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

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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

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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