Immunological Methods for the Detection of *Campylobacter* spp. – Current Applications and Potential Use in Biosensors

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1. Introduction

The genus *Campylobacter* belongs to the epsilon division of the class Proteobacteria. This genus comprises a group of bacteria that occupy diverse habitats and produce a wide range of diseases in different animal hosts (On 2001). Campylobacteriosis is a self-limited gastrointestinal illness that produces diarrhea, fever and abdominal cramps, and antimicrobial therapy is not generally indicated. However, treatment can reduce the duration and severity of illness if caught early, especially in those with the potential for severe illness, including infants, elderly, patients with underlying disease and immunocompromised individuals. In addition to acute enteritis, campylobacteriosis may result in reactive arthritis and Guillain-Barre syndrome (Hannu et al., 2002; Rees et al., 1995). The epidemiology of campylobacteriosis is unique in that most infections are sporadic. Meats, especially undercooked broiler chicken, are the main source of sporadic campylobacteriosis cases, while outbreaks are usually associated with the consumption of raw milk or unchlorinated water (Skirrow 1991). *Campylobacter* spp. are one of the leading agents of bacterial foodborne diseases worldwide (EFSA 2005, 2006). In the USA, there are approximately 12 reported cases per 100,000 persons per year, although the actual number has been estimated at 432 cases per 100,000 (Olson et al., 2008; Samuel et al., 2004). *C. jejuni* is responsible for approximately 95% of the human cases, while *C. coli* is responsible for the rest of the infections. *C. concisus*, *C. fetus*, *C. upsaliensis* and other *Campylobacter* species can cause sporadic cases.

Current methods of identification of campylobacters in stool and food samples rely on their growth on selective agar plates with and without prior broth enrichment. These methods are labor intensive and time consuming, taking four or more days for completion and require robust bacterial growth, which may limit the detection of stressed bacteria that do not grow well but are still infective. More rapid and accurate methods are necessary to identify campylobacters in stool samples in order to treat campylobacteriosis early, and to detect outbreaks as campylobacteriosis is a notifiable disease in the USA. Moreover, rapid detection of campylobacters in environmental samples is important for trace-back investigations to mitigate outbreaks and in food samples to catch contamination during commercial food processing.

During the 1960s and early 1970s, the search for alternative techniques to replace radioactive label reporters to identify biological molecules led to the development of the enzyme-linked immunosorbent assay (ELISA or EIA; Engvall and Perlmann 1971). In the mid-1970s the, the development of the hybridoma technique for monoclonal antibody synthesis (Köhler & Milstein 1975) helped expand the new field of immunodiagnosis and initiate development of immunoassays for the identification of bacterial pathogens. Since then, a variety of immunoassays have been developed for the detection of different foodborne pathogens in food and stool samples, including a few immunoassays for *Campylobacter* spp. detection. The simplest is latex agglutination, in which antibody-coated colored latex beads or colloidal gold particles are used for rapid confirmation of culture results or serotyping of culture isolates. The EIA, the most popular immunoassay used for pathogen detection in foods and stool, is typically designed as a "sandwich" assay, in which an antibody bound to a solid matrix is used to capture the bacterium and a second antibody conjugated to an enzyme then binds to the bacterium. Multi-well microplates are a commonly used solid support but other formats include dipsticks, paddles, membranes, or other solid matrices.

The lateral flow immunochromatographic method is a modified EIA, packaged in a simple device (dipstick or within a plastic casing) and used for rapid pathogen detection. Typically, "sandwich" assays are used for large analytes such as bacteria. Samples migrate from the sample pad through a conjugate pad where the target analyte binds to the antibody conjugated to colored particles. The sample is drawn across the membrane to the capture zone where the target/conjugate complex binds to immobilized antibodies producing a visible line on the membrane. To ensure a working test, the sample migrates further until it reaches the control zone, where excess conjugate is bound to produce a second visible line on the membrane. Two clear lines on the membrane are a positive result. A single line in the control zone is a negative result. Lateral flow immunoassays have many advantages including their simplicity, production of a result within 15 minutes, stability with a long shelf life even in some cases without refrigeration and their low cost, but they may have a higher threshold of detection compared to EIA.

The present chapter reviews commercial and/or published immunological methods, mainly EIA and lateral flow immunoassays used to identify species of *Campylobacter* in food and stool samples. Methods that can be dovetailed with immunoassays to increase bacterial concentration and immunoassay detection, such as sample enrichment, sample filtration and immunomagnetic separation, are described. The strengths and limitations of immunoassays for identification of *Campylobacter* spp. are reviewed, along with suggestions to improve assay performance. The detection of *Campylobacter* spp. by antibody-based biosensors, primarily optical biosensors is also briefly discussed.

2. Identification of *Campylobacter* spp. using immunoassays

The presence of campylobacters in food and stool samples is based on the growth of the bacteria on a selective agar, incubated at 42°C under a reduced oxygen atmosphere (~5% O_2). The limitations of the culture method, especially the need for up to four days to identify campylobacters (Endtz 2000), have dictated the development of culture-independent methods, including immunoassays, for the detection of *Campylobacter* spp. in clinical stool and food samples. Although a variety of immunoassays have been developed for testing clinical and food samples for *Campylobacter* spp., these assays require approval by regulatory bodies, necessitating comparison of immunoassays to culture-based methods,

considered the "reference methods." The validation of immunoassays for detection of *Campylobacter* spp. includes testing for **inclusivity**, to assure that different strains of *C. jejuni* and *C. coli* are detected, and **exclusivity**, to assure that closely related, non-target bacteria do not cross-react with the assay (Brunelle 2008). Other performance indicators used to validate assays include:

- Sensitivity: The probability that an assay will yield a positive result when the culture is positive by the reference method.
- Specificity: The probability that an assay will yield a negative result when the sample is negative by the reference method.
- Positive predictive value: The probability that a sample with a positive test contains the bacteria.
- Negative predictive value: The probability that a sample with a negative test does not contain the bacteria.

In the following sections, commercial immunoassays available to detect *Campylobacter* spp. in stool and food samples will be described.

2.1 Clinical stool samples

Several commercial immunological assays are available for identification of *C. jejuni* and *C. coli* in stool samples, including some designed to confirm culture results and others that are culture-independent. Assay formats include latex agglutination, EIAs, and lateral flow formats. Immunoassays based on latex agglutination, developed in the late 1980s, are of limited use because they can only confirm culture results, and may detect closely-related organisms (Haymann 2004). Several EIAs are commercially available for use directly with clinical stool samples, and in some studies have performed as good or better than the standard culture techniques for detecting *C. jejuni* and *C. coli*, and possibly *C. upsaliensis*, and are comparable to nucleic acid tests (Abubakar et al., 2007) but not in all cases. Of the four EIAs commercially available in the USA for direct detection of *Campylobacter* in stool specimens, two are microplate-based and two are incorporated in lateral flow devices (Table 1). These methods are reasonably rapid, from 20 minutes for the lateral flow assays to 2-4 h for the microplate assays, and identify specific *Campylobacter* antigens common to *C. jejuni* and *C. coli*.

The ProSpecT™ Campylobacter assay (Remel Inc., Lenexa, KS), a microplate sandwich EIA that uses a polyclonal antibody conjugated to horseradish peroxidase for the detection of common antigen of *C. jejuni* and *C. coli* in fecal specimens and enriched fecal cultures, has received the most scrutiny. The results are read visually or spectrophotometrically, and the analytical sensitivity is approximately 10^{5-6} colony forming units (CFU) per ml^{-1}. The test is accurate for samples stored at 4°C for several days (Dediste et al., 2003; Tolcin et al., 2000). No cross-reactivity with other major fecal bacteria or with other *Campylobacter* spp., including *C. lari* and *C. fetus*, has been identified (Endtz et al., 2000; Hindiyeh et al., 2000). The manufacturer's studies, using three sites in the USA and Canada, demonstrated a pooled sensitivity of 97-100% and specificity of 98-100% using 1,049 stool samples (Table 1). A meta-analysis of the clinical utility of the ProSpecT assay in relationship to standard culture-based methods (Abubakar et al., 2007) included four studies (Dediste et al., 2003;

Endtz et al., 2000; Hindiyeh et al., 2000; Tolcin et al., 2000) that were chosen using QUADAS, an evidence-based tool for the quality assessment of diagnostic studies (Whiting et al., 2003) (Table 1). For the pooled samples (n=2078), the specificity was 98% (95% CI: 89-100%) while the sensitivity was 89% (95% CI: 81-98%). Although the number of false positives was low,

Test Name	Sample Size (% Positive)	Sensitivity (95% CI)	Specificity (95% CI)	Positive Predictive Value	Negative Predictive Value	Source
ProSpecT™ Campylobacter Microplate (Remel, Lenexa, KS)	1049	100 (97-100)	99 (98-100)	-	-	Manufacturer
	164 (30%)	96 (87-99)	99 (95-100)	9	98	Tolcin et al., 2000
	78 (38%)	80 (62-91)	100 (93-100)	100	89	Endtz et al., 2000
	631 (3%)	89 (67-97)	99 (98-100)	80	99	Hindiyeh et al., 2000
	1205 (8.4%)	89 (82-94)	98 (97-99)	78	99	Dediste et al., 2003
	182 (34%)	95 (88-98)	94 (84-98)	96 (90, 99)	91 (81,96)	Tribble et al., 2008
	485	99	96	90	99.7	Granato et al., 2010
ImmunoCard STAT!® CAMPY (Meridian Bioscience, Inc., Cincinnati, OH)	420	98 (90-100)	95.9 (93-98)	-	-	Manufacturer
	485	98	94	89.4	99.7	Granato et al., 2010
	242 (9.5%)	90	-	70	-	Bessede et al., 2011
Premier CAMPY EIA (Meridian Bioscience)	2073	97 (89-99)	96 (95-96)	-	-	Manufacturer
	485(6%)	99.2	96	90	99.7	Granato et al., 2010*
	242 (9.5%)	90-95	-	~80	-	Bessede et al., 2011
RIDASCREEN® (R-Biopharm, Darmstadt, Germany)	259	100	99.6	93	100	Manufacturer
	1050 (9.3%)	69	87	36	97	Tissari et al., 2007
	242 (9.5%)	90	-	~89	-	Bessede et al., 2011

Table 1. Performance of selected, commercial kits for direct detection of *C. jejuni* and *C. coli* in stool samples

the positive predictive values ranged from 78-100%, indicating a possible unacceptable number of false negative results especially when screening samples with a low level of contamination. Indeed, a later study showed that when a population has high prevalence of campylobacteriosis, the ProSpecT EIA is sensitive and specific (Tribble et al., 2008). In another study, culture-based methods were less sensitive than PCR and the ProSpecT assay (Granato et al., 2010).

The PREMIER™ CAMPY microplate EIA (Meridian Bioscience, Inc, Cincinnati, OH) uses a monoclonal antibody that binds to an unspecified common antigen of *C. jejuni* and *C. coli*. The limit of detection is 10^{6-7} CFU per ml^{-1}, and according to the manufacturer's literature, this assay had a sensitivity of 97% and specificity of 96% in a study of 2,073 samples (Table 1). High specificity and sensitivity for this EIA were reported by Granato et al. (2010). Another microplate EIA, Ridascreen Campylobacter (R-Biopharm AG, Germany), also uses monoclonal antibodies but does not appear to perform as well as the manufacturer's claims (Bessede et al., 2011; Tissari et al., 2007). The lateral flow assay, ImmunoCard STAT! CAMPY (Meridian Bioscience, Inc.), uses the same monoclonal antibody as the PREMIER CAMPY assay. The capturing of the target bacteria is by an antibody colloidal complex and the assay has similar specificity to the EIA assays, but with a sensitivity of 10^7 CFU per ml^{-1} (Bessede et al., 2011; Granato et al., 2010). The Xpect *Campylobacter* assay (Remel Inc.) is a rapid assay equivalent to the ImmunoCard STAT! CAMPY assay, but preliminary results suggest that it has poor sensitivity (Fitzgerald et al., 2011).

Currently, none of the described assays can be recommended for standalone identification of campylobacters in stool samples, in part because of the limited and conflicting findings regarding the performance of these immunoassays. The performance of these EIAs is variable and suggests a high probability of non-acceptable levels of false negatives in certain situations. The Centers for Disease Control and Prevention (CDC) has recently released preliminary data concluding that EIAs should not be used as standalone tests for direct detection of *Campylobacter* in stool samples. This study investigated 2,767 stool samples with a positive *Campylobacter* prevalence of approximately 3%. Although the specificity and negative predictive values in these tests were excellent, typically >95%, the sensitivities and positive predictive values (PPV) of the four EIAs were not acceptable. For example, the ProSpecT™ Campylobacter assay exhibited a sensitivity of 84% and a PPV of 56%, the PREMIER™ CAMPY assay exhibited a sensitivity of 83% and a PPV of 52%, the ImmunoCard STAT! CAMPY exhibited a sensitivity of 73% and a PPV of 39%, and the Xpect *Campylobacter* assay exhibited a sensitivity of 74% and a PPV of 80%. Therefore, a positive EIA test alone is not sufficient to consider a case "confirmed" and laboratories must confirm positive EIA results by culture methods (Fitzgerald et al., 2011). The basis for discordant results between culture, immunoassay and PCR-based methods needs to be determined.

It is not clear whether the poor performance of these EIAs is inherent in the assays themselves or is due to lack of optimization. The fact that variations appear to be dependent on the test format and manufacturer suggests that these assays could be improved (See Section 4). A limitation for the adoption of EIAs is the prohibitive cost of adopting these rapid tests in combination with routine culture methods (Abubakar et al., 2007).

2.2 Food samples

Current identification methods for *Campylobacter* in foods rely on bacterial growth on one of a variety of selective agar plates which contain antimicrobials to allow for the growth of the target organism, a method adapted from that used for stool samples (Corry et al., 1995). To achieve the required sensitivity of 1 cell per 25 g food, a 25 g food sample is typically enriched in a broth with selective agents to inhibit the growth of competing bacteria while allowing growth of the target organism before plating on selective agar (Oyarzabal 2005; Oyarzabal et al., 2007). Besides increasing bacterial concentration, enrichment is important because campylobacters are randomly distributed and can be present in clumps or aggregates in foods, limiting direct detection. Also, enrichment ensures that stressed or injured bacteria have a chance to recover prior to plating on selective agars. Culture-based methods are the accepted methods outlined in the Food and Drug Administration (FDA)'s Bacteriological Analytical Manual (BAM), the Microbiology Laboratory Guidebook (MLG) of the Food Safety and Inspection Services of the U.S. Department of Agriculture (FSIS USDA) and the International Organization for Standardization (ISO).

These culture-based methods for food screening suffer from the same constraints as those for stool samples: low specificity resulting in false positives, and long lag-time for results (Josefsen et al., 2011). Until recently, the lack of active surveillance and regulatory efforts to control *Campylobacter* spp. in poultry meat, a common vehicle for these pathogens (FSIS USDA 2009a), delayed the development of rapid methods. However, a new performance standard aimed at limiting the prevalence of *Campylobacter*-contaminated poultry meat products in the USA requires the screening of processed carcasses for the presence of this pathogen (FSIS USDA 2009b). Thus, there is a critical need to develop and validate rapid methods for *Campylobacter* identification, including those that are antibody-based.

Although antibody-based methods would speed assay time, only a few latex agglutination assays are included in reference manuals and only for confirmation of positive *Campylobacter* isolates. The FDA's BAM (Hunt et al., 2001), suggests the use of Dryspot *Campylobacter* Test (Oxoid, Basingstoke, Hampshire, England) or Alert for *Campylobacter* (Neogen Corporation, Lansing, MI), but neither are available. The MLG of the FSIS USDA recommends the use of Campy (jcl) (Scimedx Corp., Denville, NJ) or Microgen *Campylobacter* Rapid Test (Microbiology International, Frederick, MD) for confirmation of presumptive isolates (MLG 2011). Commercial EIAs are available for culture-independent identification of campylobacters in food, but these assays have not been extensively validated. Typically applied to enriched cultures, these EIAs reduce assay time and may perform with equal specificity as culture methods, albeit with a sensitivity $> 10^4$ CFU per ml^{-1}, which is not an improvement over culture methods (Josefsen et al., 2011). Presently, a positive result by a rapid method is only regarded as a presumptive result and must be confirmed by a standard method (Hunt et al., 2001; ISO 2006; MLG 2011). The next paragraphs describe the most common commercial EIAs used with food samples.

The VIDAS/MiniVIDAS CAM (bioMerieux, Hazelwood, MO), an automated EIA for detection of thermotolerant *C. jejuni, C. coli* and *C. lari*, has received the most attention over the last decade. In this assay, food or stool samples are incubated in an enrichment broth for 48 h. After enrichment, samples are boiled for 15 minutes and aliquots are analyzed by an automated enzyme immunoanalyzer. The few published studies indicate that this method performs similarly to culture methods for the identification of naturally occurring

Campylobacter spp. in foods. Hoorfar et al. (1999) found that the MiniVIDAS CAM may provide a faster method for fecal samples from cattle and pigs with the capability of rapid screening of a large number of samples. The reported sensitivity of this assay ranged from 88-94%, which may not be adequate for screening samples with low numbers of *Campylobacter* spp. However, authors used a lower enrichment time (24 h) compared to the manufacturer's recommendation (48 h).

Borck et al. (2002) compared the MiniVIDAS and the EIAFoss, an automated EIA which is no longer available, to culture methods for the identification of *Campylobacter* spp. The reported sensitivity for both EIA assays was higher than the sensitivity of selective agar plates for a variety of turkey meat and turkey fecal samples, but sensitivity varied as a function of sample and enrichment broth. The MiniVIDAS exhibited a sensitivity of approximately 94% for fecal samples with high bacterial loads and 65% for environmental samples with lower bacterial loads, and each methods had a specificity above 93%. Paulsen et el. (2005) has also indicated that the MiniVIDAS is as good as the standard method, with a sensitivity of 97.6% and a reduction in assay time by 24 h for chilled and frozen meat. In this study, Bolton enrichment broth in modified stomacher bags was better than Preston broth for enrichment prior to immunoassay. The MiniVIDAS CAM method has been successful for the detection of *Campylobacter* spp. in tissue samples (tonsils and lymph nodes) and fecal material from pigs during processing (Nesbakken et al., 2003), in a variety of chicken parts with positive results confirmed by culture methods (Reiter et al., 2005), and in artificially-contaminated ground beef and fresh cut vegetables (Chon et al., 2011). Our experiences (OAO) with the MiniVIDAS for screening poultry meat are in accordance with the majority of reports mentioned above. However, the enrichment of the samples for 48 h is indispensable to reduce the high number of false negative samples that otherwise will be encountered (Liu et al., 2009).

Other commercial immunoassays have received less scrutiny than the MiniVIDAS CAM. The TECRA Campylobacter Visual Immunoassay (3M, St. Paul, MN) for *C. jejuni* and *C. coli* employs a 40-h aerobic enrichment in a proprietary broth followed by a 2-h EIA. For 398 broiler carcass rinses from 19 processing plants, TECRA found 317 *Campylobacter* positives, four false positives and 22 false negatives, compared to 48 false negatives with the reference method (Bailey et al., 2008). All these false negatives came from rinses of carcasses collected toward the end of the production process, suggesting that severely injured campylobacters may not recover well in the TECRA enrichment broth. In another study, the TECRA VIA was less sensitive than a PCR method for detection of campylobacters in spiked raw and processed meat and poultry, but performed similarly or better than traditional culture media (Bohaychuk 2005). The low number of false negatives indicates that the TECRA VIA may be a viable screening tool as long as follow-up tests are performed on presumptive positive samples to rule out false positives. Unfortunately, no literature was found describing the antibody target and improvements might be possible with a better choice of antibody because, in the same study, an immunoassay outperformed PCR for detection of *Salmonella*.

Two other commercially available assays for rapid testing of food samples are the Singlepath™ *Campylobacter* microwell sandwich EIA (Merck KGaA, Germany) and the NH Immunochromato *Campylobacter* (Cosmo Bio Co., Tokyo, Japan). The Singlepath was comparable to standard culture methods in a preliminary study with artificially-inoculated

meat (Hochel et al., 2004). The NH Immunochromato *Campylobacter* is a two-step enrichment assay using immunochromatographic identification with a reported sensitivity of 55 CFU per 25 g of spiked chicken meat for non-freezer stressed samples. Freezing samples decreased the sensitivity approximately 10-folds (Kawatsu et al., 2010).

Based upon the studies described above, commercial immunoassays show promise as one of the tools to speed food sample screening. However, more studies of these assays with naturally-contaminated samples from a variety of foods is necessary. The effect of bacterial stress that occurs during food processing and storage on assay performance should also be studied. Additionally, the antigen targets of the antibodies used in these assays need to be identified. A concerted effort to improve the performance of these assays should be undertaken now that a new standard for food screening poultry meat for *Campylobacter* spp. has been established in the USA.

3. Increasing concentration of campylobacters prior to immunoassay identification

Because EIA assays have a sensitivity of $\geq 10^4$ CFU per ml^{-1} or g^{-1}, there is a need to increase the number of the target organisms in food samples prior to assay. The enrichment of the sample is the most common method to increase the number of bacterial cells. No enrichment protocol is 100% selective for the organism of concern; therefore other methods are used to separate or concentrate the target bacteria from the rest of the contaminants. This section will review enrichment broths, filtration, and immunomagnetic separation as techniques to increase the number of *Campylobacter* spp. in the food samples prior to identification by EIA.

3.1 Enrichment of the samples

The enrichment of food samples is imperative for the isolation of *Campylobacter* spp. from poultry or raw milk products. The most commonly used enrichment broth is Bolton broth, which has a basal component made up of meat peptone, lactalbumin hydrolysate, yeast extract, alphaketoglutaric acid, ssodium chloride, sodium pyruvate, metabisulphite and carbonate. The use of buffered peptone water performs similarly to Bolton broth for the isolation *Campylobacter* spp., suggesting that the basal medium does not need to be rich in nutrients to support the growth and multiplication of *Campylobacter* spp. (Oyarzabal et al., 2007). An important requirement is the incubation of food samples for at least 48 h in enrichment broth before identification of positive cultures by immunoassay because 24 h incubation results in a high number of false negative samples (Liu et al., 2009).

The antimicrobials added to the basal broth are cefoperazone, trimethoprim and vancomycin, at concentrations of 20 mg per L^{-1} each, and cycloheximide, at a concentration of 50 mg per L^{-1}. Originally, Bolton broth was supplemented with 5% lysed horse blood, but research has shown that the addition of blood is not necessary for isolation of *Campylobacter* spp. from retail broiler meat. Most importantly, blood in the enrichment broth is not necessary if an EIA-based method is employed to detect positive samples (Liu et al., 2009). In the laboratory of one of the authors (OAO), a modification of Bolton broth was made by reducing the antimicrobials added to enrichment and plate media to only cefoperazone, at a concentration of 32 mg per L^{-1}, and amphotericin B at a concentration of 2 to 10 mg per L^{-1} (Liu et al., 2009; Zhou et al., 2011). Although this reduction of antimicrobials may allow for contaminants to grow, we control those contaminants by filtration prior to assay.

3.2 Use of filter membranes to separate contaminating bacteria

Filter membranes were used for the first isolation of *C. jejuni* from human stools (Dekeyser et al., 1972). In this report, the use of 0.65-μm filters was aimed at retaining most of the contaminating bacteria in fecal suspensions while allowing *Campylobacter* organisms to pass through the filter for isolation on agar plates. This differential filtration is different from most of the other filtration protocols in which the target organisms are concentrated by filter retention for subsequent identification. A search for different agar plates with more antimicrobials to control contaminants was initiated in the 1970s and by the early 1990s the filters were not used anymore, except for the isolation of less-known species of *Campylobacter* by Albert Lastovica using the Cape Town protocol (Le Roux & Lastovica 1998; Lastovica & Le Roux 2003). Although filters may not help isolate *Campylobacter* spp. from samples with low number of cells such as water samples (Diergaardt et al., 2003), filters help isolate *Campylobacter* spp. from highly contaminated samples. In enriched samples, the high motility of *Campylobacter* spp. allows for a relatively low number of cells to go through membrane filters and grow as pure colonies on plate media. Thus, filters are highly sensitive for the isolation of naturally occurring *Campylobacter* spp. present on retail broiler meat (Speegle et al., 2009).

A filtration method coupled with a sandwich EIA that uses polymyxin B sulfate (PMB) instead of an antibody to capture *C. jejuni* and *C. coli* was developed by one of the authors (CAB). PMB is used to capture Gram-negative bacteria for EIA detection of *Escherichia coli* and *Salmonella* in a variety of foods (Blais 2005, 2006). PMB binds to lipid A of the lipooligosaccharide (LOS) layer and improves bacterial capture, although treatment of bacteria with zwitterionic detergents is necessary (Blais 2005, 2006; Brooks et al., 1998). In our experiments, various surface waters and tap water were filter sterilized prior to use, and samples of each were spiked with concentrations of 0 to 10^4 CFU ml^{-1} of *Campylobacter* cells. Aliquots (500 ml) were filtered using a conventional EPA filter system with Microfil V 0.2 µm filters and then filters were added to 1 ml phosphate-buffered saline containing CHAPS detergent (3-[(3-cholamidopropyl dimethylammonio]-1-propanesulfonate) with 1-2 minute vortexing. Aliquots of 100 µl were assayed in the PMB-capture ELISA. Sampling (100 µl) of the 500 ml prior to filtration did not yield values above background. Thus, filter concentration coupled with PMB-capture ELISA was able to detect 10^2 CFUml^{-1}, compared to the typical threshold of 10^{4-5} CFU per ml^{-1}, although the net fluorescence intensity was much lower for ocean water (Fig. 1).

3.3 Concentration by immunomagnetic separation

Immunomagnetic separation utilizes magnetic spheres, such as Dynabeads™ (Dynal Biotech), coated with antibodies to capture target bacteria from a variety of matrices. Antibody-bound target cells are separated from the matrix and other bacteria by application of a magnetic field. However, pre-enrichment may still be necessary to obtain a high number of bacteria for detection. Immunocapture followed by plating on selective agar had a threshold of 10^4 CFU per g^{-1} for the detection of *Campylobacter* in ground poultry meat (Yu et al., 2001). The sensitivity did not improve when magnetic beads (Dynabeads™) were coated with a monoclonal antibody to the major 45 KDa porin and coupled with a DNA hybridization assay specific for the 23S rRNA gene of *Campylobacter* spp. (Lamoureux et al., 1997).
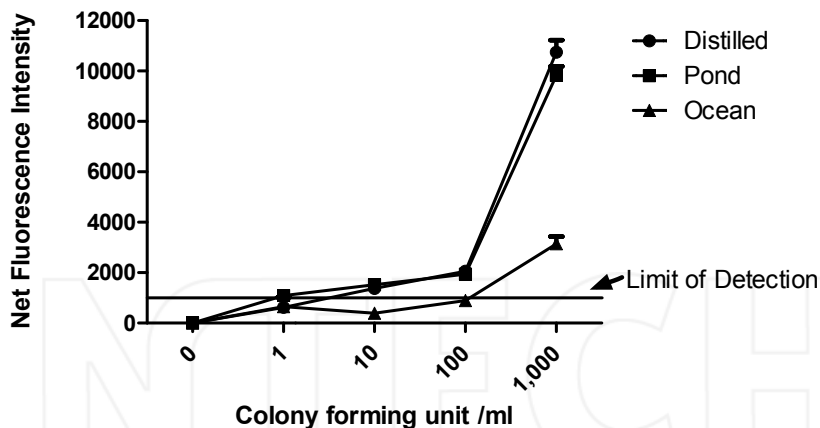
Fig. 1. Capture of *C. jejuni* on Microfil V filters prior to polymyxin B EIA detection. Various water source samples (500 ml) were spiked with different concentrations of *C. jejuni* and then filtered. Net fluorescence intensity was obtained by subtraction of background. Means ± standard error of the means (n= 6 replicates) are plotted as a function of original bacterial concentration.

A modification of immunomagnetic capture uses multiplexed magnetic microspheres, fluorescence-coded microspheres coated with antibodies, to detect bacteria by flow cytometry (MagPlex® Microspheres, Luminex, Austin, TX). Although this technique allows for rapid, multiplexed assays, the desired limit of detection is still a challenge for a variety of food matrices (Kim et al., 2010).

4. Improving antibody-based methods for *Campylobacter* spp. detection

One of the limitations of antibody-based methods for detection of pathogenic bacteria is the high threshold of detection ($\sim 10^{4-6}$ CFUml^{-1}), which results in unacceptable numbers of false negative samples especially for foods (Hochel et al., 2007). The detection limit is not as problematic for rapid detection in stool samples due to the high concentration of campylobacters in fecal samples. As discussed above, bacterial concentrations can be increased to EIA-detectable levels in food, such as retail broiler meats, by broth enrichment of up to 48 h (Liu et al., 2009), although components of enrichment broths may reduce immunoassay sensitivity (Chon et al., 2011). Improvements in EIAs such as reducing background noise or increasing the amplitude of the detection signal can improve performance. Fluorescence-based signaling improves the detection threshold by at least an order of magnitude over colorimetric assays, and the use of biosensors may allow for more efficient signaling to improve sensitivity. If sample matrices reduce EIA performance by increasing background signal, a variety of polymers, such as polyvinylpyrrolidone (Nyquist-Battie 2004) and Biolipidure^{tm} (http://www.biolipidure.com/) can reduce background noise thereby increasing the signal to noise ratio. Another factor that may improve immunoassay performance is the molecular structure of the antibodies. F(ab')$_2$ fragments, or single chain Fv recombinant antibodies because they are smaller resulting in less steric hindrance between antibody molecules, although this premise needs to be investigated.

A possible reason for the high rate of false negatives in EIAs used to detect *Campylobacter* spp. may be the choice of antibody (Hochel et al., 2007; Rice et al., 1996). Some antibodies may not be able to detect all strains of *C. jejuni* and *C. coli*. Antigenic variation is common for externally-exposed molecule, such as outer membrane proteins and LOS, which limits the inclusivity of generic antibodies (Logan & Trust, 1983; Taylor & Chang, 1987, Dubreuil et al., 1990; Hochel et al., 2004). Indeed, surface- exposed immunodominant molecules, such as flagellar proteins (Fernando 2007) and the O-antigen, undergo genetic drift to avoid immunological detection. An alternative target molecule is the core oligosaccharide of the LOS, which has been successfully targeted in EIA assays to detect *C. fetus* (Brooks 2002, 2004; Devenish 2005). High rates of continuous expression of the antigen is another important consideration, especially in *Campylobacter* cells that undergo stress during food production and processing. Detection of stressed bacteria by EIA has been shown to be reduced (Hahm & Bhunia 2006; Nyquist-Battie 2005). In this regard, the structure of the LOS, a target of many antibodies, may vary as a function of the temperature at which *Campylobacter* cells are grown (Semchenko et al., 2010). Thus, identifying stable surface-exposed molecules for detection of *C. jejuni* and *C. coli* could be an avenue to improve antibody-based detection of these pathogens.

Improvements in proteomics over the last decade have made the identification of surface molecules easier (Cordwell et al., 2008; Prokhorova et al., 2006). Once ideal surface molecules are identified, newer methods of antibody production, such as the use of recombinant proteins, peptide fragments or DNA plasmids as immunogens, should be explored. For example, identifying epitopes that are stable but specific for *C. jejuni* in FlaA protein could be undertaken (Fernando 2007), and then peptide fragments with these epitopic sequences could be used to generate antibodies. If an ideal molecular target cannot be identified, it may be necessary to use antibody cocktails to increase the inclusivity of immunoassays (Hochel et al., 2007; Rice et al., 1996).

4.1 Polyclonal versus monoclonal antibodies for *C. jejuni* and *C. coli* detection

Several polyclonal antibodies have been developed for the detection of *C. jejuni* and *C. coli*, but few have been thoroughly tested for the ability to identify most if not all strains. In one study, nine strains were screened using two commercial anti-*Campylobacter* antibodies, B6601R (Biodesign, Saco ME) and 01-92-93 (Kirkegaard and Perry Laboratories; KPL, Gathesburg, MD). Only the former antibody identified all nine *Campylobacter* strains (Wang et al., 2000). Recently, we screened polyclonal antibodies for detection of *C. jejuni* and *C. coli* using a non-sandwich indirect fluorescence ELISA. The KPL antibody 01-92-93, a rabbit anti-*Campylobacter jejuni* C1037-10 (US Biological B10), and C1037-14 (USB14) antibodies recognized all four strains of *C. coli* and all nine strains of *C. jejuni* with a limit of detection of approximately 10^6 CFU per ml^{-1} (unpublished results). The rabbit anti-*Campylobacter* spp. antibody 9-25B-PA (Cygnus Tech.) did not recognize all strains. Given the successful detection of a variety of *C. jejuni* and *C. coli* strains by the USB polyclonal antibodies and the need to determine useful antigenic targets, we performed Western blotting with two *C. jejuni* isolates (ADPH1608 and ADPH 1208, both human isolates). The USB antibodies did not bind to LOS epitopes, as Proteinase K digestion eliminated all bands. Next the surface proteins that are recognized by the USB antibodies were determined by biotinylating surface proteins of the bacteria using a non-membrane penetrant biotinylation agent, EZ-

Link Sulfo-NHS-LC-Biotin, at 5 mg per ml-1 for 1 h at room temperature, a modification of the method of Harding et al. (2007). Membrane proteins were extracted using two membrane protein isolation kits (G Bioscience Focus™ Membrane Protein Extraction kit and BioRad Ready Prep sequential extraction kit). Seven protein bands with the molecular weights of 73, 62, 43, 41, 35, 28, and 24 KDa were both biotinylated and recognized by the USB antibodies using both protein extraction kits. The molecular weight of these bands is similar to many proteins described previously as useful antibody targets for detection of campylobacters. A 62 KDa protein, possibly one of the flagellin proteins, has been mentioned as a useful by previous authors, target for campylobacter detection (Heo et al., 2009; Lu et al., 1997) and is one of the protein targets of the KPL polyclonal antibody (Rice et al., 1996). Lu et al. (1997) demonstrated that a monoclonal antibody to a62 KDa protein was able to detect campylobacters at concentrations of 10^5 CFU per ml-1 with great specificity. The 43 Kda protein is another protein that is recognized by *Campylobacter* antibodies such as the KPL antibody (Rice et al., 1996). This 43 KDa protein, bound by a monoclonal antibody developed after injecting mice with whole cell *C. coli,* was determined by MALDI-TOF mass spectrometry to be a major outer membrane protein (Qian et al., 2008a). This antibody was shown to bind to the cell surface using immunogold electron microscopy and detected 10^3 to 10^4 CFU ml-1. A recombinant major outer membrane protein (43 KDa) was used to generate a monoclonal antibody that was specific for *C. jejuni* (Qian et al., 2008b). This antibody recognized an amino acid epitope which is 97% conserved but which is predicted to be exposed to the periplasm not the cell surface. The 28 KDa protein may correspond to PEB1, a surface exposed adhesion protein that is the basis of an ELISA kit (Pei et al., 1991). Antibodies to this protein were able to detect 35 *C. jejuni* and 15 *C. coli* isolates without cross-reactivity (Pei et al., 1991). Taken all together, the 62, 43, 28 KDa proteins may be useful target molecules for *Campylobacter* immunoassays and deserve further scrutiny.

Several monoclonal antibodies have been synthesized for *Campylobacter* detection. Monoclonal antibodies target a single epitope of an antigen, which may limit their use in detection of *Campylobacter* because the epitope may not be conserved across all *Campylobacter* species and strains (Rice et al., 1996), although they exhibit greater specificity than polyclonal antibodies (Velusamy et al., 2010). Indeed, Wang et al. (2000) reported a lack of success with the following monoclonal antibodies: 1744-9029 and 1744-9006 (Biogenesis Ltd, NH), MAB001 (Harlan Sera Lab, GB), and C65701M (Biodesign International, MD). Our experiences with different monoclonal antibodies (AbD Serotec monoclonal antibodies 1745-00, 1744-8508, 1744-9059, and 1744-9109; USB C1037-02A, C1037-04, and C1037-16) support the premise that monoclonal antibodies are not ideal for ELISA detection of *Campylobacter* spp. (data not published). In contrast, the monoclonal antibody to the 62 KDa protein, discussed above, was able to detect whole cells at concentrations of 10^5 CFU pe ml-1 with excellent specificity (Lu et al., 1997). Also the monoclonal antibody to the 43 KDa protein was specific for *C. coli*, bound to the cell surface, and detected 10^3 to 10^4 CFU per ml-1 (Qian et al., 2008a). However, it is not clear whether these antibodies bind to conserved epitopes expressed under different conditions.

4.2 Capture of *C. jejuni* and *C. coli* in EIAs

The retention of bacteria may be inefficient in EIAs without the use of a capture element such as an antibody as in sandwich EIAs (Hochel et al., 2007) and thus bacteria are typically

captured by an antibody in commercial kits. The number of capture antibodies that attach to a microplate may be improved by using biotin-labelled antibodies that can attach to streptavidin coated plates. The use of microplates with bottom filters in conjunction with antibody-capture, such as multi-well filter plates (AcroWell, Pall Corp.), or hydrophobic grid membrane filters may improve capture of antibody-bacterial complexes (Tsai & Slavik 1994; Wang et al., 2000). Another approach is the colony-lift immunoassay. After bacterial colonies are grown on select agar plates, a membrane filter lifts bacteria from the plate for immunoassay. When coupled with an anti-goat polyclonal antibody, the assay was specific and detected all tests strains of *C. jejuni*, *C. coli* and *C. lari* in 18-28 h (Rice et al., 1996). However, field testing of this method has not been published to the best of our knowledge.

Novel capture molecules such as polymyxin B sulfate (PMB), which binds to lipid A of the LOS may improve bacterial capture. The limitation of this method is that treatment of bacteria with zwitterionic detergents is necessary (Blais 2005, 2006; Brooks et al., 1998). Fukuda et al. (2005) demonstrated that PMB capture was superior to antibody capture for detection of *Salmonella* in chicken. Our laboratory demonstrated that PMB-capture enhances ELISA detection of *C. jejuni* as indicated by a 10-fold improvement in the limits of detection with both the USB10 and USB14 antibodies (unpublished results). The PMB-capture ELISA with either of these antibodies detected 19 *C. jejuni* isolates obtained from a variety of sources, including human, pork, turkey and chicken. The minimum cell concentration yielding a positive assay signal was approximately 10^4-10^5 CFU per ml^{-1}, and fluorescence intensity values at 10^6 CFU per ml^{-1} were similar for all isolates with no discernable difference between the two USB antibodies (Fig. 2). The mean signal to noise ratios for the 19 isolates were 16 ± 1 (USB10) and 17±2 (USB14) at 10^6 CFU per ml^{-1}, while the signal to noise ratios for the other Gram-negative bacteria screened at 10^7 CFU per ml^{-1} were below 2.5 except for *H. pylori* (Table 2). Therefore, the assay is specific for *Campylobacter*.

5. Potential use of antibodies in biosensors

Antibodies have the potential to be used in biosensors for the detection of specific bacterial pathogens. Biosensors are defined as analytical devices that combine a bioreceptor, which is a biological or biologically derived element, with a physicochemical transducer (Turner & Newman 2005). The term *immunosensor* describes a sensor that uses antibodies as the sensing element. When antibodies are used in bisosensors, they are the bioreceptors, capturing or sensing elements, and therefore the advantages and limitations in sensitivity and specificity of traditional antibody-based detection methods apply to these biosensors.

The transducers in biosensors are the components in charge of converting the biorecognition event, which happens when antibodies associate to the antigen, into signal that can be quantified. Several different transducers have been developed. In general, transducers can be categorized into four groups: electrochemical, calorimetric, acoustic and optical (Jönsson & Scott 2005). Optical transducers can measure variables such as changes in temperature (thermometric transducers), pressure, flow, etc., and sensors based on optical transducers are called *optical sensors*.
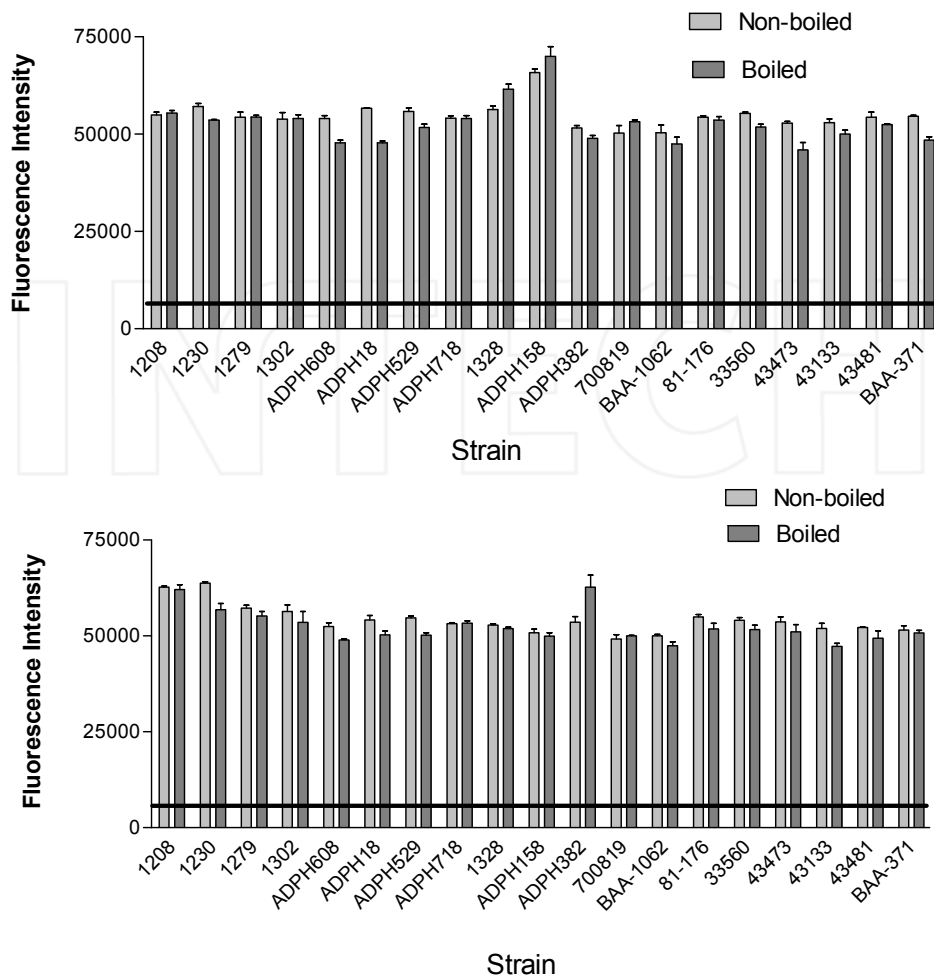
Fig. 2. Detection of 19 *C. jejuni* isolates by a polymyxin B capture ELISA. Representative bacterial colonies were suspended in phosphate buffered saline prior to assay. Fluorescence intensity at 10^6 CFU per ml^{-1} was plotted for both USB10 and USB14 antibodies. Values are means ± SEM for n=5 colonies. The limit of detection was 2.5 greater than background.

5.1 Optical sensors

The optical sensors that use labeled antibodies, e.g. antibodies tagged with fluorescent labels, are similar in design to conventional EIAs. An optical sensor that has been used extensively to detect chemical compounds and bacterial pathogens is surface plasmon resonance (SPR). SPR is an optical phenomenon involving excitation of free oscillating metal electrons and it is based on the phenomenon called *total internal reflection*. When light traveling from a medium more optically dense (refractive index n_1) penetrated into a less optically dense medium (refractive index n_2), the light is bent away from the normal plane to the boundary ($\theta_2 > \theta_1$). The exit angle

Strain	USB10 Antibody	USB14 Antibody
Aeromonas caviae	2.0 ± 0.09	0.8 ± 0.25
A. hydrophila	1.6 ± 0.05	0.74 ± 0.23
Campylobacter coli	19 ± 0.18	18 ± 2.4
C. jejuni	20 ± 0.32	19 ± 2.6
Citrobacter freundii	0.6 ± 0.02	1.4 ± 0.03
Enterobacter cloacae	0.8 ± 0.02	1.8 ± 0.15
E. coli O157:H7	0.98 ± 0.08	1.1 ± 0.01
ECOR 6	1.2 ± 0.02	1.4 ± 0.05
ECOR 15	1.8 ± 0.14	2.2 ± 0.18
EHEC 2-1	0.8 ± 0.05	1.0 ± 0.02
Helicobacter pylori	3.0 ± 0.2	4.0 ± 0.3
Klebsiella oxytoca	0.6 ± 0.01	1.5 ± 0.17
K. pneumoniae	0.5 ± 0.01	1.2 ± 0.14
Morganella morganii	0.7 ± 0.02	1.4 ± 0.09
Pantoea agglomerans	2.8 ± 0.10	1.5 ± 0.40
Plesiomonas shigelloides	0.6 ± 0.02	1.8 ± 0.30
Proteus mirabilis	0.5 ± 0.03	1.3 ± 0.06
Pseudomonas aeruginosa	1.0 ± 0.05	1.0 ± 0.03
Salmonella enterica	1.1 ± 0.07	1.4 ± 0.03
S. enteritidis	1.0 ± 0.08	1.2 ± 0.02
S. typhimurium	1.1 ± 0.08	1.5 ± 0.15
Serratia marcescens	1.7 ± 0.05	0.8 ± 0.23
V. cholerae, classical, Inaba	1.4 ± 0.10	1.5 ± 0.16
O:139	1.7 ± 0.11	2.1 ± 0.10
Vibrio mimicus	2.1 ± 0.06	2.0 ± 0.05
V. parahaemolyticus	1.6 ± 0.08	0.9 ± 0.25

Table 2. Signal to noise ratios for the polymyxin B capture ELISA for various Gram-negative bacteria compared to *C. jejuni* and *C. coli*. Representative bacterial colonies, suspended in PBS at ~ 10^7 CFU per ml^{-1} were assayed using the optimised PMB-capture ELISA. Values are means ± SEM for n=5 experiments. The limit of detection was set at 2.5 or 2.5 greater than background.

(θ_2) approaches 90° when the incident angle (θ_1) increases to a critical angle (θ_c). When the incident angle (θ_1) is equal to or greater than the critical angle (θ_c), the light will be internally reflected (Fig 3 A). The electromagnetic field component of the incident light penetrates a short distance (tens of nanometers) into the less optically dense medium to create an exponentially decaying *evanescent wave* (Fig 3 B). If the incident light is monochromatic and plane-polarized, and a thin film of metal (most frequently gold) is coated at the interface between the two different optically dense media, the photon of the evanescent wave will resonance with free oscillating electrons (plasmons) in the metal film. The evanescent wave can be used to interrogate variations on the surface structure in a distance of up to 300 nm from the surface. The use of SPR as sensor emerged from the realization that SPR signal is sensitive to changes in the refractive index, which is the bending or refraction of a beam of

light on entering a denser medium. The refractive index is influenced by the accumulation of mass on the metal surface. The optical excitation of plasmons only "when the energy of the photons of light exactly equals the quantum energy level of the plasmons", a circumstance that is called *attenuated total reflection* (Mol & Fisher 2010).

Some advantages of optical sensors include a wide dynamic range, the possibility of multiplexing to detect several analytes, and a compact, light built that allows for field applications. Another advantage that most commercial SPRs have is that the sensors are based on an open architecture and therefore the sensing element, the antibodies, can be defined by the final user and can be used without any labels to create a label-free biosensor (Ivnitski et al., 1999; Jönsson & Scott 2005). These types of sensors provide a close monitoring of the antibody/antigen reaction in a real-time manner. However, biosensors still have some limitations. For instance, there are constrains on the quantity of antibodies in an active state to capture the target bacteria that can be immobilized on the surface of a sensor. Increasing the density of antibodies is very important to increase sensitivity, but recent studies using three-dimensional aggregation of immunoglobulin G can increase the amount of antibodies by surface area and may provide a way to develop "high performance antibody biosensors" (Feng et al., 2011).
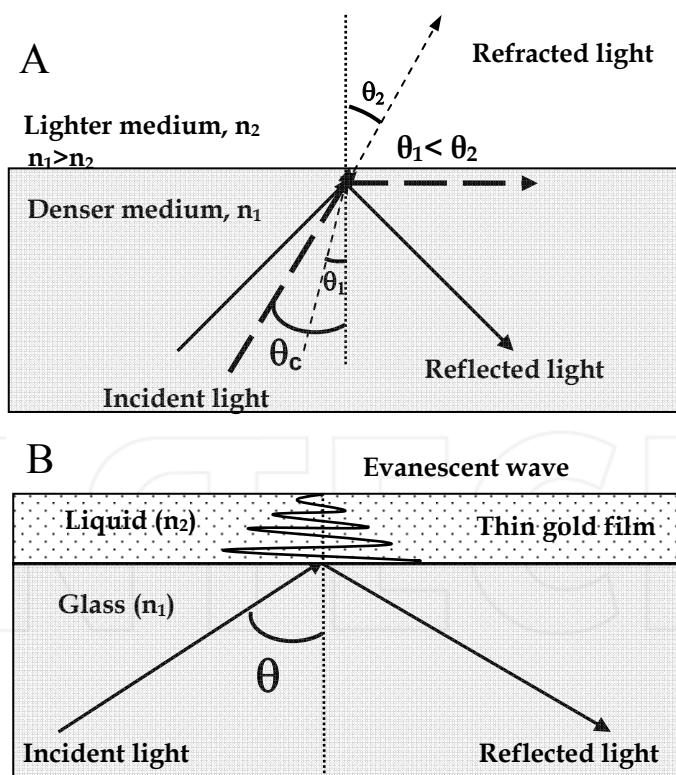

Fig. 3. The optical bases for surface plasmon resonance. Changes in refracted light (A) and the production of evanescent light (B) to interrogate the thin gold surface.

6. Conclusion

Current widely-accepted methods for identifying campylobacters in stool and food samples rely on their growth on selective agar plates with and without prior broth enrichment. These methods are labor-intensive and time-consuming. More rapid and accurate methods, including immunoassays, are necessary to identify campylobacters in stool samples, outbreaks investigations and more recently for screening processed poultry under a new performance standard established in the USA. Yet, the development of antibody-based methods for *Campylobacter* spp. has not received enough attention. At present, none of the commercially-available EIAs are recommended for standalone identification of campylobacters in stool samples, in part because of the limited and conflicting findings regarding their performances. Some of the commercial immunoassays show promise for identification of campylobacters in food although only after broth enrichment for up to 48 hours. More studies of these assays with naturally-contaminated samples from a variety of foods is necessary to validate their performance. Areas of research to improve antibody-based methods should include antibody/antigen characterization, biosensors platforms, and the dovetailing of concentration methods to improve sensitivity.

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8. References

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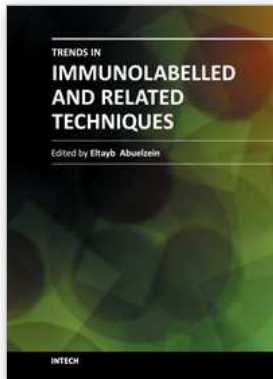
Trends in Immunolabelled and Related Techniques

Edited by Dr. Eltayb Abuelzein

The book is coined to provide a professional insight into the different trends of immunoassay and related techniques. It encompasses 22 chapters which are grouped into two sections. The first section consists of articles dealing with emerging uni-and-multiplex immunolabelled methods employed in the various areas of research. The second section includes review articles which introduce the researchers to some immunolabelled techniques which are of vital significance such as the use of the conjugates of the Staphylococcus aureus protein "A" and the Streptococcus Spps. protein "G" in immunolabelled assay systems, the use of bead-based assays and an overview on the laboratory assay systems. The book provides technological innovations that are expected to provide an efficient channel for developments in immunolabelled and related techniques. It is also most useful for researchers and post-graduate students, in all fields, where immunolabelled techniques are applicable.

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