) was purchased from Abcam Ltd (UK). Campy cefex agar and Campylobacter supplement was purchased from Acumedia Manufacturers Inc, (Baltimore, USA). Phosphate buffered saline (PBS), Tween-20, hydrogen peroxide (H₂O₂), anti-rabbit IgG (whole molecule)-alkaline phosphatase antibody produced in goat, alkaline

phosphatase yellow substrate (pNPP), bovine serum albumin (BSA) and Nunc-Immuno™ MicroWell™ 96 well solid plates were purchased from Sigma-Aldrich (Poole, UK).

Preparation of culture media for *C. jejuni*

Campylobacter jejuni was grown and maintained on Campy cefex agar as before (Masdor *et al.*, 2016). The preparation of the *C. jejuni* inoculum was carried out by sub-culturing a 48 h culture plate into Bolton broth (10 mL) in a 25 mL universal bottle. The inoculum was then grown at 42 °C for 48 h under microaerophilic conditions. After the 48 h incubation period, approximately 10 mL of the Bolton broth was then transferred into 90 mL of Bolton broth in a Duran bottle and further incubated at 42 °C for another 48 h under microaerophilic conditions. Cells were harvested by centrifugation at 10,000 $\times g$ for 15 min at 4 °C on a benchtop centrifuge (Sorvall™ Legend™ X1, Thermo Fischer Scientific, USA). The pelleted cells were then washed with PBS and the procedures were repeated three times. The bacterial cells were then re-suspended in PBS and the bacterial suspensions were adjusted to optical densities (OD) at 600 nm of between 1.6 and 2.0 to obtain bacterial concentrations between 1×10^8 and 1×10^{10} CFU/mL on a UV/VIS spectrophotometer (Perkin-Elmer Lambda 20 GenTech Scientific, Inc. USA). The bacterial concentrations were confirmed by a spread plate method. Colony forming units (CFU) on the agar plates were then counted as CFU/mL. The entire culture was then heat-killed at 70 °C for 30 min. To ensure that the cells were dead, the cells were inoculated again on Campy cefex agar and incubated at 4 °C for 48 h (under microaerophilic conditions). Bacterial suspensions were then diluted to the desired target concentrations ranging between 1×10 and 1×10^9 CFU/mL in PBS. The cells were used in the preparation of immunogen, as the standard curve plotting for *C. jejuni* and as an analyte in the development of immunosensor.

Indirect ELISA

The indirect ELISA method was carried out by coating the microtiter plates with a coating buffer (100 mM carbonate buffer, pH 9.6) containing 100 μ L of heat-killed *C. jejuni* cells (1×10^9 CFU/mL) per well and was incubated overnight at 4 °C. Heat-killed *C. jejuni* was prepared as outlined above. The control for this experiment was PBS (10 mM, pH 7.4). On the next day, the plate was emptied and washed with 200 μ L per well of a washing buffer, PBS-T (10 mM PBS, pH 7.4, with 0.05% Tween 20) for three times. Unoccupied sites were blocked with 200 μ L per well of 1% solution of BSA diluted in PBS for 2 h at 37 °C. When the incubation period was over, the wells were emptied and washed again using the above method. Both antibodies; monoclonal and polyclonal at concentrations varying from 0.01 to 100 μ g/mL diluted in the antibody diluent buffer (10 mM PBS, pH 7.4, 1% BSA and 0.05% Tween 20) in a volume of 100 μ L were added per well, and the plate was incubated for another 2 h at 37 °C.

After another washing procedure, a 1:10,000 dilution of Goat-anti rabbit IgG-AKP, secondary antibody conjugate that specific to rabbit polyclonal antibody and Goat-anti mouse IgG-AKP, conjugate that specific to mouse monoclonal antibody (both 100 μ L, diluted in antibody diluent buffer) were added to the wells and incubated for another 2 h at 37 °C. The wells were emptied and washed again using the same method above. The plate was then incubated in the dark at room temperature for 30 min after the addition of 100 μ L per well of p-nitrophenyl phosphate (pNPP), a substrate of AKP. When AKP and pNPP were reacted, a yellow water-soluble reaction product is formed. This reaction was stopped by the addition of 50 μ L of 1 M NaOH. The absorbance of the resulting yellowish solution was measured at 405 nm using a Varioskan Flash Multimode microplate reader (Thermo Fischer Scientific, USA). The entire experiment was carried out in triplicates. The results obtained were then compared.

The optimised antibody concentration was then utilised in the construction of the *C. jejuni* standard curve using the indirect ELISA. In this experiment, only polyclonal antibody at the concentration of 5 μ g/mL was utilised. The experiment was carried out by coating the microtiter plate with 100 μ L of coating buffer (100 mM carbonate buffer, pH 9.6) containing heat-killed *C. jejuni* cells (concentrations varied from 1×10^1 - 1×10^9 CFU/mL) per well and incubated overnight at 4 °C. The control for this experiment was PBS (10 mM, pH 7.4). Then 200 μ L per well of washing buffer, PBS-T was utilised to wash the wells for three times. The subsequent steps were the same as described in the previous indirect ELISA method. The entire experiment was carried out in triplicates.

Sandwich ELISA

Sandwich ELISA method was carried out in order to construct the *C. jejuni* standard curve. This method measures the amount of antigen between two layers of antibodies, capture and detector. The antigens to be measured must contain at least two antigenic sites, since at least two antibodies act in the sandwich which binds to different sites on the antigen or antibody.

The experiment was carried out by coating the microtiter plate with 100 μ L of 5 μ g/mL capture antibody (rabbit polyclonal antibody against *C. jejuni*) which has been optimised in the previous indirect ELISA method, by diluting it in 100 mM carbonate buffer (pH 9.6), and incubated overnight at 4 °C. The plate was emptied and washed with 200 μ L per well of a washing buffer, PBS-T for three times. Unoccupied sites were blocked with 200 μ L per well of 1% solution of BSA diluted in PBS and incubated for 2 h at 37 °C. The wells were then emptied and washed using the above method. A 100 μ L of diluted *C. jejuni* cells (1×10^9 CFU/mL) was added per well and incubated for another 2 h at 37 °C. The control for this experiment was PBS (10 mM, pH 7.4). After another washing procedure, a various concentration of two different detector antibodies (rabbit polyclonal and mouse monoclonal against *C. jejuni*) which were 10, 30 and 50

μ g/mL. Both antibodies were added to the wells and incubated for 2 h at 37 °C.

After another washing procedure, a 1:10,000 dilution of Goat-anti rabbit IgG-AKP and Goat-anti mouse IgG-AKP, a secondary antibody conjugate was added to the wells and incubated for another 2 h at 37 °C. The wells were again washed using the same method mentioned above. The plate was then incubated at room temperature for 30 min after the addition of 100 μ L per well of pNPP and was stopped by the addition of 50 μ L of 1 M NaOH. The absorbance of the resulting yellowish solution was measured at 405 nm as described in the previous indirect ELISA method. The entire experiment was carried out in triplicates. The results obtained were then compared.

Both optimised capture and detector antibodies concentration were then utilised in the construction of the *C. jejuni* standard curve using the sandwich ELISA method. In this experiment, the only polyclonal antibody was utilised for both capture and detector antibodies at the concentration of 5 and 50 μ g/mL respectively. The experiment was carried out by coating the microtiter plate with 100 μ L of coating buffer (100 mM carbonate buffer, pH 9.6) containing heat-killed *C. jejuni* cells (concentrations varied from 1×10^1 - 1×10^9 CFU/mL) per well and incubated overnight at 4 °C. The control for this experiment was PBS (10 mM, pH 7.4). Then 200 μ L per well of washing buffer, PBS-T was utilised to wash the wells for three times. The subsequent steps were the same as described in the above sandwich ELISA method. The entire experiment was carried out in triplicates.

Campylobacter jejuni standard plot using commercial ELISA kit

In this experiment, a commercial ELISA kit; *Campylobacter* antigen detection (in food) (Diagnostics Automation Inc, CA, USA) was utilised as a comparative method. The kit is based on a double antibody (sandwich) assay utilizing specific polyclonal antibody against *C. jejuni* coated onto microwells as the capture antibody and polyclonal antibody conjugated with peroxidase as the detector antibody. A standard curve based on detection of *C. jejuni* was developed at concentrations ranging from 1×10^1 - 1×10^9 CFU/mL. The experiment was carried out according to the instructions provided by the manufacturer. A 100 μ L aliquot of *C. jejuni* cells concentrations ranging from 1×10^1 - 1×10^9 CFU/mL were added to the appropriate wells and incubated for 30 min at 25 °C. The control for this experiment was PBS (10 mM, pH 7.4). Then, the plate was emptied and washed with 200 μ L per well of a washing buffer, containing a surfactant for three times. Two drops of a chromogen solution consisting of tetramethylbenzidine (TMB) as the substrate for the peroxidase-conjugated to the detector antibody were added and incubated for 5 min. Finally, two drops of stop solution were added and mixed gently by tapping the side of the strip holder with the index finger. The absorbance was measured at 450 nm using a Varioskan Flash Multimode Reader. The entire experiment was carried out in triplicates.

Specificity studies

The specificity test of the rabbit polyclonal antibody against *C. jejuni* was conducted with other food-borne pathogens including the Gram-negative, *Salmonella* Typhimurium, *Escherichia coli* and the Gram-positive, *Listeria monocytogenes* using the optimised sandwich ELISA method. A 100 µL of 5 µg/mL of rabbit polyclonal antibody against *C. jejuni* (capture antibody) was coated on a microtiter plate and incubated overnight at 4 °C. The plate was emptied and washed with 200 µL per well of a washing buffer, PBS-T for three times. Unoccupied sites were blocked with 200 µL per well of 1% solution of BSA diluted in PBS and incubated for 2 h at 37 °C. The wells were then emptied and washed using the above method. Then, a 100 µL of each bacterial solution (1×10⁹ CFU/mL) was used as the analyte and incubated for 2 h at 37 °C. The same polyclonal antibody was used as the detection antibody at the concentration of 50 µg/mL was diluted in 100 mM carbonate buffer (pH 9.6), added and incubated for 2 h. The subsequent steps were the same as described in the above sandwich ELISA method. The entire experiment was carried out in triplicates. The results obtained were then compared.

Reactivity against the different preparation of *C. jejuni* cells

The test for the ability of rabbit polyclonal antibody to recognise and differentiate live cells and dead cells of *C. jejuni* was performed using the sandwich ELISA method by employing two different methods of dead cells preparations; i) heating at 70 °C for 30 min and ii) addition of 0.5% formalin with the subsequent incubation at room temperature for 1 h. A 100 µL of 5 µg/mL of rabbit polyclonal antibody against *C. jejuni* (capture antibody) was coated on a microtiter plate and incubated overnight at 4°C. The plate was emptied and washed with 200 µL per well of a washing buffer, PBS-T for three times. Unoccupied sites were blocked with 200 µL per well of 1% solution of BSA diluted in PBS and incubated for 2 h at 37 °C. The wells were then emptied and washed using the above method. Then, a 100 µL of each live and dead bacterial cells solution (1×10⁹ CFU/mL) was used as the analyte and incubated for 2 h at 37 °C. The same polyclonal antibody was used as the detection antibody at the concentration of 50 µg/mL was diluted in 100 mM carbonate buffer (pH 9.6), added and incubated for 2 h. The subsequent steps were the same as described in the above sandwich ELISA method. The entire experiment was carried out in triplicates. The results obtained were then compared.

Limit of detection (LOD) calculation for the ELISA method

Calibration curves were fitted with a non-linear regression using four-parameter logistic equations (Karpinski, 1990) as showed in Equation 1.

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

where y is the absorbance obtained (nm), a and d the maximum and minimum response (nm) of calibration curve respectively, x is the concentration of bacterial cells (log CFU/mL) that produced a 50% signal response (EC50) value, c is bacterial cell concentration (log CFU/mL), 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.

Statistical analysis

Data analyses were carried out using GraphPad Prism version 5.0 available from www.graphpad.com. A one-way analysis of variance with post hoc analysis by the Tukey's test or a Student's t-test was utilised for between groups comparison. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Detection of *C. jejuni* via indirect assay

The use of the indirect ELISA assay offers several advantages such as; i) an increase in sensitivity due to the use of more than one labelled antibody per primary antibody, ii) the assay offers flexibility since a multitude of different primary detection antibodies, and secondary labelled antibody can be used, and iii) economy, as fewer labelled antibodies, will need to be prepared. In this experiment, concentrations of rabbit polyclonal and mouse monoclonal antibodies of 0.01, 0.1, 1, 5, 10, 50 and 100 µg/mL were tested with 1×10⁹ CFU/mL *C. jejuni* cells using an indirect format to find the optimum concentration of antibody in order to construct a standard curve for the detection of *C. jejuni*. Control studies were included in each experimental set, and the absorbance responses were subtracted from the control values to get the total absorbance values. The result in Figure 1 shows that the absorbance value obtained from the interaction of the rabbit polyclonal antibody against *C. jejuni* was increased dramatically when the concentration of the antibody was increased. A concentration of 5 µg/mL of the rabbit polyclonal antibody was chosen for the construction of a standard curve using the indirect ELISA since it gave an absorbance value as high as 1.582. Higher concentrations of between 10 and 100 µg/mL were not used due to economic issues although the absorbance obtained were higher. In comparison, a mouse monoclonal antibody against *C. jejuni* purchased

from Abcam (7721) shows low absorbance values of about ten to fifteen times poorer (between 0.05 to 0.1) compared to the polyclonal antibody at all concentrations even though the concentration of *C. jejuni* tested was as high as 1×10^9 CFU/mL. In addition, marginally improved absorbance at concentrations of between 5 and 100 $\mu\text{g/mL}$ was observed for the commercial monoclonal antibody. Hence, the monoclonal antibody was not utilized further for the construction of the *C. jejuni* standard curve using indirect ELISA.

Figure 1: Bar chart showing the absorbance at 405 nm of indirect ELISA format which was constructed with different concentration of rabbit polyclonal and mouse monoclonal antibodies (ranging from 0.01 to 100 $\mu\text{g/mL}$) tested with *C. jejuni* at the concentration of 1×10^9 CFU/mL. Error bars represent the average $\pm$ standard deviation of triplicates.

Figure 2: Standard curve constructed for the detection of *C. jejuni* concentrations from 1×10 to 1×10^9 CFU/mL using rabbit polyclonal antibody (5 $\mu\text{g/mL}$) through indirect ELISA method. Error bars represent the average $\pm$ standard deviation of triplicates.

Although the use of monoclonal as the capturing antibody is usually more specific and gives higher binding

affinity than polyclonal, the same cannot be said about the monoclonal antibody against *C. jejuni* as Wang *et al.* (2000) have reported that several sources of monoclonal antibody against *C. jejuni* performs poorly compared to the polyclonal antibody. The use of monoclonal can sometimes give poor performance compared to the polyclonal antibody. For example, a monoclonal preparation for the detection of the pathogen *Francisella tularensis* in the indirect format showed a difference in absorbance of 0.15 between the lowest and highest determination range whilst the polyclonal preparation gave a difference in absorbance of 0.45, with the limit of detection of 5.4×10^6 CFU/mL for polyclonal antibodies and 6.9×10^6 CFU/mL for monoclonal antibodies (Pohanka *et al.*, 2008). The difference may be attributed to the ability of *C. jejuni* to exist in two different morphologies; vibrioid or bacillary forms and coccoid form, which varies according to age and stress conditions (Kelly *et al.*, 2001).

In addition, the targeted singular surface protein, of which is a target to the monoclonal preparation might not be expressed under certain conditions, while the polyclonal preparation captures a multitude of surface protein, and hence, has a wider range of target. These different morphological forms may be sensitive to different antibody preparation. These explanations are, however, only conjectures and more experiments are needed to find the root cause of the difference in sensitivity between monoclonal and polyclonal preparations.

The construction of standard curve for the detection of *C. jejuni* through indirect ELISA method was carried out using the rabbit polyclonal antibody (5 $\mu\text{g/mL}$) against different concentrations of *C. jejuni* cells to give nine calibration standards ranging from 1×10 to 1×10^9 CFU/mL. The result in Figure 2 shows a typical sigmoidal curve based on the four-parameter logistic equation. There was no increase in binding response observed at *C. jejuni* concentrations from 1×10 to 1×10^4 CFU/mL. However, there was a slight increase in the binding response observed at *C. jejuni* concentration in between 1×10^4 and 1×10^5 CFU/mL with an absorbance response of 0.1345. The calculated LOD value was at 1.6×10^4 CFU/mL with a good correlation coefficient value of 0.9985.

Sandwich ELISA

The second most reported format of ELISA after indirect is the sandwich assay. A sandwich ELISA measures antigen between two layers of antibodies (capture and detection antibody). The target antigen must contain at least two antigenic sites capable of binding to antibodies. In the sandwich method, either monoclonal or polyclonal antibodies can be used as the capture and detection antibodies.

In this experiment, a previously optimised polyclonal antibody at 5 $\mu\text{g/mL}$ was utilised as the capture antibody. The result in Figure 3 shows that the highest absorbance value of 1.679 was obtained using a sandwich ELISA method with the combination of a captured rabbit

polyclonal antibody (5 µg/mL) against *C. jejuni* at 1×10^9 CFU/mL and a detector antibody at 50 µg/mL.

When the commercial mouse monoclonal antibody against *C. jejuni* was tested in the sandwich format using the same concentration as the polyclonal antibody, it gave eight times less response compared to the polyclonal antibody at 50 µg/mL. In addition, increasing the concentration of the monoclonal antibody from 10 to 50 µg/mL only gave an increase in response of about two times while the increase in concentrations of the polyclonal antibody from 10 to 50 µg/mL gave an increase in response of about four times (Figure 3).

Figure 3: Bar chart showing the absorbance at 405 nm of sandwich ELISA method which was constructed with rabbit polyclonal antibody against *C. jejuni* as the capture antibody (5 µg/mL) and with two different detector antibodies which were rabbit polyclonal and mouse monoclonal antibodies against *C. jejuni* at three different concentration (10, 30 and 50 µg/mL) tested with *C. jejuni* at the concentration of 1×10^9 CFU/mL. Error bars represent the average $\pm$ standard deviation of triplicates.

The use of a polyclonal antibody as a capture antibody has been shown to increase the sensitivity of the ELISA for about 10-fold compared to the monoclonal antibody (Hawkes *et al.*, 2000). In addition, a similar work using both polyclonal antibody from rabbit and monoclonal antibody from the mouse for the detection of various *Salmonella* strains in an indirect mode by Konigshofer *et al.* (2014) also showed that polyclonal antibodies have better avidity than monoclonal antibodies in detecting the bacterium.

Wang *et al.* (2000) reported that the commercial monoclonal antibodies such as 1744-9029, 1744-9006, MAB001 and C65701M from Biogenesis Ltd, NH Harlan Sera Lab and GB Biodesign International, MD, respectively, give poor results. In addition, Oyarzabal and Battie (2012) reported that several more commercial monoclonal antibodies for *C. jejuni* such as 1745-00, 1744-8508, 1744-9059, and 1744-9109; USB C1037-02A, C1037-04, and C1037-16 from AbD Serotec also showed poor results compared to polyclonal for ELISA detection of *Campylobacter* spp. In general, monoclonal exhibit greater specificity than polyclonal antibody as the former

targets a single epitope of an antigen (Velusamy *et al.*, 2010b). Despite this, the use of monoclonal antibody could limit the detection of *Campylobacter* spp. because the conservation of epitope may not occur across all *Campylobacter* species and strains (Rice *et al.*, 1996).

Based on the result above, a construction of the standard curve for the detection of *C. jejuni* through a sandwich ELISA method was carried out using the rabbit polyclonal antibody for both capture (5 µg/mL) and detection (50 µg/mL) antibodies and was tested with different concentrations of *C. jejuni* cells to give nine calibration standards ranging from 1×10^1 to 1×10^9 CFU/mL. The results obtained in Figure 4 shows a typical sigmoidal curve based on the four-parameter logistic equation. There was no increase in binding response observed at *C. jejuni* concentrations from 1×10^1 to 1×10^4 CFU/mL. However, there was a slight increase in the binding response observed at *C. jejuni* concentration in between 1×10^4 and 1×10^5 CFU/mL with an absorbance response of 0.2832. The calculated LOD value was at 1.29×10^4 CFU/mL with a good correlation coefficient value of 0.9944.

Based on the LOD obtained, the developed sandwich ELISA method is more sensitive to the commercial kit *Campylobacter* antigen detection (in food) from Diagnostics Automation Inc, CA, USA (Figure 5) with an LOD value of 1.66×10^6 CFU/mL. In addition, the developed method is also more sensitive to several similar commercial kit products in the market such as ProSpecT™ *Campylobacter* assay (Remel Inc., Lenexa, KS) for detecting *C. jejuni* in stool sample and PREMIER™ CAMPY microplate EIA (Meridian Bioscience, Inc, Cincinnati, OH) with reported LOD values of between 10^5 and 10^6 CFU/mL, and between 10^6 and 10^7 CFU/mL, respectively (Oyarzabal and Battie, 2012).

Figure 4: Standard curve constructed for the detection of *C. jejuni* concentrations from 1×10^1 to 1×10^9 CFU/mL using rabbit polyclonal antibody as a capture (5 µg/mL) and detector (50 µg/mL) antibodies through sandwich ELISA method. Error bars represent the average $\pm$ standard deviation of triplicates.

Figure 5: Standard curve constructed for the detection of *C. jejuni* concentrations using a commercial *Campylobacter* detection kit (Diagnostics Automation Inc, CA, USA). Error bars represent the average $\pm$ standard deviation of triplicate.

Cross-reactivity tests against other bacteria

The specificity of the developed rabbit polyclonal antibody against *C. jejuni* was tested with other bacterial genera such as *S. Typhimurium*, *L. monocytogenes* and *E. coli*. The results in Figure 6 shows that the developed polyclonal antibody overwhelmingly detected only *C. jejuni* with a good signal giving an absorbance value of about 2.25 whilst other bacterial genus gave absorbance values of 0.128, 0.140 and 0.238 for *E. coli*, *L. monocytogenes* and *S. Typhimurium*, respectively, whilst the control gave a low absorbance value of 0.09. When translated as a percentage of response compare to *C. jejuni* (100%), the response obtained for *E. coli*, *L. monocytogenes* and *S. Typhimurium* were 1.78, 2.36 and 6.87%, respectively (Table 1). The results indicated that the responses by other bacterial genus were relatively low.

Cross-reactivity of antibody to the antigen is usually species specific for monoclonal. For instance, a monoclonal antibody raised against *C. jejuni* recognise only *C. jejuni*. On the other hand, a polyclonal antibody raised against *C. jejuni* could also detect *C. lari* and *C. coli* (Hochel *et al.*, 2007). This is because monoclonal antibody is raised against a specific component of a bacterial epitope whilst a polyclonal antibody is raised against many epitopes- some of which are shared in bacteria resulting in non-specific interaction. There is a linear relationship between amino acid substitutions and the cross-reactivity of polyclonal because antigen on the surface of a protein appears to show sets of epitopes that are nearly continuous and overlapping (Hochel *et al.*, 2004).

A comprehensive cross-reactivity work carried out by Hochel *et al.*, (2007) indicated that polyclonal antibody produced by rabbit using heat-killed antigen from *C. jejuni* O:23 is specific to *C. jejuni* and has very low cross-

reactivity against other *Campylobacter* spp. such as *C. lari*, *C. hyointestinalis*, *C. upsaliensis* and *C. coli* and to other genera such as *E. coli*, *S. Typhimurium*, *S. enteritidis*, *Yersinia pestis*, *Enterococcus faecalis*, *Bacillus subtilis*, *Bacillus cereus* and many other bacteria suggesting that polyclonal antibody prepared from heat-killed *C. jejuni* cells can be very specific.

Figure 6: The relative response against different food-pathogen bacterial strain (*S. Typhimurium*, *L. monocytogenes* and *E. coli*) at the concentration of 1×10^9 CFU/mL using sandwich ELISA. Error bars represent the average $\pm$ standard deviation of triplicates.

Table 1: The relative response of polyclonal antibody binding against different food-pathogen bacterial strain in percentage calculation. Error represent the average $\pm$ standard deviation of triplicates.

Bacteria tested (1×10^6 CFU/mL)	% Relative activity
(a) <i>Campylobacter jejuni</i>	100 $\pm$ 0.013
(b) <i>Salmonella</i> Typhimurium	6.87 $\pm$ 0.002
(c) <i>Listeria monocytogenes</i>	2.36 $\pm$ 0.002
(d) <i>Escherichia coli</i>	1.78 $\pm$ 0.05
(e) Control (PBS at 10 mM, pH 7.4)	0.0 $\pm$ 0.002

CONCLUSION

The use of a polyclonal preparation gave a higher sensitivity compared to a monoclonal preparation for both an indirect and a sandwich ELISA-based method with the latter giving the most sensitive result showing a calculated LOD of 1.29×10^4 CFU/mL. At the same concentration of antibody, the monoclonal preparation gave very poor absorbance value that it could not be used for the detection of this pathogen under both indirect and sandwich assay formats. The results of the specificity test obtained from the developed polyclonal antibody against other bacterial genera such as *S. Typhimurium*, *L. monocytogenes* and *E. coli* tested using the polyclonal

preparation based on the sandwich ELISA method indicated that the responses by another bacterial genus were relatively low. This indicated that the developed system is highly specific towards *C. jejuni* and should be used in the future in place of monoclonal preparations for commercial development of detection kits.

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