Analytica Chimica Acta 651 (2009) 98–104

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Development of a surface plasmon resonance-based assay for the detection of *Corynebacterium pseudotuberculosis* infection in sheep

Sharon Stapleton[a,b], Bernard Bradshaw[c], Richard O'Kennedy[a,b,*]

[a] School of Biotechnology, Dublin City University, Dublin 9, Ireland
[b] National Centre for Sensor Research, Dublin City University, Dublin 9, Ireland
[c] Department of Agriculture and Food, Central Veterinary Research Laboratory, Backweston Campus, Young's Cross, Celbridge, Co. Kildare, Ireland

ARTICLE INFO

Article history:
Received 26 April 2009
Received in revised form 3 August 2009
Accepted 11 August 2009
Available online 13 August 2009

Keywords:
Corynebacterium pseudotuberculosis
Caseous lymphadenitis
Surface plasmon resonance (SPR)
Serum
Non-specific binding

ABSTRACT

Caseous lymphadenitis (CLA), a disease affecting sheep and goats, is caused by *Corynebacterium pseudotuberculosis* and is difficult to detect, especially at early stages in its development. A surface plasmon resonance-based biosensor assay for the detection of antibodies to the phospholipase D (PLD) exotoxin of *C. pseudotuberculosis* in sheep serum was successfully generated. It employed a recombinant form of PLD, which was immobilised, and all aspects of the assay including minimisation of non-specific binding, and the regeneration of the chip, were optimised. The applicability of the assay was initially demonstrated using sera collected from experimentally infected sheep and from sheep with no prior history of infection. The assay was then evaluated on a panel of clinical samples and the results obtained compared very favourably to those obtained by a double sandwich ELISA (over 90% similarity) and clearly verified its analytical value.

1. Introduction

Caseous lymphadenitis (CLA) is a disease affecting sheep and goats caused by *C. pseudotuberculosis* infection [1] and is characterised by the enlargement and suppuration of one or more lymph nodes [2]. The bacteria can infect other animal species, such as horses, causing ulcers and abscesses [3] and cows, causing mastitis and visceral lesions [4]. Human infection has also been reported, although it is usually associated with those occupationally exposed to farm animals [5].

CLA is endemic in many countries worldwide, being most prevalent in nations where intensive husbandry is practiced. It is also emerging in European countries previously 'CLA-free' as border controls are removed and the movement of animals becomes less restricted. The disease is considered to be one of the most economically important diseases of sheep and goats in the U.S., Canada and Australia [6]. Significant losses can occur when the internal lesions go undetected causing decreased milk and wool production and reproductive performance [7]. Other losses may result from the disruption of shearing time to treat a ruptured lesion or disinfect shearing equipment.

CLA was first recorded in Northern Ireland in 1999, where the source of infection was traced back to imported Scottish sheep [8]. A year later the first case was reported in the Republic of Ireland [9]. Since then there have been a number of cases of the disease reported but it is possible, due to its insidious nature, that many other cases have gone undetected.

CLA is a difficult disease to control within a flock due to a number of factors. The subclinical nature of the infection, the long incubation period and recurring nature of the disease make it extremely difficult to distinguish animals that carry the disease from those that do not. Once *C. pseudotuberculosis* has established itself within a flock, eradication is problematic, as the thick walls of the abscesses characteristic of the bacterial infection are virtually impossible to penetrate with medication [10,11]. Vaccination is used in both Australia and the U.S. as a method of disease control but commercial vaccines are not licensed in the UK and Ireland. However, vaccination would not essentially eradicate CLA from a flock but it would merely reduce the prevalence and severity of the disease. The optimal method of control of CLA is eradication of infection by identification and removal of infected carrier animals [12,13].

A variety of diagnostic tests have been described in the literature. These include an anti-beta-haemolysin test [14], a synergistic haemolysis test [12,15,16], an immunodiffusion test [17], a complement fixation assay [18], an indirect haemagglutination test [18], a microagglutination assay [19], and diagnosis using polymerase chain reaction (PCR) [20]. However, although useful, the

* Corresponding author at: School of Biotechnology, Dublin City University, Dublin 9, Ireland. Tel.: +353 1 7005319; fax: +353 1 7005412.
E-mail address: richardokennedy@gmail.com (R. O'Kennedy).

0003-2670/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.aca.2009.08.004

tests discussed can be laborious and time-consuming, and are generally unsuitable for testing large numbers of serum samples at one time. To overcome these problems, a number of enzyme-linked immunosorbent assays (ELISA) have been developed for the detection of antibodies to *C. pseudotuberculosis* [21–26]. An ELISA format originally reported by ter Laak et al. in 1992 [23] was employed for the eradication of CLA from goats over a six-year period in the Netherlands [27]. A similar programme eradicated CLA from a goat herd of 100 animals in Norway [28].

One of the most significant components of *C. pseudotuberculosis* in relation to CLA disease is its potent exotoxin, a phospholipase D (PLD). Structurally PLD is a glycoprotein with an amino acid composition similar to that of collagen [1], a molecular weight of approximately 31–35 kDa [24,29–34] and an isoelectric point (p*I*) of 9.2–9.6 [30,31]. It can be found in the bacterial cytoplasm and in smaller amounts in the cell wall. *Corynebacterium ulcerans* is the only other member of the *Corynebacterium* genus that also features PLD [35], although the molecule is known to be very similar to the toxic enzyme found in the brown recluse spider, *Loxosceles reclusa* [36]. While various antigenic proteins have been isolated from *C. pseudotuberculosis*, the PLD exotoxin was found to be one of the immunodominant antigens [33] and thus the PLD gene has been cloned [34] and expressed in *E. coli* for use in diagnostic assays [24]. The exotoxin is thought to play a vital role in the spread of the bacterium from the site of infection to regional lymph nodes [37,38].

This study was initiated to explore the suitability of a surface plasmon resonance (SPR) optical biosensor system (Biacore 3000™) to detect antibodies specific to the PLD exotoxin of *C. pseudotuberculosis* in sheep sera. Biacore™ has proved to be a valuable alternative to ELISA detection procedures as it is rapid and reproducible, allows 'real-time' detection, is label-free, fully automated and its rapid analysis makes it ideal for testing a large number of samples. Over the past decade Biacore™ technology has facilitated the detection of antibodies against various pathogenic bacteria including *E. coli* [39] and *Salmonella* [40]. However, many other sensor-based platforms have also been used for microbial analysis [41–43]. We have also successfully applied similar approaches to the detection of Listeria, drugs, toxins, fungal spores, and for studying binding of *Helicobacter pylori* to a variety of ligands [44–48]. This paper focuses on the development and evaluation of a Biacore™-based assay employing a carboxymethylated-5 (CM5) dextran chip immobilised with a PLD recombinant protein for the detection of CLA infection in a range of clinical samples from Irish herds. For the successful development of such an assay, a number of parameters were considered, including minimisation of non-specific interactions, optimisation of serum dilutions, stability of the immobilised surface for the assessment of the binding capacity and regeneration of the chip.

2. Experimental

2.1. Materials

2.1.1. Equipment and reagents

All reagents and chemicals were supplied by Sigma–Aldrich Ireland Ltd. (Dublin, Ireland), unless otherwise stated. Carboxymethyl (CM) dextran sodium salt was obtained from Fluka Chemicals (Gillingham, Dorset, England). The Biacore 3000™ instrument, research grade carboxymethylated 5 (CM5) sensor chips and an amine coupling kit containing NHS (N-hydroxysuccinimide), EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) and ethanolamine hydrochloride, were supplied by Biacore™ AB (Central Milton Keynes, UK).

2.1.2. Sheep sera

All serum samples employed in the development of the Biacore™ assay for the detection of CLA were obtained from the Central Veterinary Research Laboratory (CVRL), Abbotstown, Dublin, Ireland. A set of nine control serum samples, which had been taken from sheep 'on-site' were provided. These included five samples (GB04-013280 to GB04-013284) collected from sheep experimentally infected with CLA and four samples (GB04-013276 to GB04-013279) collected from sheep with no history of CLA infection. A panel of reference sera ($n = 92$), which had been previously tested in the Central Veterinary Institute, Lelystad, the Netherlands by an ELISA method described by Dercksen et al. [25] were also obtained from Abbotstown. All sera were dispensed in aliquots and stored at −20 °C. Working stocks were thawed and stored at 4 °C until depletion.

2.1.3. Recombinant PLD protein

The plasmid-bearing the *C. pseudotuberculosis* PLD gene (denoted pJGS90), cloned by Dr. Glenn Songer of the Department of Veterinary Science and Microbiology, University of Arizona, Tuscon, USA, was kindly donated by Dr. John Prescott of the Department of Pathobiology, Ontario Veterinary College, University of Guelph, Ontario, Canada.

2.2. Experimental techniques

2.2.1. Expression and purification of recombinant PLD protein

The pJGS90 plasmid was transformed into competent XL-10 Gold® *E. coli* cells for high-level expression of the recombinant PLD. Single colonies of XL10-Gold *E. coli* cells containing pJGS90 were inoculated into 5 mL super broth (SB) media, containing 1% (wt/vol) glucose, 10 μg mL^{-1} tetracycline, 100 μg mL^{-1} ampicillin and 25 μg mL^{-1} chloramphenicol and incubated overnight at 37 °C, with orbital shaking at 250 rpm. Each overnight culture was used to seed 500 mL of SB, containing 1% (wt/vol) glucose and the appropriate antibiotics, as before, and grown at 37 °C for 8 h with shaking at 200 rpm. The culture was then centrifuged at 3000 × *g* (Eppendorf 5810R) at 4 °C for 30 min and the supernatant was discarded. The bacterial pellet was resuspended in 500 mL of fresh SB media containing the appropriate antibiotics, but without glucose, and the cells were incubated at 30 °C with shaking at 200 rpm. Protein expression was induced with the addition of 0.05 mM isopropyl-β-D-thiogalactoside (IPTG) and cells were incubated overnight at 30 °C with shaking at 200 rpm. The following morning the culture was centrifuged as before at 3000 × *g* (Eppendorf 5810R) at 4 °C for 30 min and the supernatant was discarded. The pellet was resuspended in 20 mL of immobilised metal affinity chromatography (IMAC) column loading buffer (50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl and 10 mM imidazole). The cells were sonicated on ice and then centrifuged in Oakridge tubes at 48,000 × *g* (Beckman J2-21) at 4 °C for 30 min. The resulting cell lysate was syringe filtered (0.2 μm cut-off) and applied to an Econo-Pac™ chromatography column (Bio-Rad Laboratories Ltd., Herts, UK) containing 1 mL of packed Ni-NTA resin (Qiagen Ltd., West Sussex, UK). The $(His)_6$-tagged PLD protein was then buffer exchanged into PBS, pH 7.4, using a Vivaspin centrifugal concentrator (Sartorius AG, Germany) following purification by IMAC. Purified protein was aliquoted and stored at −20 °C. Working stocks were thawed and stored at 4 °C until depletion.

2.2.2. ELISA development

Nunc Maxisorb™ plates were coated with 100 μL of PLD, at a concentration of 10 μg mL^{-1} in PBS, pH 7.4, and incubated for 1 h at 37 °C. Plates were washed three times with PBS and then blocked with 200 μL PBS, pH 7.4, containing 4% (wt/vol) Marvel™ for 1 h at 37 °C. The plates were then washed three times with

PBS and three times with PBS containing 0.05% (vol/vol) Tween 20. CLA-positive and negative serum were diluted 1 in 50 in PBS containing 4% (wt/vol) Marvel™ and pre-incubated at 37 °C for 1 h. Following incubation, the immunoplate was washed as before and 100 μL of each diluted serum sample was added to the wells. The plate was incubated again at 37 °C for 1 h and washed as before. This was followed by addition 100 μL of a 1 in 4000 dilution of HRP-labelled rabbit anti-sheep antibody (Dako) in PBS containing 4% (wt/vol) Marvel™ and incubated for 1 h at 37 °C. Plates were again washed and a chromogenic substrate, *o*-PD (0.4 mg mL^{-1} *o*-phenylenediamine in 0.05 M phosphate citrate buffer, pH 5.0, and 0.4 mg mL^{-1} of urea hydrogen peroxidase) (Sigma–Aldrich Ireland Ltd.), was added and incubated for 30 min at 37 °C. Absorbance was read at 450 nm on a Titertek Reader.

2.2.3. Biacore™ assay development

Analysis was performed using a Biacore 3000™ instrument operated with the Biacore 3000™ Control Software package version 3.1.1. HEPES buffered saline (HBS), pH 7.4, containing 10 mM HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid), 150 mM NaCl, 3.4 mM EDTA and 0.05% (vol/vol) Tween 20, was used as the flow buffer and as a diluent throughout analyses unless otherwise stated. Buffer was freshly prepared, filtered (0.2 μm pore size) and degassed using a vacuum filtration apparatus (Millipore sintered glass filtration unit) prior to use.

2.2.3.1. Coupling of PLD to CM-dextran sensor chip surface. Preconcentration studies were carried out according to manufacturers' instructions for efficient immobilisation of the protein onto the surface of the CM5 sensor chip. Coupling of the PLD protein to the dextran surface was achieved using standard amine coupling chemistry. The carboxymethylated dextran surface was activated by mixing equal volumes of 100 mM NHS (N-hydroxysuccinimide) and 400 mM EDC (N-ethyl-N-(dimethyl-aminopropyl) carbodiimide hydrochloride) and injecting the mixture over the sensor chip surface for 8 min at a flow rate of 10 μL min^{-1}. Protein, at a concentration of 10 μg mL^{-1} in 10 mM sodium acetate buffer, pH 4.4, was injected over the activated surface for 40 min at a flow rate of 5 μL min^{-1}, allowing the activated NHS esters on the dextran surface to react with the amino groups on the protein. Unreacted NHS groups were deactivated by injecting a solution of 1 M ethanolamine, pH 8.5, over the chip surface at a flowrate of 10 μL min^{-1} for 7 min. Loosely bound protein was removed with 30 s pulses of 5 mM NaOH prior to use. A final immobilisation level equivalent to approximately 6000 Response Units (RU) was achieved.

2.2.3.2. Assay for the detection of serum antibodies to C. pseudotuberculosis. Serum samples (n = 92), diluted 80-fold in HBS containing 12 mg mL^{-1} CM dextran sodium salt, were transferred into individual glass vials. Each sample was injected at a flowrate of 5 μL min^{-1} for 2 min and the response recorded 30 s after the end of the injection. This was followed by an injection of 20 mM NaOH at a flowrate of 10 μL min^{-1} for 30 s, which allowed the complete regeneration of the chip surface. All ninety-two samples were tested in triplicate and results were normalised by expressing their respective response unit values as a percentage of the positive control, which was included after every twenty samples analysed.

3. Results and discussion

3.1. Assessment of non-specific binding

The possibility of non-specific interactions between the serum antibodies and the dextran surface of the sensor chip is greatly increased due to the complexity and polyclonal nature of the serum matrix. A single positive (GB04-013283) and single negative (GB04-013279) control serum were used investigate the degree of non-specific binding of the serum antibodies to the dextran matrix. Control sera, diluted 30-fold, were simultaneously injected over both the blank CM-dextran surface (reference flow cell) and the PLD protein-immobilised dextran surface. Throughout analyses, the final report response was corrected for the response of the reference flow cell. Sera was injected over the sensor surface at a flowrate of 5 μL min^{-1} for 2 min and the binding response monitored 30 s after each injection. Both the positive and negative control sera displayed a significant non-specific response. Responses of 1,266RU and 39RU were observed when the positive and negative controls were injected over the reference flow cell surface, respectively, as illustrated in Figs. 1(A) and 2(A). However, this non-specific interference was greatly reduced with the addition of carboxymethyl (CM)-dextran sodium salt, at a concentration of 12 mg mL^{-1}, to the HBS diluent buffer (Figs. 1(B) and 2(B)).

3.2. Regeneration of the chip surface

Reproducible regeneration of the sensor chip is vital when using Biacore™ as a diagnostic tool. The immobilised chip surface should be used and regenerated without significant loss of assay sensitivity. The greater the number of samples analysed per flow cell, the greater the reduction in the cost of the assay. Thus mild regeneration conditions are essential for sustainable and optimal use with a large sample series. A number of regeneration solutions were evaluated according to the manufacturers' instructions but offered no improvements compared to the sodium hydroxide solution. When sera samples were diluted 40-fold, the normalised response for the positive and negative controls was found to be 640 and 25RU, respectively. The serum was passed over the sensor chip surface at a flowrate of 5 μL min^{-1} for 2 min and the response recorded 30 s after the end of injection. This was followed by an injection of 20 mM sodium hydroxide at a flowrate of 10 μL min^{-1} for 30 s, which allowed for the complete regeneration of the chip surface (Fig. 3).

The efficiency of the regeneration process was then evaluated by performing multiple (*i.e.* twenty) binding-regeneration cycles of the single positive control (GB04-013283) to the PLD-immobilised surface. Over the twenty binding-regeneration cycles, an increase of 14.5% in response signal was observed, indicating that the regeneration solution was not effective in completely removing all bound serum antibodies (data not shown). This accumulation of serum antibodies on the surface would ultimately affect the ability of other sera antibodies to bind the immobilised PLD protein. However, more concentrated sera samples would require harsher solutions to fully regenerate the chip surface and consequently could result in a decrease of the amount of immobilised ligand and would ultimately affect the lifetime of the PLD-immobilised surface. It was therefore decided to use a higher dilution of sera in subsequent analyses, which would require less stringent regeneration conditions.

3.3. Optimisation of sheep serum dilution

Serial two-fold dilutions of the sheep positive and negative controls were prepared to determine which dilution enabled optimal discrimination between positive and negative sera. Dilutions, ranging from 10- to 160-fold were prepared in HBS, containing 12 mg mL^{-1} CM dextran, and injected over the chip surface. From the results it can be observed that even at a dilution as high as 1/160, the PLD SPR-based assay was sensitive enough to enable discrimination between positive and negative sera (Fig. 4). Results also demonstrated the linearity of the assay, where each two-fold dilution demonstrated roughly a two-fold decrease in response units.

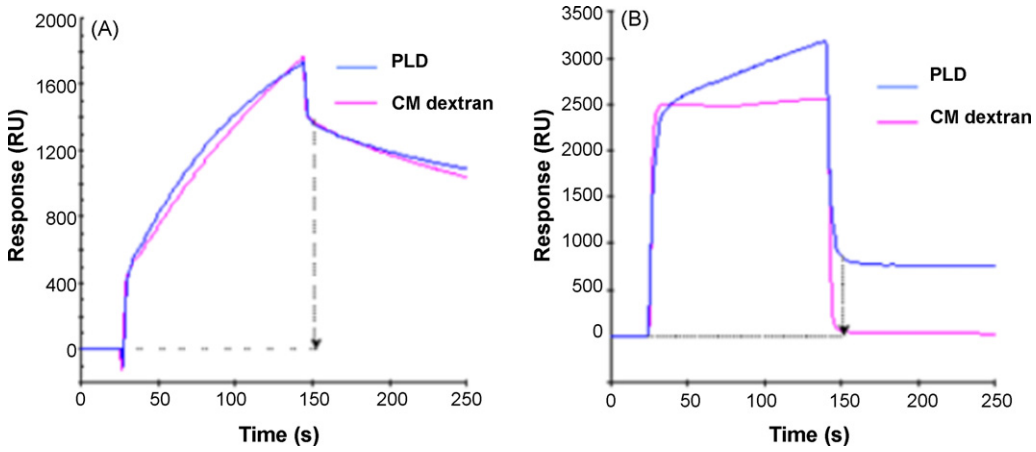


Fig. 1. Sensograms demonstrating the difference in response of the positive serum control (A) without or (B) with CM dextran sodium salt. The serum, at a dilution of 1/30, was passed over the PLD-immobilised surface and the reference dextran surface at a flowrate of 5 L min^{-1} for 2 min. (A) Approximately 1394RU was observed when the positive control was passed over the immobilised PLD surface. However, a response of 1266 units was observed when the serum was passed over the unactivated dextran surface indicating a high degree of non-specific binding. (B) Following addition of CM dextran to the sample diluent, a response of 769RU and 30RU was observed when the positive control was passed over the immobilised and reference flow cell, respectively.

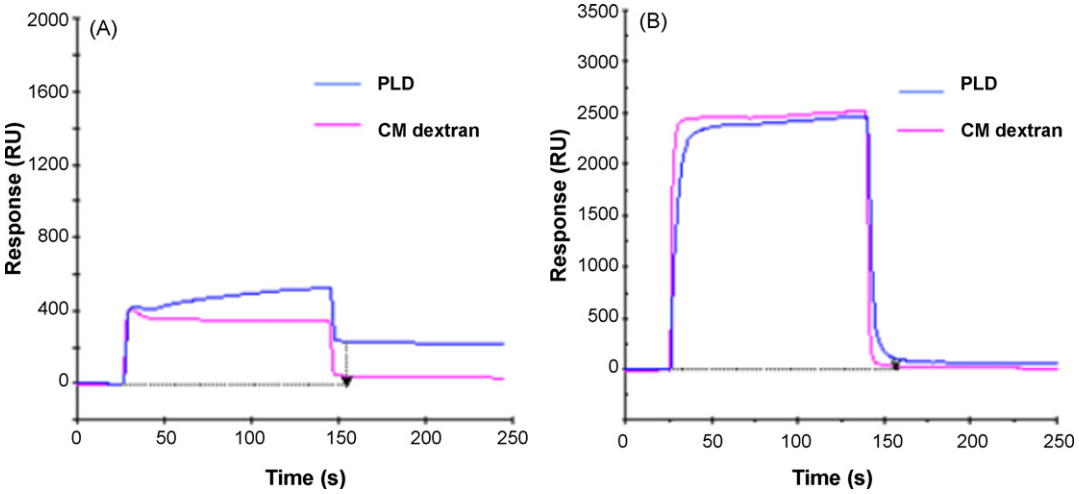


Fig. 2. Sensograms demonstrating the difference in response of the negative serum control (A) without or (B) with CM dextran sodium salt. The serum, at a dilution of 1/30, was passed over the PLD-immobilised surface and the reference dextran surface at a flowrate of 5 L min^{-1} for 2 min. (A) Approximately 225RU was observed when the negative control was passed over the immobilised PLD surface. However, a response of 39 units was observed when the negative control was passed over the unactivated dextran surface indicating a high degree of non-specific binding of the serum antibodies to the dextran surface. (B) Following addition of CM dextran to the sample diluent, a response of 70RU and 24RU was observed when the negative control was passed over the immobilised and reference flow cell, respectively.

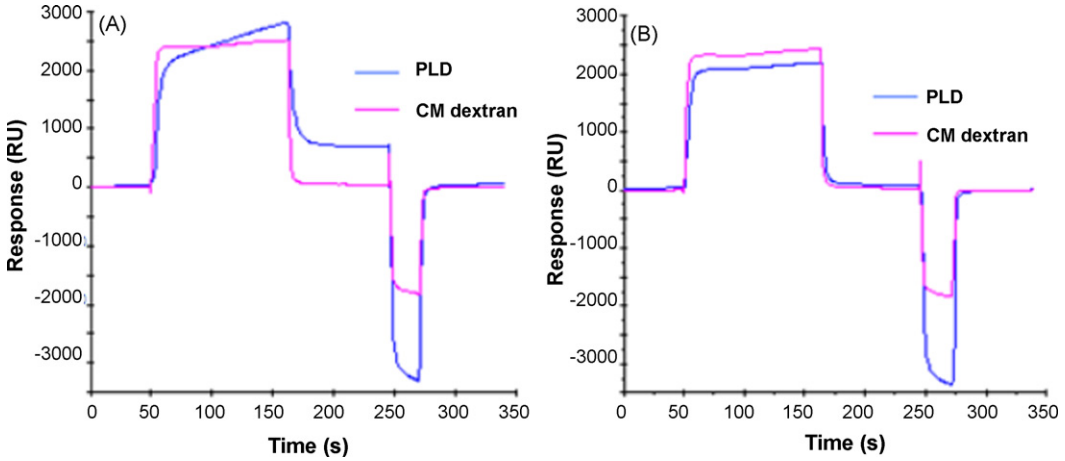


Fig. 3. Sensograms showing (A) the positive and (B) the negative sera being passed over the chip surface. The sera was passed over at a flowrate of 5 μL min^{-1} for 2 min, the specific serum antibodies bound to PLD and the surface was regenerated with 5 μL of 20 mM NaOH, which allowed complete regeneration of the surface.

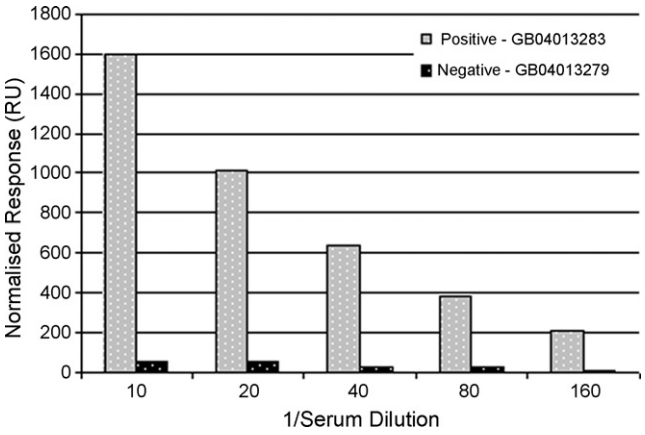


Fig. 4. Optimisation of the serum dilution for use in the PLD Biacore™ assay. Serum dilutions, ranging from 1/10 to 1/160, were tested in order to determine which concentration enabled optimal discrimination between the positive and negative serum controls.

To ensure that the same linearity of response, as found with the control sera, would be observed when testing the reference sera, eighteen of the reference samples were also examined. Coefficients of variation (CVs) were determined to below 20% when samples were assayed in triplicate at a 40- and 80-fold dilution. At a dilution of 1/80 the response from the positive control was found to be approximately 400R), which could be easily distinguished from the response from the negative serum control (approximately 20RU). Thus, it was decided to use sera at this dilution in subsequent analyses. The selection of this dilution was based on the combination of the need to have adequate discriminating response levels on Biacore™, the need to dilute to minimise non-specificity reactions while retaining the capacity to detect antibody binding and the requirement to keep regeneration conditions as mild as possible to ensure long term repeatability and longevity of the immobilised protein chip surface.

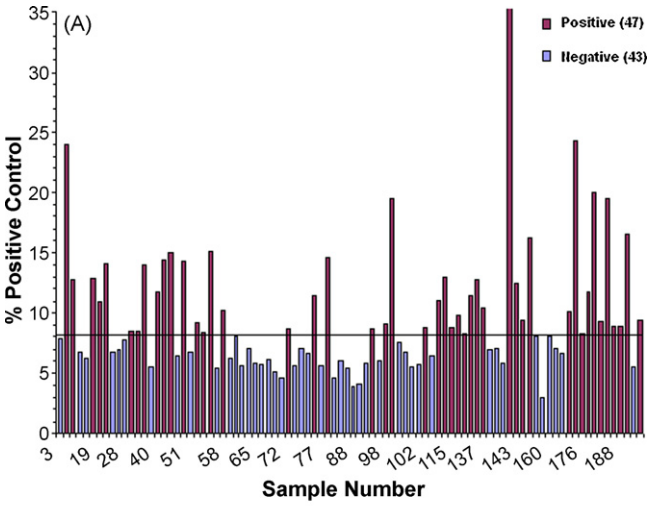
3.4. Optimised Biacore™ assay

Serum samples ($n = 92$), diluted 80-fold in HBS containing 12 mg mL^{-1} CM dextran sodium salt, were injected over the sensor surface at a flowrate of 5 μL min^{-1} for 2 min and the response recorded 30 s after the end of injection. This was followed by a 5 μL injection of 20 mM NaOH, which allowed the complete regeneration of the chip surface. All ninety-two samples were tested in triplicate and results were normalised by expressing their respective response unit values as a percentage of the positive control (Fig. 5(A)).

Results revealed the non-specific binding responses of the sera were relatively constant in terms of response units with an average of approximately 20RU. Intra-assay coefficients of variation ranged from 0.33 to 9.76%, with a single outlier at 13.48%. 83% of all the ninety-two samples tested had a CV of less than 5%. All ninety-two sera samples were tested in a continuous run, but with negative and positive controls included after every twenty serum samples. In total 306 analyses per flow cell were conducted in a continuous series with a decrease of 21% of the binding response of the positive control observed following 264 binding-regeneration cycles (*i.e.* equivalent to analysing 80 serum samples).

3.5. Cut-off point determination

The cut-off point, which determines the number of false-positive and false-negative results, was determined as the average response of the CLA-negative control throughout the run plus three



(B)

		Reference animals: Lelystad-Positive	Reference animals: Lelystad-Negative
Test Result	Positive	37 (A)	12 (B)
	Negative	6 (C)	37 (D)
		Diagnostic sensitivity $\frac{A}{A+C} = \frac{37}{43} = 86\%$	*Diagnostic specificity* $\frac{D}{D+B} = \frac{37}{49} = 76\%$

Fig. 5. (A) Characterisation of the Biacore™ assay performance. The assay was assessed for use as a diagnostic assay for caseous lymphadenitis (CLA) in sheep serum samples. The assay was performed using a panel of 44 previously determined Lelystad ELISA-positive and 48 ELISA-negative reference samples. The absorbance value of each sample was recorded at 405 nm and the mean plotted as a percentage of the mean absorbance obtained for the positive control sample. Using a cut-off point of 8.1%, 49 of the serum samples tested were found to be positive and 43 negative. Two samples, number 4 and 10, which were not included on the bar chart, gave a response as high as 169.3% and 53.83% of the positive control, which correlated with the response from the same samples tested previously in the indirect ELISA. (B) Diagnostic sensitivity (DSn) and diagnostic specificity (DSp) calculations for the PLD Biacore™ assay. Results yielded a diagnostic sensitivity of 86% and specificity of 76%.

times the standard deviation. This value was found to be 8.1% when data was normalised by expressing the respective response units as a percentage of the positive control. Applying this cut-off, forty-nine of the samples were determined positive and forty-three negative (Fig. 5(A)).

To assess the sensitivity and specificity of the Biacore™ assay, results were compared to that obtained from the ELISA performed in Lelystad. 86% of the sera that tested positive in the Lelystad ELISA also tested positive in the SPR assay. While 76% of those that tested negative in the Lelystad ELISA were also found to be negative in the SPR assay (Fig. 5(B)). Results were also compared to an indirect PLD ELISA (Section 2.2.2, Fig. 6), which had also been developed in parallel using the same format (*i.e.* an immunoplate was coated with the recombinant PLD protein and bound serum antibodies were detected using an HRP-labelled rabbit anti-sheep antibody). It was calculated that 95.5% of serum samples that gave a positive result in this ELISA also gave a positive result in the Biacore™ assay. Overall 91.3% of the negative results obtained in the Biacore™ assay gave the same result as that found in the indirect PLD ELISA. Of the ninety two sera analysed, only eight samples gave conflicting results in the Biacore™ assay, six of these were within ±1% of the cut-off point, so therefore could be considered inconclusive.

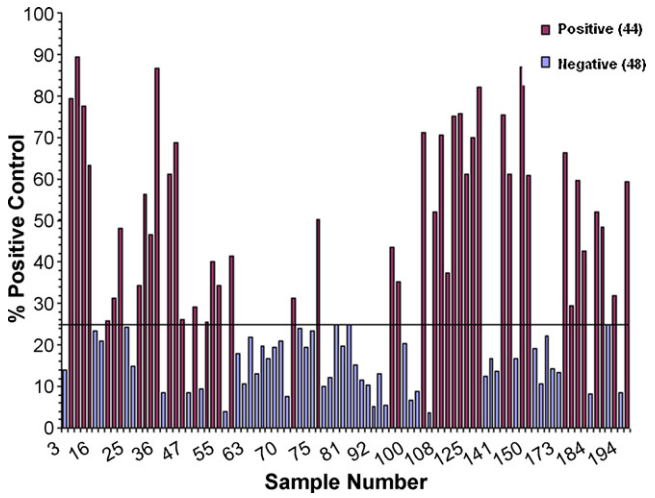


Fig. 6. Characterisation of the PLD ELISA performance. The PLD indirect ELISA was assessed for use as a diagnostic assay for caseous lymphadenitis (CLA) in sheep serum samples. The indirect ELISA was performed using a panel of 44 previously determined Lelystad ELISA-positive and 48 ELISA-negative reference samples. The absorbance value of each sample was recorded at 405 nm and the mean plotted as a percentage of the mean absorbance obtained for the positive control sample. Applying the cut-off point of 25%, 44 of the serum samples tested were found to be positive and 48 negative.

3.6. Evaluation of the sensor-based assay

This paper illustrates the potential of an SPR biosensor-based system (Biacore 3000™) as a serological diagnostic marker for CLA infection. The Biacore has proved to be a valuable alternative to ELISA procedures and facilitates automated, label-free detection of biomolecules in "real-time". A high degree of non-specific binding of the serum antibodies to the CM dextran surface of the chip was observed. However, this was overcome with the addition of CM dextran sodium salt to the serum diluent. Feasibility studies carried out on the nine control sera indicated the significant potential of the Biacore™ assay for the detection of *C. pseudotuberculosis* specific antibodies. The binding capacity and regeneration of the chip surface was also assessed. At a higher dilution of sera (1/80), a less concentrated solution of NaOH (*i.e.* 20 mM) could be used for complete regeneration of the chip surface while still obtaining a high response (approximately 400RU) for the positive control.

The optimised assay format was subsequently evaluated on a panel of reference sera collected from Irish herds, where all ninety-two samples were tested in triplicate in a continuous run. The non-specific binding responses of the sera were found to be relatively constant with an average response of approximately 20RU. The assay proved to be reproducible with intra-assay CVs of all sera analysed found to be below 10%, with the exception of one sample with a value 13.48%. 83% of all serum samples tested had a CV of less than 5%. In total 306 analyses per flow cell were conducted successfully in a continuous series before a significant decrease (>20%) was observed for the binding capacity of the positive control.

To establish the sensitivity and specificity of the assay a cut-off point of 8.1% was determined as the average response of the negative sera control throughout the analyses plus three times the standard deviation. Applying this cut-off value, forty-nine of the samples were determined positive and forty-three negative. When compared to the Lelystad ELISA results, a DSn of 86% and a DSp of 76% were calculated. Comparing the results to that found in the indirect PLD ELISA, it was calculated that 95.5% of serum samples that gave a positive result in the ELISA also gave a positive result in the SPR assay. Overall 91.3% of the results obtained in the Biacore™ assay gave the same result as that found in the ELISA. Of the ninety two sera analysed, only eight samples gave conflicting results in the Biacore™ assay. Six of these were within ±1% of the cut-off point, so therefore could be considered inconclusive.

Comparing the results from all three assays (*i.e.* Lelystad ELISA, indirect PLD ELISA and SPR assay), it was found that 80% of the samples determined as positive in the Lelystad ELISA, were also found to be positive in both the PLD ELISA and Biacore™ assay. Seven of these nine samples gave conflicting results in both the ELISA and Biacore™ assay (*i.e.* found to be negative in both assays). All of these samples, except one, gave results that could be considered inconclusive or invalid. Results obtained for two of these samples were found to have CV values above 20% when tested by the Lelystad ELISA, suggesting the positive result may be questionable. Four of the samples were found to have values within close proximity of the cut-off point established for either the ELISA or SPR assay, so therefore could be considered inconclusive. Two samples gave conflicting results in the ELISA and Biacore™ so therefore could not be determined either definitely positive or negative. Finally one sample was determined definitely negative in both the ELISA and Biacore™ assay, with values well below the cut-off points.

All data analysis was based on previous results obtained from the Lelystad ELISA and estimated DSn and DSp values are entirely dependent upon the reliability of the Lelystad assay, which like all assays for this disease, has inherent variability.

4. Conclusions

The original double-antibody sandwich ELISA developed in Lelystad by ter Laak et al. [23], claimed a specificity of 99.9% and sensitivity of 100%. However, there were a number of inconclusive samples taken from CLA-positive flocks. Dercksen et al. [25] later evaluated the assay and reported a sensitivity of 51 ± 6% and a specificity of 97 ± 2%, which differs greatly of what was previously reported by ter Laak et al. [23]. Dercksen and colleagues also modified the assay to improve sensitivity and specificity, which were found to be 79 ± 5% and 99 ± 1% in sheep, respectively. The number of false-positive results were thought to be due to the possible cross-reactivity of the toxin-specific serum, used as the coating antigen, with other bacteria including other *Corynebacterium* species, *Listeria monocytogenes* or *Mycobacterium avium* subsp. *paratuberculosis*. However, the double-antibody sandwich ELISA, modified by Dercksen et al. [25], was further investigated by Malone and colleagues in 2006. In this case, the sensitivity and specificity of the ELISA was found to be 88% and 55%, respectively. These figures differ greatly from the previously determined diagnostic sensitivity and specificity of 79 ± 5% and 99 ± 1%, respectively, reported by Dercksen and colleagues. Although they had suggested that false-positive results could be due to possible cross-reactivity with other bacteria, Malone et al. [8] found no evidence of these bacterial infections in any of the flocks examined. To the best of our knowledge this is the first time a SPR-based assay has been reported for the diagnosis in CLA in sheep. Results, presented in this paper, indicate the potential of the assay for diagnosis of CLA based on humoral response to PLD and its prospective application in a veterinary diagnostic laboratory for the detection of antibodies to a range of infectious agents for clinical analysis. Future work arising from this body of research could entail additional validation of the assay employing a larger number of serum samples and subsequent application to determine the prevalence of CLA disease in Ireland.

Acknowledgements

The financial support provided by the Department of Agriculture & Food and Dublin City University is gratefully acknowledged.

References

[1] C.C. Brown, H.J. Olander, Vet. Bull. 57 (1) (1987) 1.
[2] B.E. Schreuder, E.A. ter Laak, D.P. Dercksen, Vet. Rec. 135 (8) (1994) 174.
[3] H.D. Knight, J. Am. Vet. Med. Assoc. 155 (2) (1969) 446.
[4] P.J. Watson, B.E. Preece, Vet. Rec. 148 (21) (2001) 663.
[5] M.M. Peel, G.G. Palmer, A.M. Stacpoole, T.G. Kerr, Clin. Infect. Dis. 24 (2) (1997) 185.
[6] M.W. Paton, I.R. Rose, R.A. Hart, S.S. Sutherland, A.R. Mercy, T.M. Ellis, J.A. Dhaliwal, Aust. Vet. J. 71 (2) (1994) 47.
[7] S.G. Stoops, H.W. Renshaw, J.P. Thilsted, Am. J. Vet. Res. 45 (3) (1984) 557.
[8] F.E. Malone, S.A. Fee, E.M. Kamp, D.C. King, G.J. Baird, K.M. O'Reilly, F.E.A. Murdock, Irish Vet. J. 59 (1) (2006) 19.
[9] S. O'Doherty, M. Prendergast, M. Scanlon, W.L. Schofield, M.L. Doherty, M. Frame, H.F. Bassett, Irish Vet. J. 53 (12) (2000) 631.
[10] K.T. Maddy, J. Am. Vet. Med. Assoc. 122 (913) (1953) 257.
[11] G. Baird, Vet. Rec. 140 (23) (1997) 611.
[12] C.C. Brown, H.J. Olander, C. Zometa, S.F. Alves, Am. J. Vet. Res. 47 (7) (1986) 1461.
[13] J.F. Prescott, P.I. Menzies, Y.T. Hwang, Vet. Microbiol. 88 (3) (2002) 287.
[14] M.M. Zaki, Res. Vet. Sci. 9 (6) (1968) 489.
[15] H.D. Knight, Cornell Vet. 68 (1978) 220.
[16] D.H. Burrell, Res. Vet. Sci. 28 (2) (1980) 190.
[17] D.H. Burrell, Res. Vet. Sci. 28 (2) (1980) 234.
[18] M.T. Shigidi, Br. Vet. J. 135 (2) (1979) 172.
[19] P.I. Menzies, C.A. Muckle, Can. J. Vet. Res. 53 (3) (1989) 313.
[20] B. Çetinkaya, M. Karahan, E. Atil, R. Kalin, T. De Baere, M. Vaneechoutte, Vet. Microbiol. 88 (1) (2002) 75.
[21] L.R. Maki, S.H. Shen, R.C. Bergstrom, L.D. Stetzenbach, Am. J. Vet. Res. 46 (1) (1985) 212.
[22] S.S. Sutherland, T.M. Ellis, A.R. Mercy, M. Paton, H. Middleton, Aust. Vet. J. 64 (9) (1987) 263.
[23] E.A. ter Laak, J. Bosch, G.C. Bijl, B.E. Schreuder, Am. J. Vet. Res. 53 (7) (1992) 1125.
[24] P.I. Menzies, C.A. Muckle, Y.T. Hwang, J.G. Songer, Small Rumin. Res. 13 (2) (1994) 193.
[25] D.P. Dercksen, J.M. Brinkhof, T. Dekker-Nooren, K. Maanen, C.F. Bode, G. Baird, E.M. Kamp, Vet. Microbiol. 75 (2) (2000) 167.
[26] J. Kaba, L. Kutschke, G. Gerlach, Vet. Microbiol. 78 (2) (2001) 155.
[27] D.P. Dercksen, E.A. ter Laak, B.E. Schreuder, Vet. Rec. 138 (10) (1996) 237.
[28] K. Nord, G. Holstad, L.O. Eik, H. Gronstol, Acta Vet. Scand. 39 (1) (1998) 109.
[29] J.A. Ellis, D.A. Hawk, K.W. Mills, D.L. Pratt, Vet. Immunol. Immunopathol. 28 (3–4) (1991) 289.
[30] A.L. Hodgson, P. Bird, I.T. Nisbet, J. Bacteriol. 172 (3) (1990) 1256.
[31] T.Y. Hsu, H.W. Renshaw, C.W. Livingston Jr., J.L. Augustine, D.L. Zink, B.B. Gauer, Am. J. Vet. Res. 46 (5) (1985) 1206.
[32] P.I. Menzies, Y.T. Hwang, J.F. Prescott, Vet. Microbiol. 100 (1–2) (2004) 129.
[33] C.A. Muckle, P.I. Menzies, Y. Li, Y.T. Hwang, M. van Wesenbeeck, Vet. Microbiol. 30 (1) (1992) 47.
[34] J.G. Songer, S.J. Libby, J.J. Iandolo, W.A. Cuevas, Infect. Immun. 58 (1) (1990) 131.
[35] L. Barksdale, R. Linder, I.T. Sulea, M. Pollice, J. Clin. Microbiol. 13 (2) (1981) 335.
[36] A.W. Bernheimer, B.J. Campbell, L.J. Forrester, Science 228 (4699) (1985) 590.
[37] J.G. Songer, Trends Microbiol. 5 (4) (1997) 156.
[38] M.D. Piontkowski, D.W. Shivvers, J. Am. Vet. Med. Assoc. 212 (11) (1998) 1765.
[39] M.B. Medina, L. Van Houten, P.H. Cooke, S.I. Tu, Biotechnol. Technol. 11 (1997) 173.
[40] B.G.M. Jongerius-Gortemaker, R.L.J. Goverde, F. van Knapen, A.A. Bergwerff, J. Immunol. Methods 266 (1–2) (2002) 33.
[41] T.D. Gibson, O. Prosser, J. Hulbert, R. Marshall, P. Corcoran, P. Lowery, E.A. Ruck-Keene, S. Heron, Sens. Actuators B: Chem. 44 (1–3) (1997) 413.
[42] M. Seidel, R. Niessner, Anal. Bioanal. Chem. 391 (2008) 1521.
[43] P. Leonard, S. Hearty, J. Brennan, L. Dunne, J. Quinn, T. Chakraborty, R. O'Kennedy, Enzyme Microb. Technol. 32 (1) (2003) 3.
[44] P. Leonard, S. Hearty, J. Quinn, R. O'Kennedy, J. Food Prot. 68 (4) (2005) 728.
[45] J. Brennan, P. Dillon, R. O'Kennedy, J. Chromatogr. B 18 (217) (2003) 2.
[46] L. Dunne, S. Daly, A. Baxter, S. Haughey, R. O'Kennedy, Spectrosc. Lett. 38 (3) (2006) 229.
[47] P. Skottrup, S. Hearty, H. Frokiaer, P. Leonard, J. Hejgaard, R. O'Kennedy, M. Nicolaisen, A. Justesen, Biosens. Bioelectron. 22 (2007) 2724.
[48] E.P. Reeves, T. Ali, P. Leonard, S. Hearty, R. O'Kennedy, F.E. May, B.R. Westley, C. Josenhans, M. Rust, S. Suerbaum, A. Smith, B. Drumm, M. Clyne, Gastroenterology 135 (6) (2008) 2043.